

Investigating post-harvest insect-microbe interactions and their ramifications for global food
quality, human health, and insect behavior

by

Marco A. Ponce

B.S., Kalamazoo College, 2019

AN ABSTRACT OF A DISSERTATION

submitted in partial fulfillment of the requirements for the degree

DOCTOR OF PHILOSOPHY

Department of Entomology
College of Agriculture

KANSAS STATE UNIVERSITY
Manhattan, Kansas

2023

Abstract

Microbes are ubiquitous and play important ecological roles. While research has largely focused on arthropods and microbes separately, less attention has been given to their interactions in the post-harvest supply chain. As a result, my aims focused on *S. oryzae* and *L. serricorne* and were to 1) evaluate the vectoring capacity of both species for microbes across time, 2) elucidate the behavioral response of each species to microbially mediated volatile emissions in grain, and 3) determine how foraging preferences affect development and fitness among habitats of different quality by each species. To assess the vectoring capacity, both species originated from individuals placed immediately in a novel food patch, or given 24–72 h in a sterilized environment prior. Vectored microbes were imaged after 3–5 d. Increasing dispersal time resulted in multiple-fold reductions in microbial growth by *L. serricorne*, but not *S. oryzae*. This was likely attributable to differences in baseline mobility by each species. In addition, we identified 14 taxa and nine genera. Over half of the samples were from *Aspergillus* spp. Our data suggests that there is a more persistent risk of microbial contamination by *S. oryzae* than by *L. serricorne*. In elucidating the behavioral response of *S. oryzae* to microbial volatiles, two stages (sexual and asexual) of *A. flavus* were inoculated onto wheat and compared to uninoculated, or UV-sterilized wheat. Using headspace collection coupled with GC-MS, the sexual life stage of *A. flavus* had the most unique emissions of microbial volatiles. This translated to a higher interaction with kernels containing the *A. flavus* sexual life stage by *S. oryzae* in comparison to the asexual life stage. Grain volatiles were more important for attraction at longer distances. In another set of trials, wheat was inoculated with either *Fusarium verticillioides* or *A. flavus* and compared to uninoculated grain or UV-sterilized grain. Headspace collection revealed that the *Fusarium*- and *A. flavus*-inoculated grain produced significantly different volatiles compared to

sanitized grain or the positive control, which translated to changes in close-range foraging by *L. serricorne*, with a greater frequency of entering and spending time *A. flavus*-inoculated kernels compared to other treatments. Microbial volatiles were found to be attractive to *L. serricorne* at longer distances in pitfall traps. Overall, this suggests that microbial volatiles are important for close- and long-range orientation of *L. serricorne*. To determine how foraging preferences affected fitness, comparisons between habitats inoculated with *A. flavus*, uncontaminated, or autoclaved in surrogate food patches after 3 d of foraging by *S. oryzae* and *L. serricorne* were performed. Insects were released into the dispersal apparatus with habitats located at opposite ends. Our study found that microbes modulated the preference and progeny production of *L. serricorne*, which dispersed to the uninfested habitats, while *S. oryzae* showed a preference to reproduce and dispersed to habitats inoculated with *A. flavus*. In addition, *S. oryzae* produced progeny that were significantly larger in grain uncontaminated with *A. flavus*, compared to progeny produced in microbially rich environments. My data suggests that microbial contamination modulates fitness and development.

Investigating post-harvest insect-microbe interactions and their ramifications for global food
quality, human health, and insect behavior

by

Marco A. Ponce

B.S., Kalamazoo College, 2019

A DISSERTATION

submitted in partial fulfillment of the requirements for the degree

DOCTOR OF PHILOSOPHY

Department of Entomology
College of Agriculture

KANSAS STATE UNIVERSITY
Manhattan, Kansas

2023

Approved by:

Co-Major Professor
Dr. Rob Morrison

Approved by:

Co-Major Professor
Dr. Tania N. Kim

Copyright

© Marco Ponce 2023.

Abstract

Microbes are ubiquitous and play important ecological roles. While research has largely focused on arthropods and microbes separately, less attention has been given to their interactions in the post-harvest supply chain. As a result, my aims focused on *S. oryzae* and *L. serricorne* and were to 1) elucidate the behavioral response of each species to microbially mediated volatile emissions in grain, 2) evaluate the vectoring capacity of both species for microbes across time, and 3) determine how foraging preferences affect development and fitness among habitats of different quality by each species. In elucidating the behavioral response of *S. oryzae* to microbial volatiles, two stages (sexual and asexual) of *A. flavus* were inoculated onto wheat and compared to uninoculated, or UV-sterilized wheat. Using headspace collection coupled with GC-MS, the sexual life stage of *A. flavus* had the most unique emissions of microbial volatiles. This translated to a higher interaction with kernels containing the *A. flavus* sexual life stage by *S. oryzae* in comparison to the asexual life stage. Grain volatiles were more important for attraction at longer distances. In another set of trials, wheat was inoculated with either *Fusarium verticillioides* or *A. flavus* and compared to uninoculated grain or UV-sterilized grain. Headspace collection revealed that the *Fusarium*- and *A. flavus*-inoculated grain produced significantly different volatiles compared to sanitized grain or the positive control, which translated to changes in close-range foraging by *L. serricorne*, with a greater frequency of entering and spending time *A. flavus*-inoculated kernels compared to other treatments. Microbial volatiles were found to be attractive to *L. serricorne* at longer distances in pitfall traps. Overall, this suggests that microbial volatiles are important for close- and long-range orientation of *L. serricorne*. To assess the vectoring capacity, both species originated from individuals placed immediately in a novel food patch, or given 24–72 h in a sterilized environment prior. Vectored

microbes were imaged after 3–5 d. Increasing dispersal time resulted in multiple-fold reductions in microbial growth by *L. serricorne*, but not *S. oryzae*. This was likely attributable to differences in baseline mobility by each species. In addition, we identified 14 taxa and nine genera. Over half of the samples were from *Aspergillus* spp. Our data suggests that there is a more persistent risk of microbial contamination by *S. oryzae* than by *L. serricorne*. In determining how foraging preferences affected fitness, comparisons between habitats inoculated with *A. flavus*, uncontaminated, or autoclaved in surrogate food patches after 3 d of foraging by *S. oryzae* and *L. serricorne* were performed. Insects were released into the dispersal apparatus with habitats located at opposite ends. Our study found that microbes modulated the preference and progeny production of *L. serricorne*, which dispersed to the uninfested habitats, while *S. oryzae* showed a preference to reproduce and dispersed to habitats inoculated with *A. flavus*. In addition, *S. oryzae* produced progeny that were significantly larger in grain uncontaminated with *A. flavus*, compared to progeny produced in microbially rich environments. My data suggests that microbial contamination modulates fitness and development.

Table of Contents

List of Figures	xii
List of Tables	xviii
Acknowledgements	xx
Dedication	xxii
Chapter 1 - A systematic review of the behavioral responses by stored-product arthropods to individual or blends of microbially-produced volatile cues	1
Abstract	2
Introduction.....	3
Materials and Methods.....	7
Results.....	8
<i>Behavioral response by stored-product arthropods to complex blends of MVOCs</i>	8
<i>Positive behavioral responses</i>	9
<i>Negative behavioral responses</i>	9
<i>Net neutral behavioral responses</i>	13
<i>Behavioral response by stored-product arthropods to individual or known mixtures of MVOCs</i>	13
<i>Positive behavioral responses</i>	14
<i>Negative behavioral responses</i>	15
<i>Net neutral behavioral responses</i>	18
<i>Response to known mixtures of MVOCs</i>	19
Discussion.....	19
<i>Response by stored-product arthropods to complex blends of MVOCs</i>	19
<i>Response by stored-product arthropods to individual or known mixtures of MVOCs</i>	21
<i>Stored-product arthropods as vectors and ecosystem engineers for mycoflora</i>	27
<i>Biases and limitations of reviewed studies</i>	28
<i>Future directions in understanding response by stored-product arthropods to MVOCs</i>	31
<i>Conclusions</i>	32
References.....	34

Chapter 2 - Grain inoculated with different growth stages of the fungus, <i>Aspergillus flavus</i> , affect the close-range foraging behavior by a primary stored product pest, <i>Sitophilus oryzae</i> (Coleoptera: Curculionidae)	48
Abstract	49
Introduction.....	50
Materials and Methods.....	54
<i>Experimental Insects and Source of Grain</i>	54
Fungal Isolation, Culturing, and Identification.....	54
<i>Treatments</i>	56
<i>Four-way still-air olfactometer</i>	57
<i>Ethovision</i>	58
<i>Small Release-Recapture Trapping Assay</i>	60
<i>Large Release-Recapture Trapping Assay</i>	61
<i>Headspace Volatile Collection</i>	62
<i>Gas chromatography coupled with mass spectrometry</i>	63
Results.....	64
<i>Four-way olfactometer assay</i>	64
<i>Ethovision Assay</i>	65
<i>Release-Recapture Assays</i>	66
<i>Headspace Assay</i>	66
Discussion.....	67
References.....	76
Chapter 3 - Attraction, mobility, and preference by <i>Lasioderma serricorne</i> (F.) (Coleoptera: Ptinidae) to microbially-mediated volatile emissions by two species of fungi in stored grain	96
Abstract.....	97
Introduction.....	98
Materials and Methods.....	101
<i>Source Insects</i>	101
<i>Fungal Morphotype Culturing</i>	102
<i>Treatments</i>	103

<i>Microbial Morphotype Sequencing</i>	104
<i>4-Way Olfactometer</i>	106
<i>Movement Assay</i>	106
<i>MVOC Headspace Collection</i>	108
<i>MVOC GC-MS Analysis</i>	109
<i>Statistical Analysis</i>	110
Results.....	112
<i>Fungal Morphotype Sequencing</i>	112
<i>Four-Way Olfactometer</i>	112
<i>Movement Assay Overall Measures</i>	113
<i>Movement Assay in Kernel Zones</i>	113
<i>Movement Assay in Arena Halves</i>	115
<i>Release-Recapture Assay</i>	116
<i>Headspace Volatiles Collected from Inoculated Grain</i>	117
Discussion.....	117
References.....	124
Chapter 4 - A Microbial vectoring capacity by an internal- and external-infesting stored product insect after varying dispersal periods between novel food patches: An underestimated risk	145
Abstract.....	146
Introduction.....	147
Materials and Methods.....	152
<i>Experimental Insects and Source of Grain</i>	152
<i>Vectoring Assay for Sitophilus oryzae and Lasioderma serricorne</i>	153
<i>Individuals from Microbially-Enriched Environments for Vectoring Assay</i>	155
<i>Field-Collected Individuals for Vectoring Assay</i>	156
<i>Microbial Morphotype Sequencing</i>	157
<i>Statistical Analysis</i>	158
Results.....	159
<i>Effects of Dispersal on Lasioderma serricorne and Sitophilus oryzae</i>	159
<i>Effects of Dispersal from a Microbially-Enriched Environment by Sitophilus oryzae</i>	160

<i>Effects of Population Origin on Vectoring Capacity</i>	161
<i>Effects of Species on Vectoring Capacity</i>	162
<i>Community Composition of Microbes through Sequencing</i>	162
Discussion	163
References	169
Chapter 5 - Beetles choosing habitats poorly: Foraging preferences of adult cigarette beetle and rice weevil do not match progeny production in novel food patches with and without microbes.....	186
Abstract	187
Introduction.....	188
Materials and Methods.....	192
<i>Experimental Insects and Source of Grain</i>	192
<i>Grain Treatment Preparation</i>	193
<i>Dispersal and Progeny Production Habitat Preference Assay</i>	195
<i>Progeny Morphometric</i>	196
<i>Statistical Analysis</i>	196
Results.....	197
<i>Dispersal Preference Assay</i>	197
<i>Progeny Production Preference Assay</i>	197
<i>Morphometric Analysis</i>	198
Discussion	198
References.....	202

List of Figures

Figure 1-1. Breakdown of the methodology (left) and variables (right) used in n = 140 tests documenting the behavioral response by stored-product arthropods to complex blends of microbial cues (top) and the n = 243 tests of individual MVOCs in the primary literature from 43 studies (bottom).....	10
Figure 1-1-2. Chemical structures of the most commonly tested MVOCs against stored-product arthropods, which collectively represent 60% of the n = 185 tests of individual components in the literature. Functional groups are highlighted in red, asterisks chiral centers.....	18
Figure 1-1-3. Hypothetical and documented behavioral responses of arthropods to MVOC dosages. Most common responses: changing, neutral-positive, neutral-negative (96% of cases, n = 66).....	24
Figure 2-1. Experimental 4-way still-air olfactometer assay: 10 g of each treatment in odor source chambers, single <i>Sitophilus oryzae</i> placed in central release chamber. 2-minute decision time, defined as walking 2.54 cm toward an arm.	88
Figure 2-2. Schematic of test arenas for video-tracking assay with insects placed in the center. Ethovision software used for tracking. Treatment areas (blue) and control areas (grey) illustrated.....	89
Figure 2-3. Percent of <i>S. oryzae</i> choosing different semiochemical treatments in olfactometer assay with porous glass barriers or holes. No significant differences among treatments observed.	90
Figure 2-4. Movement parameters of <i>S. oryzae</i> in Ethovision assay. Semiochemical treatments did not significantly affect velocity or distance moved.	91
Figure 2-5. Mean frequency of <i>S. oryzae</i> entering different kernel zones in Ethovision assay. <i>S. oryzae</i> entered M2 treatment zone significantly more compared to other treatments.	92
Figure 2-6. Cumulative duration of <i>S. oryzae</i> in treatment and control zones in Ethovision assay. M2 treatment resulted in significantly longer duration in kernel zone compared to other treatments.	93
Figure 2-7. Percent of <i>S. oryzae</i> recaptured in release-recapture assay using pitfall traps. Grain traps had significantly higher captures compared to negative control, but MVOCs did not affect capture.....	94

Figure 2-8. Non-metric multidimensional scaling ordination of headspace samples based on compound emissions. Treatments showed significant differences from each other.....	95
Figure 3-1. Habitus images of morphotype 1 (left, <i>Aspergillus flavus</i> -inoculated grain) and morphotype 2 (right, <i>Fusarium verticillioides</i> -inoculated grain) at 10× magnification (SMZ-18, Nikon Inc., Tokyo, Japan) on grain after inoculation procedure.	136
Figure 3-2. 3D Habitus images of <i>Aspergillus flavus</i> at 10× magnification (SMZ-18, Nikon Inc., Tokyo, Japan) used to inoculate grain in assays. <i>A. flavus</i> is characterized by its green, filamentous morphology and visible spores.	137
Figure 3-3. Preference shown in the four-way, still-air olfactometer using the mean percentage of <i>L. serricorne</i> adults choosing a stimulus ($\pm$ SE) after given a 4-min opportunity (top panel) and their mean time to decision ($\pm$ SE, s; bottom panel) based on the semiochemical treatment to which they were exposed in the laboratory at the USDA-ARS Center for Grain and Animal Health Research in Manhattan, KS. A total of $n = 200$ adults replicate were tested in this assay. Bars with shared letters are not significantly different from each other (Tukey HSD, $\alpha = 0.05$). Abbreviations: Neg. Ctrl – negative control; UV-grain – UV-sanitized grain; P. Ctrl – uninoculated grain; FS-grain – <i>Fusarium verticillioides</i> -inoculated grain; AF-grain – <i>A. flavus</i> -inoculated grain.	138
Figure 3-4. Metrics from the movement assay assessing the effects of MVOCs on mean distance moved ($\pm$ SE, top panel) and the mean instantaneous velocity ($\pm$ SE, bottom panel) by adult <i>L. serricorne</i> over a period of 30 min using a network camera coupled with Ethovision in the laboratory at the USDA-ARS Center for Grain and Animal Health Research in Manhattan, KS. There were a total of $n = 20$ replicates per treatment, translating to 50 hours of data in this assay. Bars with shared letters are not significantly different from each other (Tukey HSD, $\alpha = 0.05$). Abbreviations: Neg. Ctrl – negative control; UV-grain – UV-sanitized grain; P. Ctrl – uninoculated grain; FS-grain – <i>Fusarium verticillioides</i> -inoculated grain; AF-grain – <i>A. flavus</i> -inoculated grain.	139
Figure 3-5. MVOC effects on adult <i>L. serricorne</i> behavior in kernel zones. Treatment (dark orange bars) and control (dark yellow bars) metrics from movement assay. Frequency of entering each kernel zone (top), duration spent in each zone (middle), and latency to finding each zone (bottom). Conducted at USDA-ARS Center for Grain and Animal Health Research. $n = 20$ adults per treatment, totaling 50 hours of data. Statistical comparisons:	

Tukey HSD ($\alpha = 0.05$) and paired t-tests (Bonferroni correction). Abbreviations: Neg. Ctrl – negative control; UV-grain – UV-sanitized grain; P. Ctrl – uninoculated grain; FS-grain – *Fusarium verticilloides*-inoculated grain; AF-grain – *A. flavus*-inoculated grain..... 140

Figure 3-6. Treatment and control zone metrics for adult *L. serricorne* in a movement assay using video-tracking. Effects of MVOCs on zone frequency, cumulative duration, and latency. USDA-ARS Center for Grain and Animal Health Research, Manhattan, KS. n=20 adults per treatment, 30 min duration. Significant differences indicated by letters. Abbreviations: Neg. Ctrl – negative control; UV-grain – UV-sanitized grain; P. Ctrl – uninoculated grain; FS-grain – *Fusarium verticilloides*-inoculated grain; AF-grain – *A. flavus*-inoculated grain..... 141

Figure 3-7. The mean percentage of adult *L. serricorne* recaptured in the release-recapture assay in response to MVOCs and grain used as lures in commercially-available monitoring pitfall traps, with a total of 20 adult, mixed-sex *L. serricorne* released in large plastic bins and placed in a large, walk-in environmental chamber under constant abiotic conditions and given 24 h to respond at the USDA-ARS Center for Grain and Animal Health Research in Manhattan, KS for a total of n = 8 replicate releases per treatment. Bars with shared letters are not significantly different from each other (Tukey HSD, $\alpha = 0.05$). 142

Figure 3-8. Non-metric multi-dimensional scaling ordination plot based on pairwise Bray-Curtis similarity index calculated between each combination of volatile emissions (ng h⁻¹ g⁻¹ grain) by samples after headspace collection for 3 h from 20 g of grain that was UV sanitized for 10 min (blue circles), inoculated with *A. flavus* (yellow circles), inoculated with *Fusarium* spp (red circles), or uninoculated (brown circles). There were n = 7 replicates per semiochemical treatment. Polygons represent convex hulls drawn between samples and are color coded similarly to treatments. There were a total of n = 1000 permutations, and stress was < 0.10, enabling adequate interpretation. Abbreviations: UV-grain – UV-sanitized grain; P. Ctrl – uninoculated grain; FS-grain – *Fusarium verticilloides*-inoculated grain; AF-grain – *A. flavus*-inoculated grain..... 143

Figure 3-9. Mean emissions rate from the semiochemical treatments (in ng h⁻¹ g⁻¹ grain) during headspace collection in 500-mL capacity containers after adsorption to Porapak-Q volatile collection trap, then elution with 150 μ l dichloromethane. Bars with shared letters are not significantly different from each other (Tukey HSD, $\alpha = 0.05$). Abbreviations: UV-grain –

UV-sanitized grain; P. Ctrl – uninoculated grain; FS-grain – *Fusarium verticilloides*-inoculated grain; AF-grain – *A. flavus*-inoculated grain..... 144

Figure 4-1. Habitus images of factitious foraging patches from the vectoring assay showing that microbes are transmitted physically by *S. oryzae* (left orange and green boxes) and *L. serricorne*, (right red box) but at decreasing frequency and diversity with increasing dispersal periods (0 h, 24 h, 72 h) prior to entering a foraging patch. *Sitophilus oryzae* originally from laboratory colonies (orange box) or *Aspergillus flavus*-inoculated grain (e.g., microbially-enriched environments; green box). The longer that *S. oryzae* and *L. serricorne* were in foraging patches (3 vs. 5 d), the greater the microbial growth under constant conditions (30°C, 60% RH, and 14:10 L:D photoperiod). Pictures are representative images taken from a total of n = 30 replicates for each dispersal period and foraging time in a food patch. 180

Figure 4-2. Mean grayscale value (e.g., surrogate measure of microbial growth) for microbes vectored by *L. serricorne* (top panel) and *S. oryzae* (bottom panel) adults from the colony population after they were given a dispersal period of 0, 24, or 72 h and then placed in factitious foraging patches for 3 d (green bars) or 5 d (yellow bars), which consisted of potato dextrose agar dishes that were photographed using a 3D imaging system and processed with ImageJ. There were a total of n = 30 replicate adults tested per bar. Bars with shared letters are not significantly different from each other (Tukey HSD, $\alpha = 0.05$). The grayscale value can range from 0, indicating no microbial growth, to 255, indicating the full agar dish was covered with microbes. 181

Figure 4-3. Mean species richness (e.g., surrogate measure of microbial diversity) for microbes vectored by *L. serricorne* (top panel) and *S. oryzae* (bottom panel) adults from the colony population after they were given a dispersal period of 0, 24, or 72 h and then placed in factitious foraging patches for 3 d (green bars) or 5 d (yellow bars), which consisted of potato dextrose agar dishes that were photographed using a 3D imaging system and processed with ImageJ. There were a total of n = 30 replicate adults tested per bar. Bars with shared letters are not significantly different from each other (Tukey HSD, $\alpha = 0.05$). Species richness represents the number of microbial morphotypes in each food patch. 182

Figure 4-4. Mean grayscale value (e.g., surrogate measure of microbial growth; top panel) or mean species richness (e.g., surrogate measure of microbial diversity; bottom panel) for

microbes vectored by *S. oryzae* from *A. flavus*-inoculated grain after they were given a dispersal period of 0, 24, or 72 h and then placed in factitious foraging patches for 3 d (green bars) or 5 d (yellow bars), which consisted of potato dextrose agar dishes that were photographed using a 3D imaging system and processed with ImageJ. There were a total of $n = 30$ replicate adults tested per bar. Bars with shared letters are not significantly different from each other (Tukey HSD, $\alpha = 0.05$). The grayscale value can range from 0, indicating no microbial growth, to 255, indicating the full agar dish was covered with microbes.

Species richness represents the number of microbial morphotypes in each food patch. 183

Figure 4-5. Mean grayscale value (e.g., surrogate measure of microbial growth; top panel) or mean species richness (e.g., surrogate measure of microbial diversity; bottom panel) for microbes vectored by *S. oryzae*, depending on population source (from *A. flavus*-inoculated grain, AF; laboratory colonies, Colony; or field-captured adults, Field) after they were given a dispersal period of 0, 24, or 72 h and then placed in factitious foraging patches for 3 d (green bars) or 5 d (yellow bars), which consisted of potato dextrose agar dishes that were photographed using a 3D imaging system and processed with ImageJ. There were a total of $n = 30$ replicate adults tested per bar. Bars with shared letters are not significantly different from each other (Tukey HSD, $\alpha = 0.05$). The grayscale value can range from 0, indicating no microbial growth, to 255, indicating the full agar dish was covered with microbes.

Species richness represents the number of microbial morphotypes in each food patch. 184

Figure 4-6. Community composition of microbes derived via sequencing the ITS4/5 ribosomal subunit from environmental fungi vectored and dispersed on PDA dishes by *S. oryzae* and *L. serricorne*. Percentages displayed for each taxa portrayed in circle. *Aspergillus* spp.... 185

Figure 5-1. Dispersal apparatus representation..... 210

Figure 5-2. The mean number of *Lasioderma serricorne* adults dispersing to a novel food resource after 72 h in environmental chambers at 30°C and 65% RH in pairwise comparisons among food patches with 20 g of *Aspergillus flavus*-inoculated (AF), unmanipulated wheat (Ctrl, as positive control), or autoclaved and surface sterilized wheat (UV). Each bar represents the mean of 10 replicates, with 30 adults released in each replicate. for treatment combination. Abbreviations: ns – not significant (with a Bonferroni-corrected α -threshold using a t-test), ** - $0.01 < P < 0.001$, *** - $P < 0.0001$ 211

Figure 5-3. The mean number of *Sitophilus oryzae* adults dispersing to a novel food resource after 72 h in environmental chambers at 30°C and 65% RH in pairwise comparisons among food patches with 20 g of *Aspergillus flavus*-inoculated (AF), unmanipulated wheat (Ctr, as positive control), or autoclaved and surface sterilized wheat (UV). Each bar represents the mean of 10 replicates, with 30 adults released in each replicate. for treatment combination. Abbreviations: ns – not significant (with a Bonferroni-corrected α -threshold using a t-test), ** - $0.01 < P < 0.001$, *** - $P < 0.0001$ 212

Figure 5-4. The mean progeny production by *Lasioderma serricorne* adults that dispersed to each novel food patch after 60 d of incubation at 30°C and 65% RH. The novel food patches consisted of 20 g of grain inoculated with *Aspergillus flavus*—(AF), unmanipulated wheat (Ctrl –as positive control), or autoclaved and surface sterilized wheat (UV). Each bar represents the mean of 10 replicates, with 30 adults released in each replicate. for treatment combination. Abbreviations: ns – not significant (with a Bonferroni-corrected α -threshold using a t-test), ** - $0.01 < P < 0.001$, *** - $P < 0.0001$ 213

Figure 5-5. The mean progeny production by *Sitophilus oryzae* adults that dispersed to each novel food patch after 60 d of incubation at 30°C and 65% RH. The novel food patches consisted of 20 g of grain inoculated with *Aspergillus flavus*—(AF), unmanipulated wheat (Ctrl –as positive control), or autoclaved and surface sterilized wheat (UV). Each bar represents the mean of 10 replicates, with 30 adults released in each replicate. for treatment combination. Abbreviations: ns – not significant (with a Bonferroni-corrected α -threshold using a t-test), ** - $0.01 < P < 0.001$, *** - $P < 0.0001$ 214

Figure 5-6. Mean body length ($\pm$ SE, mm) of progeny produced after 60 d by *Sitophilus oryzae* that dispersed to habitats inoculated with *Aspergillus flavus* (AF), and food patches with unmanipulated wheat (Ctrl) after an initial 72 h period at 30C and 65% RH in an environmental chamber. Bars represent the means of 200 randomly selected adults from each habitat, and bars with shared letters are not significantly different from each other (t-test, $\alpha = 0.05$). A habitus image above bar shows size difference between adult *S. oryzae* in a side-by-side comparison. 215

List of Tables

Table 1-1. Summary of the percent documented occurrences in the primary literature of attraction (+), repellency (–), or no statistical difference from included controls (○) by each of the following stored-product arthropod species among complex blends of microbial cues.	11
Table 1-2. Summary of the percent of documented occurrences in the primary literature of attraction (+), repellency (–), or no statistical difference from included controls (○) across 16 stored-product arthropods to each of the following complex blends of microbial cues. .	12
Table 1-3. Summary of the percent of documented occurrences in the primary literature of attraction (+), repellency (–), or no statistical difference from included controls (○) by each of the following stored-product arthropod species among individual MVOCs.....	14
Table 1-4. The behavioral response among 19 stored-product arthropod species from the primary literature to the following specific MVOCs, including documented attraction (+), repellency (–), or no statistical difference from included controls (○).....	16
Table 1-5. Non-exhaustive and non-mutually exclusive hypotheses for the role of MVOCs in driving the behavior of stored-product arthropods described from the primary literature and in the current review.	25
Table 2-1. List of semiochemical treatments and their amounts used in each assay during experiments at the USDA-ARS Center for Grain and Animal Health Research in Manhattan, KS from Jun-Sep 2020.....	85
Table 2-2. Mean emission rates (ng · g ⁻¹ · h ⁻¹) and percent contribution to total emissions of identified compounds in headspace from different grain treatments. Fungal-inoculated grain showed significantly higher and more complex emissions compared to UV-sterilized or untreated grain (n=7 replicates per treatment).	86
Table 3-1. List of semiochemical treatments and the amounts used in each assay during the study.....	131
Table 3-2. Summary of headspace volatiles emitted (in ng h ⁻¹ g ⁻¹ grain) from grain that was uninoculated (P. ctrl), surface sanitized with UV-radiation for 10-min (UV-grain), or inoculated with <i>A. flavus</i> or <i>Fusarium</i> spp. for periods of 3 hours using a push headspace	

collection systems. There were n=7 replicates per treatment and mean emission rates per compound are portrayed.	132
Table 4-1. Summary of ANOVA models for mean grayscale value (microbial growth) and microbial diversity (e.g., species richness) after the introduction of <i>L. serricorne</i> or <i>S. oryzae</i> on PDA agar dishes after 0, 24, or 72 h dispersal from laboratory colonies.....	175
Table 4-2. Summary of ANOVA models for mean grayscale value (microbial growth) and microbial diversity (e.g., species richness) after the introduction of <i>S. oryzae</i> on PDA agar dishes after 0, 24, or 72 h dispersal from grain inoculated with <i>A. flavus</i>	176
Table 4-3. Summary of ANOVA models for mean grayscale value (microbial growth) and microbial diversity (e.g., species richness) after the introduction of <i>S. oryzae</i> from different populations (colony, <i>A. flavus</i> -inoculated grain, and field) on PDA agar dishes after a 0, 24, or 72 h dispersal period.	177
Table 4-4.. Summary of microbial identifications from each species via sequencing the ribosomal ITS4/5 subunit.....	178
Table 5-1. Summary of t-tests with Bonferroni correction for number of <i>Lasioderma serricorne</i> and <i>Sitophilus oryzae</i> dispersing to new habitats.	208
Table 5-2. Summary of t-tests with Bonferroni correction for progeny production by <i>Lasioderma serricorne</i> and <i>Sitophilus oryzae</i> dispersing in new habitats.....	209

Acknowledgements

I would like to express my deepest gratitude to all those who have supported and guided me throughout the journey of completing this dissertation. In particular, I am immensely grateful to those who have encouraged me to never give up, even when circumstances are not in favor of succeeding. And just because I love my native language; nunca se me olvida, el famoso dicho del “**Sí se puede!**” First and foremost, I am profoundly grateful to my advisors, Dr. Rob Morrison and Dr. Tania Kim for their unwavering support, invaluable guidance, and intuitive feedback. Their expertise and encouragement have been instrumental in shaping this research and my academic growth.

I would like to extend my sincere appreciation to the members of my dissertation committee, Dr. Thomas Phillips, Dr. Erin Scully, Dr. Yoonseong Park, and Dr Christos Athanassiou. Their expertise from different aspects of research, valuable input, and constructive criticism have significantly enriched this study. I am thankful to the faculty and staff from the Department of Entomology at Kansas State University for fostering a conducive academic environment. Their dedication to excellence in education and research has provided me with numerous opportunities for intellectual and personal development.

I want to take a moment to acknowledge those who helped me during my earlier career as a high school/undergraduate student. Without your constant support, I would not have been able to reach and accomplish my academic goals. A special thank you goes to Pablo Roncoroni and Cynthia Rettenmund, who have believed in me since day one. I truly admire your kindness and dedication to making your students succeed, no matter the circumstances. I also want to thank those faculty professors that I met during my undergraduate years at Kalamazoo College, specially to Natalia Calvalho-Pinto, Dr. Francisco Villegas, Dr. Jeffry Bartz, Dr. Binney Girdler,

Dr. Santiago Salinas, Karen Berthel, and to Dr. Ann Fraser, the one that introduce me to the wonderful field of Entomology.

A pesar de la distancia que nos separa, estoy en deuda con mi familia por su amor incondicional, aliento constante y creencia inquebrantable en mis habilidades. Su apoyo ha sido el pilar de fuerza que me ha impulsado hacia adelante durante los momentos difíciles. Despite the distance that separates us, I am indebted to my family for their unconditional love, constant encouragement, and unwavering belief in my abilities. Their support has been the pillar of strength that propelled me forward during challenging times.

My heartfelt appreciation goes to my friends who became part of my family; Alejandro Garnica and his family, Jose Covarrubias and his family, Adan Cernas, Jose Lopez, Guillermo Lomeli, Sally Lopez, Hannah Quellhorst, Andres Holguin, Sabita Ranabhat, Rachel Wilkins, Jackie Maille, Yvonne Sarmiento, Dora Gallo and her family, Andrea Salazar, Daniela Gonzalez, Alexis Susunaga, Miguel Salazar, Claudio Salazar, and all my colleagues who have supported me throughout this journey. Their companionship, nerdy or funny discussions, quality of time, and shared experiences have made this endeavor more enjoyable and memorable.

In conclusion, I am grateful for the collective support, guidance, and encouragement that I have received throughout this research endeavor. This dissertation would not have been possible without the invaluable contributions of all those mentioned above. Gracias!

Dedication

This dissertation is dedicated to all first-generation students who have braved unfamiliar paths and overcome adversity in pursuit of knowledge. **Your courage and resilience inspire us.** You have shattered barriers, defied expectations, and shown the transformative power of education. May this work stand as a tribute to your journeys, struggles, and triumphs. You are pioneers, lighting the way for future generations. In honor of your strength and determination, this dissertation is dedicated to all first-generation students, who embody the spirit of possibility and the pursuit of a brighter future.

Esta tesis está dedicada a todos los estudiantes de primera generación que han enfrentado caminos desconocidos y aun así superado la adversidad en busca del conocimiento. **Su valentía y resiliencia nos inspiran.** Han roto barreras, desafiado expectativas y demostrado el poder transformador de la educación. Que este trabajo sea un tributo a sus viajes, luchas y triunfos. Son pioneros, iluminando el camino para las generaciones futuras. En honor a su fortaleza y determinación, esta tesis está dedicada a todos los estudiantes de primera generación, quienes encarnan el espíritu de posibilidad y la búsqueda de un futuro más brillante.

Chapter 1 - A systematic review of the behavioral responses by stored-product arthropods to individual or blends of microbially- produced volatile cues

Published as: Ponce, M. A., Kim, T. N., & Morrison III, W. R. (2021). A Systematic Review of the Behavioral Responses by Stored-Product Arthropods to Individual or Blends of Microbially Produced Volatile Cues. *Insects*, 12(5), 391.

<https://doi.org/10.3390/insects12050391>

Abstract

Microbes are ubiquitous and play important ecological roles in a variety of habitats. While research has been largely focused on arthropods and microbes separately in the post-harvest supply chain, less attention has been paid to their interactions with each other. Up to this point, there has been no attempt to systematically describe the patterns of behavioral responses by stored-product insects to microbially-produced volatile organic compounds (MVOCs). Thus, our aims were to evaluate whether stored-product arthropods were primarily and significantly attracted, repelled, or had a net neutral effect (e.g., unaffected or mixed) by MVOCs presented as 1) complex headspace blends, or 2) single constituents and known mixtures. In total, we found 43 articles that contained 384 sets of tests with different combinations of methodology and/or qualitative findings, describing the behavioral responses of 24 stored-product arthropod species from two classes, four orders, and 14 families to 58 individual microbial compounds and the complex headspace blends from at least 78 microbial taxa. A total of five and four stored-product arthropod species were significantly attracted and repelled by MVOCs across odor sources, respectively, while 13 were unaffected or exhibited mixed effects. We summarize the biases in the literature, including that the majority of tests have occurred in the laboratory with a limited subset of methodology and has largely only assessed the preference of adult arthropods. Finally, we identify foundational hypotheses for the roles that MVOCs play for stored-product arthropods, identify gaps in research and future directions, while highlighting that the behavioral responses to MVOCs are complex, context-, and taxon-dependent, which warrants further investigation.

Keywords: postharvest; chemical ecology; attract-and-kill; semiochemicals; microbially-produced volatile organic compounds; integrated pest management; stored products; mycotoxins, fungi

Introduction

Stored products are essential for the global economy and feeding the planet's growing population [1]. Each year, the US stores over 16 billion bushels ($\approx 407,264,000$ MT) of corn, soybean, and wheat alone [2]. The retail value for just wheat flour in the US is \$40 billion [3]. Together, arthropods and microbes represent the two most serious threats to the success of the post-harvest supply chain in delivering nutritious, safe, and sustainable food and food products to consumers and other end users. Globally, losses from both insects and microbes exceed \$100 billion annually [4]. While arthropods contribute to governmentally-regulated tolerances on insect-damaged kernels and insect fragments, they also result in commodity damage, including contributing to weight loss or downgrades in quality [5]. The presence and spread of microbes in the post-harvest environment is equally undesirable, with potential public health implications [6], and including "off-odor" grain [7], and other deleterious effects on grain quality [8].

Microbes are ubiquitous and play important ecological roles in a variety of habitats. In most environments, microbes are found near the base of the trophic system, and are responsible for recycling nutrients through a vast "brown web" of decomposers that includes fungi and bacteria [9]. In post-harvest environments, deterioration of protected commodities that represent a large investment of resources is obviously not desirable. As a result, food facilities adopt a variety of sanitation and drying protocols to preserve the quality of commodities [10]. The moisture in food facilities is generally kept very low, with grain moisture for wheat hovering at about 10% or less to suppress the growth and spread of microbes in the post-harvest

environment. However, even when appropriate safeguards are maintained, there may still be issues with “musty” or “off-odor” grain, which has become an increasing complaint by industry. There have been attempts in recent years to classify some these sets of volatiles as biomarkers of commodity quality through a variety of methods [11].

In characterizing the microbial community of grain, community composition is typically a complex assembly of species and is highly variable over time. For example, in warehouse-stored coffee beans, *Saccharomycetales* sp., *Meyerozyma guilliermondii*, and *Pichia kluyveri* dominated in the first six months, but then was dominated by *Wallemia* sp. thereafter [12]. However, the major genera of stored product pathogens include *Alternaria*, *Aspergillus*, *Fusarium*, and *Penicillium* spp., which collectively produced at least 18 different mycotoxins hazardous to human health [13], even in trace amounts [14].

While research has been largely focused on arthropods and microbes separately in the post-harvest supply chain, less attention has been paid to their interactions with each other. However, there is an increasing recognition that insects and microbes jointly contribute to grain quality issues. For example, Ponce et al. [15] found that the presence of microbes increased the fitness of the rice weevil, *Sitophilus oryzae* (L.) (Coleoptera: Curculionidae) at high temperatures, while the presence of both types of pests modified abiotic conditions in ways that were mutually favorable. When the related *Sitophilus zeamais* (Motschulsky) (Coleoptera: Curculionidae) was reared in the presence of the mycotoxin fumonisin B, there was higher progeny production and grain damage than when the mycotoxin was absent [16]. The ham mite, *Tyrophagus putrescentiae* (Schrank) (Acari: Acaridae), was documented vectoring eight fungal species, including *Aspergillus clavatus* and *Penicillium citrinum*, among others [17]. A total of 7–9 stored-product insect taxa in maize stores and in the field were found to have spores

belonging to *Aspergillus*, *Fusarium*, *Penicillium* spp., and other fungal taxa on their cuticles [18]. Thus, it appears that stored-product arthropods and microbes may have reciprocal interactions in post-harvest environments.

In addition to their effects on insect population growth and grain quality, microbes may also play important ecological roles by mediating behavioral interactions. For example, microbes are an important source of behaviorally-active semiochemicals for insects (reviewed in [19]). Microbial cues may support long-distance habitat-finding, short-range host-finding, as well as support mate-location and reproduction. For example, the microbially-produced organic compound (e.g. MVOCs, hereafter), 1-octen-3-ol, synergized attraction to traps with conspecific aggregation pheromone for *Cryptolestes ferrugineus* Stephens (Coleoptera: Laemophloeidae) [20]. Prior work has demonstrated that the red flour beetle, *Tribolium castaneum* (Herbst) (Coleoptera: Tenebrionidae), used fungi associated with cotton seeds for short-range resource location [21].

Evolutionarily, modern-day stored-product arthropods are descended from species that historically used animal caches of seeds (e.g. [22]) and/or already stressed woody species as food sources (e.g. [23]). It is possible that in the damp environments harboring animal seed caches (e.g. in the ground), fungal cues may have been an indicator of presence of food to stored-product arthropods. Others have even hypothesized that this behavior may result in a genetically conserved response to fungal volatiles by stored-product arthropods [24]. In addition, secondary stored-product arthropods are only able to access nutrients and develop on commodities where the bran has broken or is no longer intact, suggesting light colonization by fungi may actually allow secondary pests a chance to take advantage of intact kernels that would otherwise be unusable. These life history constraints results in an additional ecological and evolutionary

rationale for attraction of secondary stored-product arthropods to fungal volatiles. Indeed, the red flour beetle, *Tribolium castaneum* (Herbst) (Coleoptera: Tenebrionidae) was found to be attracted to cultures of *Aspergillus niger* and many other fungi [25], suggesting some support for this rationale.

Along with confirmatory studies on the importance of MVOCs to the attraction and orientation by stored-product arthropods, there are published examples suggesting that MVOCs may also have roles as repellents or as neutral stimuli. For instance, though Ahmad et al. [21] found attraction to fungi associated with cotton seeds by *T. castaneum*, Van Winkle et al. [8] found no support for the use of fungal volatiles associated with wheat by the same species. Further, others found 1,315 species oriented to traps baited in agricultural landscapes with the yeast, *Aureobasidium pullulans*, but very few oriented to traps with *Penicillium expansum* [26], suggesting that the use of MVOCs is not haphazard by taxa. Moreover, work has found that some MVOCs may act as repellents and thereby disrupt orientation of the granary weevil, *Sitophilus granarius* (L.) (Coleoptera: Curculionidae), to its food source [27]. Each of these suggest that the behavioral response by insects to MVOCs may not be universal to all microbial species and compounds. However, up to this point, there has been no attempt to systematically describe the patterns of behavioral response by stored-product arthropods to MVOCs. Thus, our aims in this study are to evaluate whether stored-product arthropods are primarily attracted, repelled, or unaffected by MVOCs presented as 1) complex headspace blends or 2) single constituents or known component mixtures. We hypothesized that MVOCs may be more important to secondary than primary stored-product arthropods, because they may signal a usable resource to the former but not the latter. In addition, we identify other potential hypotheses for

observed patterns, as well as gaps and biases in the literature elucidating MVOC-mediated behavioral changes in stored-product arthropods.

Materials and Methods

A systematic search of the literature using Google Scholar, (<https://scholar.google.com/>) and Web of Science was used to identify studies that examined the effects of individual compounds or mixtures of MVOCs on the behavioral responses of stored-product arthropods. Stored-product arthropods were defined as those insects and arachnids attacking stored, durable commodities in the post-harvest supply chain at any of the successive links, including storage, transportation, processing, and marketing. Where applicable, we parsed studies into component experiments where behavioral responses or other factors such as type of assays or measured variables may have differed (e.g. dosage, compound, etc.). We classified each test as resulting in statistically significant attraction (+), repellence (−), or neither (○) compared to a negative or positive control. We excluded any studies lacking appropriate negative or positive controls, lacking replication, or lacking sufficient details on the identity of tested substrates to enable appropriate interpretation. Terms used to search databases included the following singly and/or in combination: “fungal”, “volatiles”, “stored products”, “insect behavior”, “insect-microbe”, “interactions”, “semiochemicals”, “mycotoxin”, “behavioral response”, “attraction”, and “postharvest”, and combinations thereof. In addition, we kept track of methodology used for tests, response variables, target insect, insect stage, and microbial taxon. We split our analysis up between tests with complex (but usually uncharacterized) blends of MVOCs, and those with known individual or known component mixtures of MVOCs. We summarized responses by arthropod species, as well as by microbial cue in order to understand generalizability of pattern across other categories, and performed a χ^2 -test in instances with at least three documented tests

in the literature to determine whether there were deviations from the null hypothesis of equal frequency of attraction (+), repellency (-), or net neutral responses (○) to MVOCs. Finally, we performed post-hoc comparisons to determine whether there were any behavioral differences between the frequency of stored-product arthropods attracted, repelled, or with net neutral responses to individual MVOCs compared to known mixtures of MVOCs. We used R Software for all statistics [28], with $\alpha = 0.05$. Our search of the literature identified 134 articles that addressed the broad topic of insect-microbe interactions, and 43 articles addressing specifically the behavioral response of stored-product arthropods to MVOCs.

Results

In total, we found 43 articles that contained 384 sets of tests with different combinations of methodology and/or qualitative findings (Table S1, Table S2). Overall, the behavioral response of 24 stored-product arthropod species from two classes, four orders, and 14 families to 58 individual microbial compounds and the complex headspace blends from at least 78 microbial taxa was assessed. While studies may have indirectly tested the microbial cues from bacteria in complex headspaces along with fungal cues, no study to date has specifically evaluated the behavioral responses of stored-product arthropods to bacteria alone.

Behavioral response by stored-product arthropods to complex blends of MVOCs

In total, there were 140 tests documenting the behavioral response of 16 arthropod taxa to an average of 5 ± 1.6 (mean $\pm$ SE) complex blends of microbial cues per taxon (Table S1). The behavioral responses of stored-product arthropods were assessed to complex blends of MVOCs through the combined use of 2-way olfactometers, 4-way olfactometers, and petri dish assays in 75% of cases (Figure 1, top-left panel). A total of 91% of the behavioral responses were

evaluated in the laboratory. In characterizing behavior, 71% of tests used preference as the primary response variable (Figure 1, top-right panel).

Positive behavioral responses

There have been a variety of studies documenting attraction by various combinations of stored-product arthropods to complex blends of microbial cues (Table S1). Overall, complex blends of microbial cues resulted in significant attraction or repellency relative to no response by arthropod taxa ($\chi^2 = 5.89$; $df = 2$; $P < 0.05$), with 41% of tests documenting attraction and 34% documenting repellency. In particular, there was significantly more documented attraction across complex blends of microbial cues by three stored-product insect species than expected *a priori*, including by *Carpophilus hemipterus* (Coleoptera: Nitidulidae), *Cryptolestes ferrugineus* (Coleoptera: Laemophloeidae), and *Plodia interpunctella* (Hübner) (Lepidoptera: Pyralidae) (Table 1). Significantly more attraction across stored-product arthropod taxa was exhibited to complex blends of microbial cues emitted from *Alternaria* spp. and *Saccharomyces cerevisiae* (Table 2), both of which are fungi.

Negative behavioral responses

There was also a subset of stored-product arthropods that were consistently and significantly repelled by complex microbial cues. A biological control agent, *Lariophagus distinguendus* (Förster) (Hymenoptera: Pteromalidae), and a stored product booklouse, *Liposcelis bostrychophila* Badonnel (Psocoptera: Liposcelididae) were both significantly and consistently repelled by MVOCs (Table 1). Odor sources tested against these species included biodegrading paper, *Eurotium amstelodami* and *Ulocladium botrytis* for the book louse, and two species of *Aspergillus* sp. for the biocontrol agent. Although it was not significantly repelled across all complex MVOCs, the ham mite, *Tyrophagus putrescentiae* (Acari: Acaridae) was

repelled by most species of *Aspergillus* sp. (Table S1). By contrast, *Tribolium confusum* du Val (Coleoptera: Tenebrionidae) was both repelled and attracted to many species of *Penicillium*. Because the behavioral responses of stored-product arthropods were often context-dependent, microbial taxon-dependent, and associated with experimental conditions, there were no consistently repellent MVOCs across stored-product arthropod taxa (Table 2).

Figure 1-1. Breakdown of the methodology (left) and variables (right) used in n = 140 tests documenting the behavioral response by stored-product arthropods to complex blends of microbial cues (top) and the n = 243 tests of individual MVOCs in the primary literature from 43 studies (bottom).

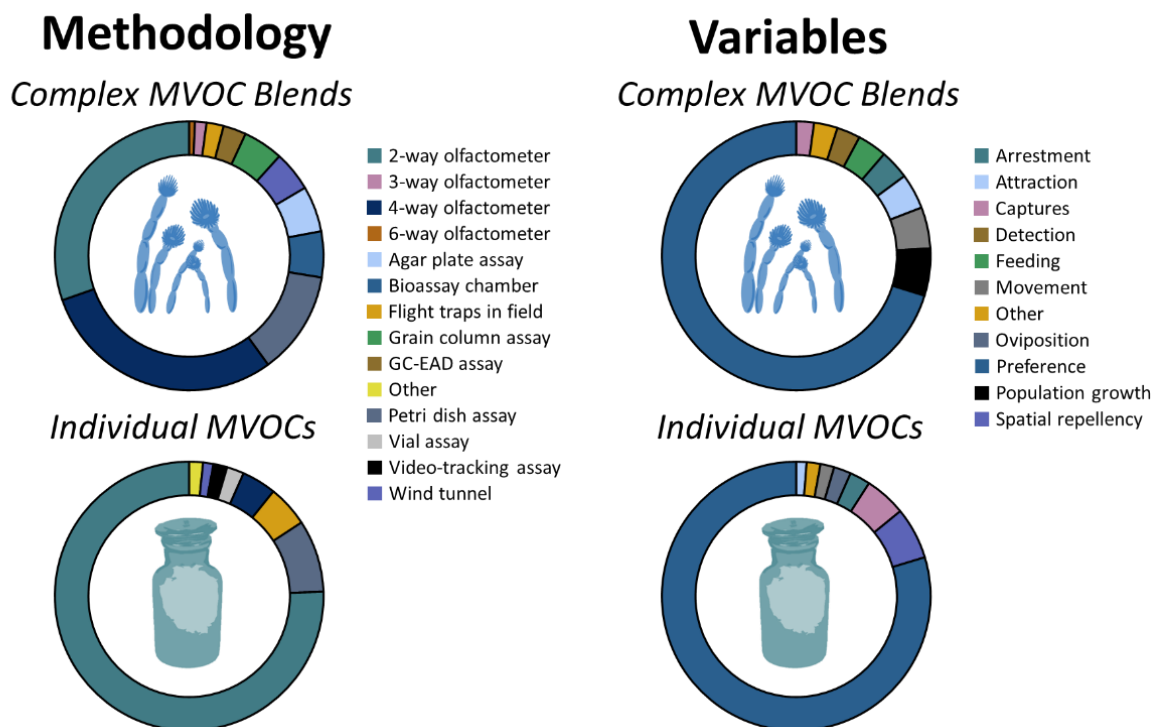


Table 1-1. Summary of the percent documented occurrences in the primary literature of attraction (+), repellency (–), or no statistical difference from included controls (○) by each of the following stored-product arthropod species among complex blends of microbial cues.

Arthropod Taxon	Behavioral Response			χ^2	P	N	Type of Pest ¹
	–	○	+				
<i>Acanthoscelides obtectus</i>	25	0	75	3.5	0.17	4	1°
<i>Carpophilus hemipterus</i>	0	0	100	10.0	0.01	5	2°
<i>Carpophilus humeralis</i>	0	0	100	-	-	1	2°
<i>Cryptolestes ferrugineus</i>	0	14	86	8.9	0.01	7	2°
<i>Cynaesus angustus</i>	0	0	100	-	-	1	2°
<i>Lariophagus distinguendus</i>	100	0	0	10.0	0.01	5	wasp
<i>Liposcelis bostrychophila</i>	75	25	0	7.0	0.03	8	2°
<i>Oryzaephilus mercator</i>	0	0	100	-	-	2	2°
<i>Oryzaephilus surinamensis</i>	50	0	50	-	-	2	2°
<i>Plodia interpunctella</i>	0	0	100	8.0	0.02	4	2°
<i>Sitophilus zeamais</i>	80	0	20	5.2	0.07	5	1°
<i>Tenebrio molitor</i>	43	0	57	3.7	0.16	7	2°
<i>Tribolium castaneum</i>	14	55	31	10.4	0.01	42	2°
<i>Tribolium confusum</i>	46	21	33	3.8	0.15	39	2°
<i>Typhaea stercorea</i>	0	0	100	-	-	1	2°
<i>Tyrophagus putrescentiae</i>	50	0	50	4.0	0.14	8	2°

Note: arthropods showing a sig. behavioral response bolded & italicized

¹ Abbreviations: 1° – primary pest, 2° – secondary pest.

Table 1-2. Summary of the percent of documented occurrences in the primary literature of attraction (+), repellency (–), or no statistical difference from included controls (○) across 16 stored-product arthropods to each of the following complex blends of microbial cues.

Microbial Taxon	Behavioral Response ¹			χ^2	<i>P</i>	N
	–	○	+			
<i>Alternaria</i> sp. (extract, plated)	0	0	100	6.0	0.05	3
<i>Aspergillus</i> sp. ²	48	22	30	2.4	0.30	23
Brewer's yeast	25	0	75	3.5	0.17	4
Assorted Fungal Genera ³	19	31	50	1.6	0.45	16
Unspecified Fungi	14	46	41	3.9	0.14	22
<i>Fusarium</i> sp. (culture, plated, extracts, grains inoculated)	35	18	47	2.2	0.33	17
<i>Penicillium</i> sp. (pure culture, inoculated grain)	46	20	34	4.4	0.11	41
<i>Saccharomyces cerevisiae</i> (culture and inoculated food)	0	0	100	6.0	0.05	3
<i>Trichoderma harzianum</i> (multiple strains)	20	20	60	1.6	0.45	5
<i>Ulocladium botrytis</i> (two strains, multiple sources)	66	33	0	4.0	0.14	6
Net behavioral response to microbial cues	34	24	41	5.8	0.05	140

¹ Behavioral response by 16 stored-product arthropods, including *Acanthoscelides obtectus*, *Carpophilus hemipterus*, *Carpophilus humerus*, *Cryptolestes ferrugineus*, *Cynaesus angustus*, *Lariophagus distinguendus*, *Liposcelis bostrychophila*, *Oryzaephilus mercator*, *Plodia interpunctella*, *Sitophilus zeamais*, *Tenebrio molitor*, *Tribolium castaneum*, *Tribolium confusum*, *Typhaea stercorea*, and *Tyrophagus putrescentiae*.

² The genera *Aspergillus*, *Fusarium*, and *Penicillium* have been collapsed into a single row to allow potential generalizations.

³ Genera include *Beauveria*, *Byssoschamlys*, *Candida*, *Cephalosporium*, *Cladosporium*, *Epicoccum*, *Helminthosporium*, *Hypocrea*, *Microsporium*, *Nigrospora*, *Rhizopous*, *Scopulariopsis*, *Streptomyces*, and *Trichophyton* sp.

Net neutral behavioral responses

Overall, in responding to complex blends of MVOCs, 63% of stored-product arthropods had a net neutral behavioral response (e.g. no statistically significant deviation from null). Most of the behavior demonstrated by stored-product arthropods in this category were either nearly equally mixed between one response and another (in 38% of species), or the taxon had low replication of tests in the literature (in 31% of species), with the latter preventing robust statistical evaluation among those subset of cases (Table 1). However, in one case, there was actually significantly more net neutral responses than expected at random, and this was by *T. castaneum*. In particular, the response by *T. castaneum* to complex blends of MVOCs was highly indifferent (Table 1), highlighting its generally ambivalent response to other stimuli. In addition, 80% of the MVOC sources for which there was sufficient replication showed a roughly equal mixture of attraction, repellency, and net neutral responses by stored-product arthropods. The reasons for the large proportion of stored-product arthropods with neutral or mixed responses to MVOCs is discussed further below.

Behavioral response by stored-product arthropods to individual or known mixtures of MVOCs

In total, there were 243 sets of tests documenting the behavioral response of 19 arthropod taxa to an average of 7 ± 1.5 (mean $\pm$ SE) individual microbial compounds per taxon (Table S2). Overall, the responses by stored-product arthropods to 26 individual microbial compounds were evaluated in this study. In particular, the behavioral responses of stored-product arthropods were assessed to individual MVOCs through the use of 2-way olfactometers and petri dish assays in 84% of cases (Figure 1, bottom-left panel), while a total of 93% of tests were performed in the

laboratory. In characterizing the behavior of stored-product arthropods, 84% of tests used preference as the primary measure of behavioral response (Figure 1, bottom-right panel).

Positive behavioral responses

Some stored-product arthropods had consistently positive responses to individual MVOCs. For example, overall *C. hemipterus*, *T. confusum*, and *T. putrescentiae* significantly preferred or were attracted to individual or known mixtures of microbial compounds (Table 3). Compounds tested against these species included a quaternary mixture of ethyl acetate, acetaldehyde, 2-pentanol, and 3-methyl-1-butanol, as well as an 18-component mixture of known volatiles mimicking *S. cerevisiae*-inoculated banana, and 11 individual microbial compounds (Table S2). When MVOCs were tested individually, there was fairly little consistency in attraction among stored-product arthropods, except in the case of 3-methyl-1-butanol, which elicited more attraction and neutral behavioral responses than repellency ($\chi^2 = 22.5$; $df = 2$; $P < 0.0001$; Table 4).

Table 1-3. Summary of the percent of documented occurrences in the primary literature of attraction (+), repellency (–), or no statistical difference from included controls (○) by each of the following stored-product arthropod species among individual MVOCs.

Arthropod Taxon	Behavioral Response			χ^2	<i>P</i>	N	Type of Pest ¹
	–	○	+				
<i>Ahasverus advena</i>	27	40	33	0.41	0.82	15	2°
<i>Callosobruchus maculatus</i>	33	50	17	1.06	0.59	6	1°
<i>Carpophilus hemipterus</i>	0	0	100	6.09	0.05	3	2°
<i>Cathartus quadricollis</i>	40	50	10	2.72	0.26	10	2°
<i>Cryptolestes ferrugineus</i>	20	46	34	2.96	0.23	35	2°
<i>Holepyris sylvanidis</i>	25	25	50	0.52	0.77	4	wasp
<i>Lariophagus distinguendus</i>	50	50	0	-	-	2	wasp
<i>Oryzaephilus mercator</i>	20	40	40	1.15	0.56	15	2°
<i>Oryzaephilus surinamensis</i>	20	40	40	1.15	0.56	15	2°
<i>Plodia interpunctella</i>	13	50	38	1.75	0.42	8	2°
<i>Sitophilus granarius</i>	43	45	12	15.46	0.0004	69	1°
<i>Sitophilus zeamais</i>	74	0	26	19.7	0.0001	23	1°

<i>Tenebrio molitor</i>	75	25	0	3.55	0.17	4	2°
<i>Tribolium castaneum</i>	22	50	28	2.42	0.30	18	2°
<i>Tribolium confusum</i>	0	0	100	8.12	0.02	4	2°
<i>Trogoderma granarium</i>	50	50	0	-	-	2	2°
<i>Trogoderma inclusum</i>	50	50	0	-	-	2	2°
<i>Trogoderma variabile</i>	100	0	0	-	-	1	2°
<i>Tyrophagus putrescentiae</i>	0	0	100	14.2	0.001	7	2°
Net Behavioral Response	34	37	28	3.8	0.1	243	

¹ Abbreviations: 1° – primary pest, 2° – secondary pest.

Negative behavioral responses

Two stored-product arthropods had negative behavioral responses to MVOCs when tested individually. These included *S. granarius* and *S. zeamais* (Table 3), and were consistently negative or neutral among 33 individual or known mixtures of MVOCs (Table S2). *Sitophilus zeamais* was repelled by individual MVOCs almost twice as much as *S. granarius*. Overall, among stored-product arthropod taxa, (*E*)-2-hexenal generally elicited repellency significantly more than attraction or neutral responses in about two-thirds of documented cases.

Table 1-4. The behavioral response among 19 stored-product arthropod species from the primary literature to the following specific MVOCs, including documented attraction (+), repellency (–), or no statistical difference from included controls (○).

Microbial Compound ¹	Behavioral Response			χ^2	<i>P</i>	N
	–	○	+			
<i>(E)</i> -2-hexenal (with or without wheat)	64	7	29	7.11	0.03	14
(<i>E,E</i>)-2,4-decadienal (with or without wheat)	50	0	50	2.06	0.36	4
(<i>E,E</i>)-2,4-heptadienal	33	33	33	0.001	1.00	3
(<i>E,E</i>)-2,4-nonadienal (with or without wheat)	50	0	50	2.06	0.36	4
1-butanol	25	50	25	0.46	0.80	4
1-octen-3-ol (multiple isomers, with or without maize)	44	33	22	1.35	0.51	18
1-octen-3-one	23	31	46	1.11	0.57	13
1-pentanal	33	33	33	0.001	1.00	3
1-pentanol	50	25	25	0.46	0.80	4
2,3-butanedione	67	0	33	2.05	0.36	3
2-decanone (with or without wheat)	50	50	0	2.06	0.36	4
2-heptanone (with or without wheat)	50	0	50	2.06	0.36	4
2-hexanone (with or without wheat)	50	0	50	2.06	0.36	4
2-pentanone (with or without wheat)	67	0	33	2.05	0.36	3
2-phenylethanol	31	23	46	1.16	0.56	13
3-methyl-1-butanol	11	42	47	22.5	0.0001	19
3-octanone	35	35	29	0.10	0.95	17
butanal (with or without wheat)	50	0	50	2.06	0.36	4
deoxynivalenol	75	0	25	3.58	0.17	4
ethanol	0	50	50	2.06	0.36	6
fumonisin B extract	33	67	0	2.05	0.36	3
heptanal (with or without wheat)	50	0	50	2.06	0.36	4
hexanol (with or without wheat)	50	0	50	2.06	0.36	4
nonanal	0	33	67	2.05	0.36	3

octanol (3-, 1-)	35	29	35	0.16	0.92	17
oleic acid	50	17	33	1.06	0.59	6
<i>Net Behavioral Response to Compounds</i>	<i>37</i>	<i>25</i>	<i>37</i>	<i>6.09</i>	<i>0.05</i>	<i>185</i>

¹ Some compounds may have multiple sources apart from their role as microbial volatiles, including being emitted by grain and other biological sources.

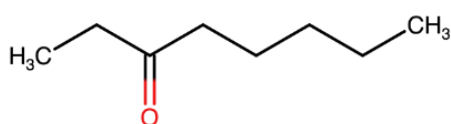
² Compounds were left off the table if they had fewer than three documented occurrences in the primary literature.

Net neutral behavioral responses

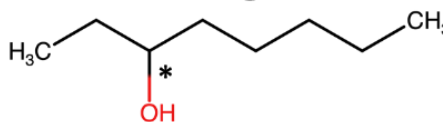
Similar to complex blends, most of the behavior exhibited by stored-product arthropods to individual or known mixtures of MVOCs were either nearly equally mixed between amongst attraction, repellency, and neutrality (in 47% of species; Table 3), or the species had low replication of tests in the literature as was the case for 26% of species. The latter prevented robust statistical evaluation for those particular species (Table 3). In total, 24 individual MVOCs (or 92% of volatiles with sufficient replication) elicited mixed or multiple responses among stored-product arthropods (Table 4).

Figure 1-1-2. Chemical structures of the most commonly tested MVOCs against stored-product arthropods, which collectively represent 60% of the $n = 185$ tests of individual components in the literature. Functional groups are highlighted in red, asterisks chiral centers.

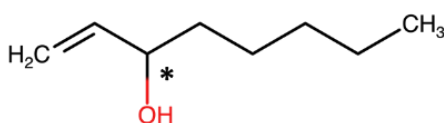
Most commonly tested fungal volatiles



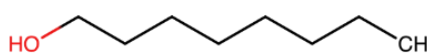
3-octanone



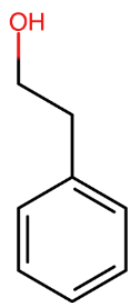
3-octanol



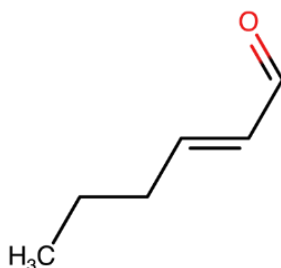
1-octen-3-ol



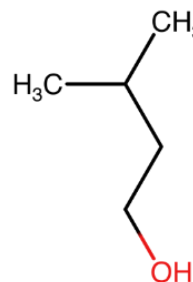
1-octanol



2-phenylethanol



(*E*)-2-hexenal



3-methyl-1-butanol

Response to known mixtures of MVOCs

There were $n = 22$ documented instances of known mixtures of MVOCs tested for the behavioral response of stored-product arthropods (Table S2). The median number of components in each mixture was three MVOCs. Overall, stored-product arthropods were attracted, repelled, or neither to mixtures of MVOCs in 23%, 32%, and 45% of the cases. Stored-product arthropods were significantly more likely to show a net neutral behavioral response (45% in known component mixtures vs. 25% with individual MVOCs; $\chi^2 = 5.71$; $df = 1$; $P < 0.02$), but not attraction (23% vs. 37%; $\chi^2 = 3.27$; $df = 1$; $P < 0.07$) or repellency (37; $\chi^2 = 0.36$; $df = 1$; $P < 0.55$) when MVOCs were presented in mixtures compared to individual MVOCs.

Discussion

Response by stored-product arthropods to complex blends of MVOCs

We found robust evidence for the use of complex blends of MVOCs as stimuli by stored-product arthropod, but roughly equal numbers were repelled or attracted, depending on specific microbial taxon, arthropod species, microbial isolate, and methodology. Interestingly, some stored-product arthropods appear to have fairly idiosyncratic responses to the blends of volatiles from microbes. For example, in one study, *T. confusum* was found to have no preference between isolate 5583 of *Penicillium viridicatum* and whole wheat, but was found to be significantly repelled by three other isolates of the same fungus (isolates 60, 5572, and 963; [29]). This suggests that other factors might be contributing to the interaction of stored-product arthropods and microbes, such as possibly environmental context, physiological status, priming, or learning.

A common hypothesis in the primary literature is that secondary stored-product arthropods are more likely to respond to MVOCs, since some colonization of a commodity by

microbes may be an indicator that broken grain is nearby or that intact kernels may be weakened sufficiently to be used (e.g. [8,24]). This hypothesis appears to be supported with some exceptions, because seven secondary pest species had significant responses to MVOCs compared to three primary pests, and 71% of that subset of secondary pest species responded to MVOCs positively. However, this finding may be a function of research effort in assessing secondary and primary pests in the literature, with 3.5-fold more secondary pests assessed than primary pests assessed in the literature. In the future, the number of species tested should be expanded to include more species with primary pest life histories in stored products.

Interestingly, while many studies have expounded on the importance of MVOCs for *T. castaneum* in particular (e.g. [21,30–32]) , we found most (over half) of its documented responses were net neutral. Ambivalence to stimuli by a key stored-product arthropod is an important point, because as several studies have noted [24,32–34] most lures perform poorly in robustly attracting *T. castaneum*. While attraction was documented in a third of cases, the results here suggest that the behavioral response of *T. castaneum* to MVOCs is not indiscriminate, and may be more complex than previously anticipated. While this pattern of behavioral response does not preclude the use of MVOCs for manipulating the behavior of this important cosmopolitan and damaging insect, it means more work will need to be undertaken to determine where, to which microbes, and under what conditions *T. castaneum* is attracted to MVOCs. It is possible that *T. castaneum* only responds to a subset of fungi associated with just a handful of commodities.

Overall, we found evidence for significantly fewer net neutral responses to complex blends of MVOCs, with roughly half of documented cases exhibiting attraction by stored-product arthropods and half repellency. At any given time, a grain mass may have multiple

microbial species growing, and may even contain different strains of the same species. In practice, complex microbial species assembly in grain may mean that stored-product arthropods are exposed to very different blends of MVOCs simultaneously. Some of these may have positive and negative effects, and some of these may be antagonistic when binding on the olfactory receptors of arthropods. Antagonistic effects on the behavior of stored-product arthropods appear unavoidable given the large and diverse assemblage of microbes associated with different commodities [11]. Thus, in the future, it will be important to elucidate how the behavioral responses of stored-product arthropods are altered by headspace with different species richness and/or community composition of microbes. Better elucidating the chemical ecology surrounding microbial-grain-insect interactions will enable an improved understanding of how microbial community assembly affects the behavior of arthropods in postharvest environments.

Response by stored-product arthropods to individual or known mixtures of MVOCs

Many of the individual or mixtures of MVOCs had contrasting effects depending on concentration, and often producing a net neutral behavioral effect when aggregated in the tables for this review. However, the dose at which a given MVOC was behaviorally-active in manipulating the behavior of stored-product arthropods showed remarkably consistent patterns, with a variety of behavioral response curves possible and somewhat smaller subset documented (e.g., Figure 3). A behavioral response with a significantly negative or positive effect at intermediate dosages was the most and only appreciably frequent among the “changing response” family of curves, comprising 21% of the $n = 66$ cases (e.g. parabolic blue curves). These patterns may arise if moderate mold growth has attractive qualities and MVOCs indicate the presence of mates, usable resources, or preferred microclimate, while it may be repellent if MVOCs indicate overcrowding, compromised resources, or poor oviposition locations. Which

one of these is communicated by the MVOC may depend on the particular arthropod, microbial species, prior experience, or physiological status of the individual. Prior work has demonstrated that oviposition by stored-product arthropods generally varies in accordance with the suitability of the substrate [35]. Thus, a possible hypothesis for this response may be that high concentrations of MVOCs represent excess colonization and growth of microbes, which may be inferior oviposition sites, because fungal growth could be detrimental to developing larvae inside kernels.

Neutral-positive behavioral response curves with attraction at high or low dosages comprised a quarter of documented cases (Figure 3; teal lines, left and middle columns). A possible hypothesis for why a secondary pest stored-product arthropod taxon may fall into the neutral-positive grouping with attraction at high but not low dosages of the MVOC may include that a higher concentration is indicative of robust growth by microbes sufficient to compromise the bran of a kernel. An example of this is *Oryzaephilus surinamensis* (Coleoptera: Silvanidae), which was attracted to 1-octen-3-one at high concentrations (0.1–100 ug), but not lower concentrations (0.0001–0.01 µg) [36]. Likewise, *P. interpunctella* was attracted to 1-hexanol at high concentrations [37], but not at lower ones, while the same was also true of *Cryptolestes ferrugineus* and 3-methylbutanol [36]. The MVOCs β-caryophyllene, benzaldehyde, and pentadecane were found to have a neutral-positive effect on *T. putrescentiae*, playing an important role in host-location [38]. Prior work has found that the two chemosensory proteins TputCSP1 and TputCPS12 were responsible for recognizing the host volatiles and MVOCs (–)-alloaromadendrene, 2-methylnaphthalene, and cyclopentadecane [39].

By contrast, neutral-negative behavioral response curves with repellence at high or low dosages comprised 46% of documented cases, or almost twice the number of neutral-positive

cases. These patterns emerged for primary pests like *S. zeamais*, which may be repelled by high concentrations of fungal volatiles such as (*E*)-2-hexenal and 1-pentanol, but have neutral responses when those MVOCs are present in lower concentrations, placing them in the neutral-negative grouping [40]. Overall, the two primary pests, *S. granarius* and *S. zeamais* exhibited significantly neutral-negative responses to individual MVOCs, including (*E*)-2-hexenal, 1-hexanol, 1-butanol, 1-octanol, 1-pentanal, 1-pentanol, and others [27,40–43].

There may also be other explanations for the response curves apart from those discussed above (Table 5). For example, there may be innate attraction or repulsion to some compounds [27], learning of habitat cues from the natal environment [44], or physiological constraints in the response of a given taxon to a particular MVOC. In the possible behavioral manipulation of stored-product arthropods by microbes, others have hypothesized *T. molitor* may be preferentially attracted to infected kernels with *Fusarium*, because it facilitates dispersal of the fungus, and the chemical phenotype in the fungus responsible for attraction was found to be under positive selection [45]. Further studies should determine how widespread possible behavioral manipulation of stored-product arthropods is by microbes found on grain.

Figure 1-1-3. Hypothetical and documented behavioral responses of arthropods to MVOC dosages. Most common responses: changing, neutral-positive, neutral-negative (96% of cases, n = 66).

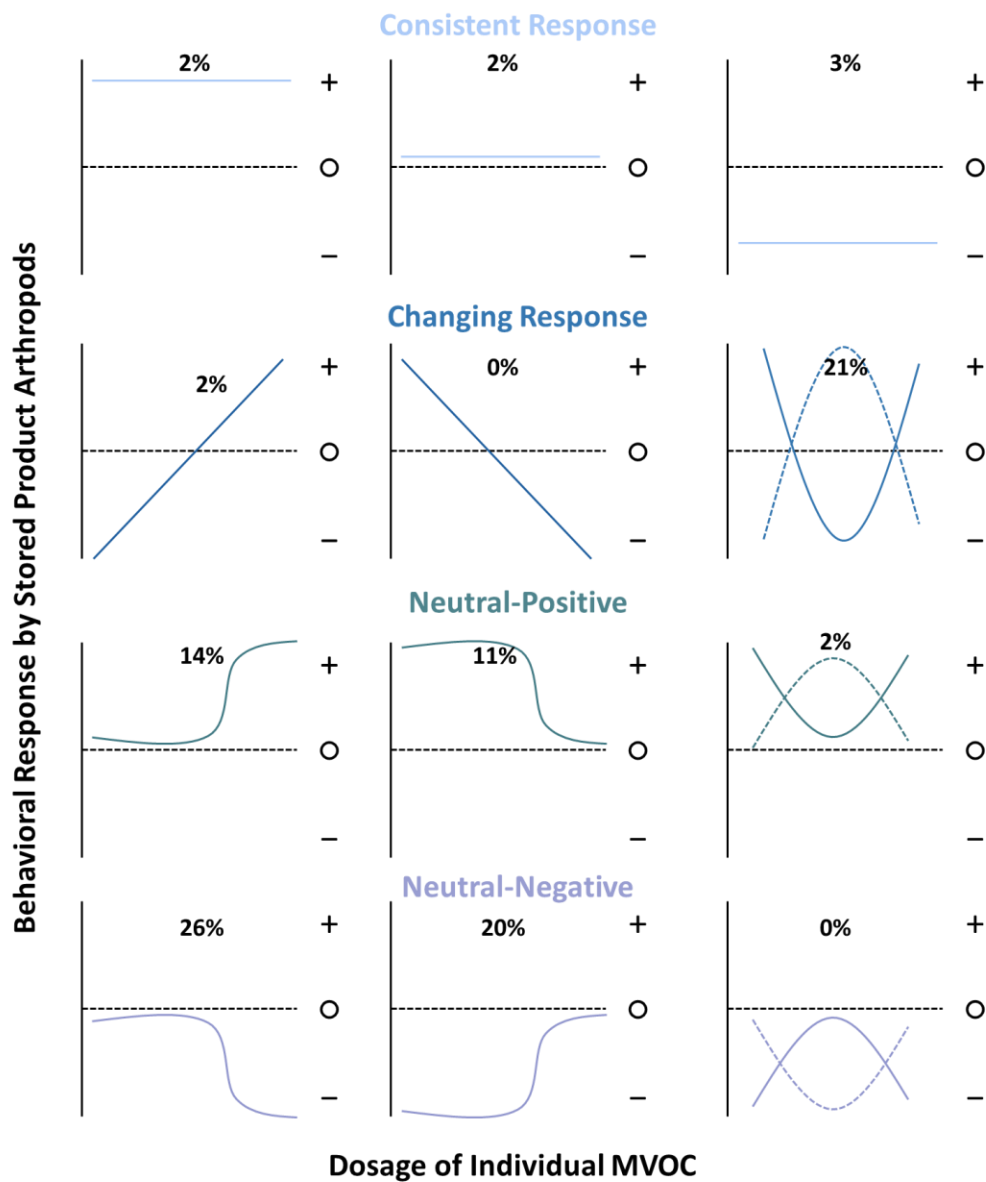


Table 1-5. Non-exhaustive and non-mutually exclusive hypotheses for the role of MVOCs in driving the behavior of stored-product arthropods described from the primary literature and in the current review.

No.	Short Title	Description	Type of Explanation	Type of Stored Product Pest	Predicted Insect Behavioral Response	Source
1	Host-finding hypothesis	MVOCs used as host-finding kairomones	Eco	1° & 2°	+	[36]
2	Convergent evolution with insects	Similarity in identity of MVOCs to behaviorally-active compounds for stored-product arthropods is the result of convergent evolution	Evo	1° & 2°	+	[36]
3	Convergent evolution with grains	Similarity in identity of MVOCs to compounds from headspace of grains is the result of convergent evolution	Evo	n/a	+	[36]
4	Microbe advantage hypothesis	Attractive kairomone-producing microbes may be advantaged by having insects serve as vectors, improve microclimate, feeding on dead insects etc.	Eco/Evo	1° & 2°	+	[16,36,45]
5	Secondary pest benefit hypothesis	MVOCs indicates a resource that may not otherwise be usable to secondary pests because of life history	Eco	2°	+	Multiple sources but see [8,30,46]
6	Symbiotic or non-host hypothesis	Microbes may form symbiotic relationships with stored-product arthropods, or commodity may not be a good host material; MVOCs generally not related to commodity	Eco	1° & 2°	+	[47]

7	Insect advantage hypothesis	Insects may be advantaged by surviving longer, having lower mortality, upregulating metabolic processes, and/or experiencing higher progeny production through as yet determined mechanisms	Eco/Evo	1° & 2°	+	[47–49]
8	Mycotoxin tolerance hypothesis	Stored-product arthropods may be able to tolerate the negative consequences of exposure to mycotoxins in order to benefit from other associations with microbes	Eco/Evo	1° & 2°	○	Based on data in [16]
9	Marking pheromone	Some MVOCs similar to insect pheromones might play a role at very low dosages as a marking pheromone to repel conspecifics	Eco/Evo	1° & 2°	–	[36,50]
10	Grain protection hypothesis	MVOCs will generally repel stored-product arthropods, and their production can possibly be induced by grain as a form of protection	Eco/Evo	n/a	–	[51]
11	Primary pest harm hypothesis	MVOCs indicate degraded environment and poor oviposition options for offspring	Evo	1°	–	This contribution
12	Innate response hypothesis	Attraction or repellency to MVOCs is genetically conserved	Evo	1° & 2°	+/-	[27]
13	Learned response hypothesis	Attraction or repellency to MVOCs is learned from natal environment or experience	Eco	1° & 2°, wasps	+/-	[44]

Abbreviations: Eco – ecological rationale or timeframe, Evo – evolutionary, fitness, or genetic rationale, 1° – primary stored-product pests, 2° – secondary stored-product pests.

Stored-product arthropods as vectors and ecosystem engineers for mycoflora

The postharvest environment can be thought of as a successional ecosystem [52], and as ecosystem with primary insect colonizers using intact kernels, followed by colonization of secondary pests that can take advantage of broken kernels and grain dust. Prior work has shown that these species may bring their own suite of microbes, with specific spores associated with the cuticles of each individual [18,52]. For example, the fungal community in bagged coffee was found to shift from being dominated by *Sacharomycetales* during the first six months to dominated by *Wallemia* sp. thereafter [12]. Thus, the microbial community may vary dynamically over time with successive arrivals and dispersal events, which in turn will change the MVOCs emitted by a particular commodity.

Indeed, an increasing amount of research has demonstrated the importance of stored-product arthropods in mediating the mycoflora present in postharvest environments. For example, feeding by insects such as *Sitotroga cerealella* (Olivier) (Lepidoptera: Gelechiidae) on grain kernels was shown to provide a favorable environment for the growth of *F. verticillioides*, as well as promote production of fumonisin B1 [53,54]. Hotspots of insect infestation in stored grain may significantly elevate the moisture and temperature in the environment, leading to microbial growth and deterioration of the durable commodities [55]. In comparing communities pure or mixed colonies of *S. zeamais* and *Prostephanus truncatus* (Horn) (Coleoptera: Bostrichidae), prior work has found when both species were present, there was greater microbial growth among the grain [56]. A positive association was found between the survival rate of *R. dominica* and *S. oryzae* in grain, and aflatoxin levels [57]. Propagules of *F. proliferatum* and *F. culmorum* were traceable in *T. molitor* feces 20 days after feeding [45]. Even the ham mite, *T. putrescentiae* was found to disseminate *Fusarium* spp. among stored grain [58].

However, benefits may also accrue to stored-product arthropods by the presence of mycoflora. For example, Ponce et al. [15] found that the presence of both *S. oryzae* and *Aspergillus* spp. in a grain mass altered the abiotic conditions in ways that were favorable to both, and also resulted in elevated progeny production by *S. oryzae* compared to when microbes were absent. In addition, others have found a positive correlation between insect survival rate and moisture contents of wheat grain [57]. Given the benefits to both microbes and stored-product arthropods by their combined presence and activity in grain, it is clearly reasonable that MVOCs modulate arthropod foraging behavior, even if we have found that it is often taxon-, compound-, and context-dependent.

Biases and limitations of reviewed studies

As we reviewed the literature, there are some obvious limitations to the available studies. In many of the studies evaluating the behavioral response of stored-product arthropods to complex blends of MVOCs, there is often little or no effort to characterize the composition of those headspace emissions, or if this is done for a microbial taxon, it happens separately from collection of behavioral data. Increasingly, it is important to not just know the behavioral implications of semiochemicals, but to have specific information on their identity and how they vary among treatments as well. For example, Van Winkle et al. [8] demonstrated not only attraction by the primary pest, *Rhyzopertha dominica* (F.) (Coleoptera: Bostrichidae), but also went further and described the composition of the MVOCs being tested. Others have also provided similarly extensive information and paired it to behavioral data (e.g. [38]). Pairing behavioral data with GC/MS and other chemical data in the future will help provide a more comprehensive picture of which volatiles may be involved in the behavioral response by stored-product arthropods to certain microbial taxa.

Another significant limitation in the literature is the lack of diversity in behavioral measures of arthropod response to MVOCs. For example, 70–80% of the behavioral responses in all the studies was assessed with preference as a proxy, usually through a 2-choice olfactometer, but sometimes with a simple petri-dish assay. However, preference is an imperfect and incomplete measure of behavioral response by stored-product arthropods to MVOCs. Importantly, preference for stimuli is often dependent on the context in which a decision is made by arthropods [59,60], and the “preferred” choice may represent the best of two poor options. As a case in point, in an olfactometer assay, Wright et al. [29] found that *T. confusum* was significantly repelled by 14 isolates of *Pencillium* sp. when choosing against whole wheat, but found the same isolates were significantly and highly preferred when choosing against autoclaved wheat. Others have noted that different behavioral assays will result in different conclusions about the same behavioral processes to the same stimuli [61], and thus using a variety of experimental approaches will help better elucidate how stored-product arthropods use microbial cues. With some exceptions [62,63], there was a notable lack of studies assessing long-distance attraction, distinguishing between arrestment and attraction, and including other important variables such as movement, and whether stored-product arthropods detect many of these microbial cues through GC-EAD. Finally, it is likely that the petri-dish assays were only testing spatially-mediated effects of MVOCs, which is far less useful information when trying to find novel behaviorally-relevant semiochemicals for use in traps or management programs. The reason for this is because if a volatile is only active in a small area around where it is located, it is not as useful for manipulating behavior from a distance.

Another severe methodological limitation for >90% of the studies were that only laboratory assays were included. While laboratory studies have their place, including allowing

researchers to eliminate extraneous stimuli to determine mechanisms of behavior, it is likewise important to bring the research back into the field to assess whether behavior observed in the laboratory is likely under realistic conditions. Even including assessments under semi-field or semi-natural conditions is helpful in relating laboratory data back to realistic conditions. While some studies assessed microbial cues in traps in the field (e.g. [20]), this could be improved going forward.

We found that individual MVOCs resulted in fewer neutral responses than mixtures. However, this may be an artifact of the low number of mixtures evaluated for just a small subset of species in the literature. A meta-analysis on attraction to lures by herbivores in agroecosystems found that there was greater attraction to mixtures of compounds than individual components [64]. When designing mixtures, it is often important to take into account the relative abundance of compounds in natural headspace in order to evoke similar behavioral responses. In the future, it may be useful to systematically test whether mixtures of MVOCs may be more effective than individual constituents.

Furthermore, while some of the literature report the ratio or identity of stereoisomer compounds tested, most of the studies did not. Which stereoisomers are used may present an additional source of variation in the data from this review. It is well known that some arthropods, especially moths, may respond to only one specific isomer, or if they respond to multiple isomers, they must be in the correct ratio [65,66]. However, the specific stereoisomer may not be as important for other species [67,68]. Thus, it will be important for future studies to note which specific stereoisomers are used in studies documenting behavioral response of stored-product arthropods in order to allow for appropriate interpretation.

Finally, with the exception of a handful of studies [69–71], the majority of the tests conducted in the literature have been with adult stored-product arthropods. While adults are typically thought of as the dispersing life stage in stored products when designing management strategies [72], grain is readily moved through human-mediated transportation, and even immature secondary pests have a strong dispersal capacity (e.g. *Trogoderma variabile*: [73]), as well as an ability to react differently to volatiles [70]. In the future, other life stages other than adults should be incorporated in tests of behavioral response to MVOCs.

Future directions in understanding response by stored-product arthropods to MVOCs

There have been relatively few studies evaluating how mycotoxins, an important health hazard in the food supply in some parts of the world [14], affect foraging by stored-product arthropods. We have included the few studies that could be found on this in the current study (e.g. [16,47,51,74]), but the lack of breadth in testing of both different mycotoxins and different arthropod species prevents robust generalization. Nonetheless, we do know from these studies that the mycotoxin, deoxynivalenol (DON), apparently reduced locomotory activity of *Tenebrio molitor*, as well as induced dose-dependent changes in physiological activity of larvae [74]. Other studies have linked insect activity with increased mycotoxin production in grain [53,57], while some studies have shown that there may be tolerance to mycotoxins [16]. Expanding testing of mycotoxins for how they affect the behavior, development, and growth of stored-product arthropods is a natural next step.

While the studies that have been performed have given us an extensive understand of some of the preferred MVOCs by stored-product arthropods, future work must expand the characterization of behavior to include other measures. For example, to characterize the behavior of *T. granarium* and *T. variabile*, three behavioral assays were used that included a wind tunnel,

olfactometer, and petri dish assay, which tested attraction, preference, and arrestment, respectively [69]. Combining these tests with others that track movement of insects (e.g. [75]), and include semi-field and field evaluations of behavior will be helpful in characterizing the full range of behavior elicited by MVOCs as well as understanding response under realistic conditions.

Recently, work has shown that neurogenesis of mushroom bodies in *T. castaneum* has is affected by the environment from day four of adulthood and onwards [76]. This has implications for how olfactory environment may affect proliferation of neurons, and it is currently unknown how MVOCs in the environment may affect this process. Others have recently made calls to better integrate molecular, genetic, behavior, and chemoecological data to speed applications for semiochemical-mediated, behaviorally-based management for stored-product arthropods [77]. There is little to no mechanistic understanding of how MVOCs encode changes in the behavior or olfaction of stored-product arthropods molecularly, genetically or in protein expression. At a minimum, using genetic tools such as barcoding will allow for the identification and better characterization of the microbial community on grain to determine which microbial species are most affecting stored-product insect behavior [78]. Taking an integrative approach to the study of MVOCs in the future will be increasingly important to determine new potential targets for RNAi or other novel technology.

Conclusions

In systematically reviewing the literature on the behavioral responses of stored-product arthropods to complex blends and individual MVOCs, overall we have found robust support for attraction among complex blends of MVOCs, and dose-dependent attraction or repulsion for individual MVOCs that follow typified response patterns. The following stored-product

arthropod species were specifically attracted, including *C. hemipterus*, *C. ferrugineus*, *P. interpunctella*, *T. confusum*, and *T. putrescentiae*. By contrast, *L. distinguendus*, *L. bostrychophila*, *S. granarius*, and *S. zeamais* were repelled, while it was much more likely *T. castaneum* had a net neutral response. Attraction to individual MVOCs by stored-product arthropods was often dose-dependent and compound-specific. The most common behavioral response curves took the form of neutral-positive or neutral-negative, with the latter twice as frequent in the subset of the dataset where multiple dosages had been evaluated. The published literature had some important limitations, including being mostly focused on assessing preference and have been mostly confined to laboratory assays. In the future, characterization of behavior should include other measures beyond preference such as movement, flight, and field data. Overall, our review has revealed that the effect of MVOCs on the foraging behavior is complex, taxon-, and context-dependent, and it will require more work to tease apart mechanisms driving responses of different species. Future directions include evaluating the behavioral response of stored-product arthropods to mycotoxins; integrating across disciplines to speed research progress; and, expanding the behavioral and biological parameters to characterize the effect of MVOCs.

References

1. FAO *The State of Food Security and Nutrition in the World*; Rome, Italy, 2018;
2. USDA-NASS *Grain stocks (January 2020)*; Washington, D.C., 2020;
3. USDA-NASS Quickstats v.2.0 Available online:
https://www.nass.usda.gov/Quick_Stats/.
4. Wacker, F. Food waste and food losses - Importance of international partnerships and research. In *Proceedings of the 12th International Working Conference on Stored Product Protection*; Adler, C., Blank, C., Fuerstenau, B., Kern, P., Mueller-Blenkle, C., Eds.; Julius-Kuhn Institut: Berlin, Germany, 2018; p. 31.
5. Hagstrum, D.W.; Subramanyam, B. *Fundamentals of Stored-Product Entomology*; AACC International: St. Paul, 2006;
6. Hubert, J.; Stejskal, V.; Athanassiou, C.G.; Throne, J.E. Health hazards associated with arthropod infestation of stored products. *Annu. Rev. Entomol.* **2018**, *63*, 553–573, doi:10.1146/annurev-ento-020117-043218.
7. Schnürer, J.; Olsson, J.; Börjesson, T. Fungal volatiles as indicators of food and feeds spoilage. *Fungal Genet. Biol.* **1999**, *27*, 209–217.
8. Van Winkle, T.; Ponce, M.; Quellhorst, H.; Albin, C.E.; Bruce, A.; Kim, T.N.; Zhu, K.Y.; Morrison, I.W.R. Microbial volatile organic compounds mediate attraction by a primary but not a secondary stored-product insect pest in wheat. *J. Chem. Ecol.* **2021**, *In press*.
9. Brabcová, V.; Štursová, M.; Baldrian, P. Nutrient content affects the turnover of fungal biomass in forest topsoil and the composition of associated microbial communities. *Soil Biol. Biochem.* **2018**, *118*, 187–198, doi:10.1016/j.soilbio.2017.12.012.

10. Morrison III, W.R.; Bruce, A.; Wilkins, R.V.; Albin, C.; Arthur, F.H. Sanitation improves stored product pest management. *Insects* **2019**, *10*, 77.
11. Tiwari, S.; Kate, A.; Mohapatra, D.; Tripathi, M.K.; Ray, H.; Akuli, A.; Ghosh, A.; Modhera, B. Volatile organic compounds (VOCs): Biomarkers for quality management of horticultural commodities during storage through e-sensing. *Trends Food Sci. Technol.* **2020**, *106*, 417–433, doi:10.1016/j.tifs.2020.10.039.
12. Broissin-Vargas, L.M.; Snell-Castro, R.; Godon, J.J.; González-Ríos, O.; Suárez-Quiroz, M.L. Impact of storage conditions on fungal community composition of green coffee beans *Coffea arabica* L. stored in jute sacks during 1 year. *J. Appl. Microbiol.* **2018**, *124*, 547–558, doi:10.1111/jam.13656.
13. Tripathi, A.; Alam, A. Mycotoxins, mycotoxicosis, and managing mycotoxin contamination: A review. In *Bio-management of Postharvest Diseases and Mycotoxigenic Fungi*; Sharma, N., Bhandari, A.S., Eds.; CRC Press: Boca Raton, FL, 2021.
14. Omotayo, O.P.; Omotayo, A.O.; Mwanza, M.; Babalola, O.O. Prevalence of mycotoxins and their consequences on human health. *Toxicol. Res.* **2019**, *35*, 1–7, doi:10.5487/TR.2019.35.1.001.
15. Ponce, M.; Kim, T.N.; Morrison, W.R. The interaction between *Sitophilus oryzae* (Coleoptera: Curculionidae) and *Aspergillus flavus* jointly affects microclimate, grain quality, and each other's fitness. *Environ. Entomol.* **2021**, *in press*.
16. Usseglio, V.L.; Dambolena, J.S.; Martinez, M.J.; Zunino, M.P. The role of fumonisins in the biological interaction between *Fusarium verticillioides* and *Sitophilus zeamais*. *J. Chem. Ecol.* **2020**, *46*, 1059–1068, doi:10.1007/s10886-020-01220-3.

17. da Silva, G.L.; Esswein, I.Z.; Heidrich, D.; Dresch, F.; Maciel, M.J.; Pagani, D.M.; Valente, P.; Scroferneker, M.L.; Johann, L.; Ferla, N.J.; et al. Population growth of the stored product pest *Tyrophagus putrescentiae* (Acari: Acaridae) on environmentally and medically important fungi. *Exp. Appl. Acarol.* **2019**, *78*, 49–64, doi:10.1007/s10493-019-00370-8.
18. Lamboni, Y.; Hell, K. Propagation of mycotoxigenic fungi in maize stores by post-harvest insects. *Int. J. Trop. Insect Sci.* **2009**, *29*, 31–39, doi:10.1017/S1742758409391511.
19. Davis, T.S.; Crippen, T.L.; Hofstetter, R.W.; Tomberlin, J.K. Microbial volatile emissions as insect semiochemicals. *J. Chem. Ecol.* **2013**, *39*, 840–859, doi:10.1007/s10886-013-0306-z.
20. Stevens, M.M.; Wood, R.M.; Mo, J. Monitoring flight activity of *Cryptolestes ferrugineus* (Coleoptera: Laemophloeidae) in outdoor environments using a commercial pheromone lure and the kairomone 1-octen-3-ol. *J. Stored Prod. Res.* **2019**, *83*, 227–235, doi:10.1016/j.jspr.2019.07.005.
21. Ahmad, F.; Daglish, G.J.; Ridley, A.W.; Burrill, P.R.; Walter, G.H. Short-range resource location by *Tribolium castaneum* Herbst (Coleoptera: Tenebrionidae) demonstrates a strong preference for fungi associated with cotton seed. *J. Stored Prod. Res.* **2013**, *52*, 21–27, doi:10.1016/j.jspr.2012.09.003.
22. Wright, V.F.; Fleming, E.E.; Post, D.; Wright, F. Survival of *Rhyzopertha dominica* (Coleoptera, Bostrichidae) on fruits and seeds collected from woodrat nests in Kansas. *J. Kansas Entomol. Soc.* **1990**, *63*, 344–347.
23. Borgemeister, C.; Tchabi, A.; Scholz, D. Trees or stores? The origin of migrating *Prostephanus truncatus* collected in different ecological habitats in southern Benin. *Entomol. Exp. Appl.* **1998**, *87*, 285–294.

24. Morrison, W.R.; Scully, E.D.; Campbell, J.F. Towards developing behaviorally-based integrated pest management programs for stored-product insects. *Pest Manag. Sci.* **2021**, in press.
25. McFarlane, D. The role of fungi and their associated volatiles in the ecology of *Tribolium castaneum*, The University of Queensland, 2018.
26. Davis, T.S.; Landolt, P.J. A survey of insect assemblages responding to volatiles from a ubiquitous fungus in an agricultural landscape. *J. Chem. Ecol.* **2013**, *39*, 860–868, doi:10.1007/s10886-013-0278-z.
27. Germinara, G.S.; De Cristofaro, A.; Rotundo, G. Repellents effectively disrupt the olfactory orientation of *Sitophilus granarius* to wheat kernels. *J. Pest Sci. (2004)*. **2015**, *88*, 675–684, doi:10.1007/s10340-015-0674-y.
28. Team, R.C. R: A language and environment for statistical computing Available online: <https://www.r-project.org/>.
29. Wright, V.F.; Harein, P.K.; Collins, N.A. Preference of the confused flour beetle for certain *Penicillium* isolates. *Environ. Entomol.* **1980**, *9*, 213–216, doi:10.1093/ee/9.2.213.
30. Ahmad, F.; Daglish, G.J.; Ridley, A.W.; Walter, G.H. Responses of *Tribolium castaneum* to olfactory cues from cotton seeds, the fungi associated with cotton seeds, and cereals. *Entomol. Exp. Appl.* **2012**, *145*, 272–281, doi:10.1111/eea.12012.
31. Seifelnasr, Y.E. Olfactory response of red flour beetles, *Tribolium castaneum* (Herbst), to various forms of wheat, millet, and a fungus as determined by a light-sensitive apparatus, Kansas State University, 1981.
32. Dooley, M.; Peel, A.D.; Wakefield, M. The responses of *Tribolium castaneum* to wheat germ oil and fungal produced volatiles. In Proceedings of the 12th International Working

Conference on Stored Product Protection (IWCSPP) in Berlin, Germany, October 7-11; 2018; pp. 129–138.

33. Savoldelli, S.; Trematerra, P. Mass-trapping, mating-disruption and attracticide methods for managing stored-product insects: Success stories and research needs. *Stewart Postharvest Rev.* **2011**, *3*, 7, doi:10.2212/spr.2011.3.7.

34. Campbell, J.F. Attraction of walking *Tribolium castaneum* adults to traps. *J. Stored Prod. Res.* **2012**, *51*, 11–22, doi:10.1016/j.jspr.2012.06.002.

35. Gerken, A.R.; Campbell, J.F. Oviposition and development of *Tribolium castaneum* Herbst (Coleoptera: Tenebrionidae) on different types of flour. *Agronomy* **2020**, *10*, 1593, doi:10.3390/agronomy10101593.

36. Pierce, A.M.; Pierce, H.D.; Borden, J.H.; Oehlschlager, A.C. Fungal volatiles: semiochemicals for stored-product beetles (Coleoptera: Cucujidae). *J. Chem. Ecol.* **1991**, *17*, 581–597.

37. Būda, V.; Apšegaitė, V.; Blažytė-Čereškienė, L.; Butkienė, R.; Nedveckytė, I.; Pečiulytė, D. Response of moth *Plodia interpunctella* to volatiles of fungus-infected and uninfected wheat grain. *J. Stored Prod. Res.* **2016**, *69*, 152–158, doi:10.1016/j.jspr.2016.08.001.

38. Li, H.P.; Yang, W.J.; Qu, S.X.; Pei, F.; Luo, X.; Mariga, A.M.; Ma, L. Variation of volatile terpenes in the edible fungi mycelia *Flammulina velutipes* and communications in fungus-mite interactions. *Food Res. Int.* **2018**, *103*, 150–155, doi:10.1016/j.foodres.2017.10.031.

39. Qu, S.X.; Ma, L.; Li, H.P.; Song, J. Di; Hong, X.Y. Chemosensory proteins involved in host recognition in the stored-food mite *Tyrophagus putrescentiae*. *Pest Manag. Sci.* **2016**, *72*, 1508–1516, doi:10.1002/ps.4178.

40. Zunino, M.P.; Herrera, J.M.; Pizzolitto, R.P.; Rubinstein, H.R.; Zygadlo, J.A.; Dambolena, J.S. Effect of selected volatiles on two stored pests: The fungus *Fusarium verticillioides* and the maize weevil *Sitophilus zeamais*. *J. Agric. Food Chem.* **2015**, *63*, 7743–7749, doi:10.1021/acs.jafc.5b02315.
41. Germinara, G.S.; De Cristofaro, A.; Rotundo, G. Behavioral responses of adult *Sitophilus granarius* to individual cereal volatiles. *J. Chem. Ecol.* **2008**, *34*, 523–529, doi:10.1007/s10886-008-9454-y.
42. Niewiada, A.; Nawrot, J.; Szafrank, J.; Szafrank, B.; Synak, E.; Jeleń, H.; Wąsowicz, E. Some factors affecting egg-laying of the granary weevil (*Sitophilus granarius* L.). *J. Stored Prod. Res.* **2005**, *41*, 544–555, doi:10.1016/j.jspr.2004.11.001.
43. Herrera, J.M.; Pizzolitto, R.P.; Zunino, M.P.; Dambolena, S.; Zygadlo, J.A. Effect of fungal volatile organic compounds on a fungus and an insect that damage stored maize. *J. Stored Prod. Res.* **2015**, *62*, 74–80, doi:10.1016/j.jspr.2015.04.006.
44. Mbata, G.N.; Shu, S.; Phillips, T.W.; Ramaswamy, S.B. Semiochemical cues used by *Pteromalus cerealellae* (Hymenoptera: Pteromalidae) to locate its host, *Callosobruchus maculatus* (Coleoptera: Bruchidae). *Ann. Entomol. Soc. Am.* **2004**, *97*, 353–360, doi:10.1093/aesa/97.2.353.
45. Guo, Z.; Pfohl, K.; Karlovsky, P.; Dehne, H.W.; Altincicek, B. Dissemination of *Fusarium proliferatum* by mealworm beetle *Tenebrio molitor*. *PLoS One* **2018**, *13*, e0204602, doi:10.1371/journal.pone.0204602.
46. Ahmad, F.; Daglish, G.J.; Ridley, A.W.; Burrill, P.R.; Walter, G.H. Short-range resource location by *Tribolium castaneum* Herbst (Coleoptera: Tenebrionidae) demonstrates a strong

- preference for fungi associated with cotton seed. *J. Stored Prod. Res.* **2013**, 52, 21–27, doi:10.1016/j.jspr.2012.09.003.
47. Lamboni, Y.; Hell, K. Propagation of mycotoxigenic fungi in maize stores by post-harvest insects. *Int. J. Trop. Insect Sci.* **2009**, 29, 31–39.
 48. Schulthess, F.; Cardwell, K.F.; Gounou, S. The effect of endophytic *Fusarium verticillioides* on infestation of two maize varieties by lepidopterous stemborers and coleopteran grain feeders. *Phytopathology* **2002**, 92, 120–128, doi:10.1094/PHYTO.2002.92.2.120.
 49. Blackmer, J.L.; Phelan, P.L. Effect of physiological state and fungal inoculation on chemically modulated host-plant finding by *Carpophilus hemipterus* and *Carpophilus lugubris*. *Entomol. Exp. Appl.* **1991**, 61, 33–43, doi:10.1111/j.1570-7458.1991.tb02393.x.
 50. Averill, A.L.; Prokopy, R.J. Factors influencing release of host-marking pheromone by *Rhagoletis pomonella* flies. *J. Chem. Ecol.* **1988**, 14, 95–111, doi:10.1007/BF01022534.
 51. Usseglio, V.L.; Pizzolitto, R.P.; Rodriguez, C.; Zunino, M.P.; Zygadlo, J.A.; Areco, V.A.; Damolena, J.S. Volatile organic compounds from the interaction between *Fusarium verticillioides* and maize kernels as a natural repellents of *Sitophilus zeamais*. *J. Stored Prod. Res.* **2017**, 73, 109–114, doi:10.1016/j.jspr.2017.08.001.
 52. Dunkel, F. V. The relationship of insects to the deterioration of stored grain by fungi. *Int. J. Food Microbiol.* **1988**, 7, 227–244, doi:10.1016/0168-1605(88)90042-6.
 53. Cao, A.; Santiago, R.; Ramos, A.J.; Marín, S.; Reid, L.M.; Butrón, A. Environmental factors related to fungal infection and fumonisin accumulation during the development and drying of white maize kernels. *Int. J. Food Microbiol.* **2013**, 164, 15–22, doi:10.1016/j.ijfoodmicro.2013.03.012.

54. Fandohan, P.; Hell, K.; Marasas, W.F.O.; Wingfield, M.J. Infection of maize by *Fusarium* species and contamination with fumonisin in Africa. *African J. Biotechnol.* **2003**, *2*, 646–665.
55. Sinha, R.P. *Food in India: An analysis of the prospects for self-sufficiency by 1975-76.*; Oxford University Press: London, 1961;
56. Quellhorst, H.E.; Athanassiou, C.G.; Bruce, A.; Scully, E.D.; Morrison, I.W.R. Temperature-mediated competition between the invasive larger grain borer (Coleoptera: Bostrichidae) and the cosmopolitan maize weevil (Coleoptera: Curculionidae). *Environ. Entomol.* **2020**, *49*, 255–264.
57. Khan, T.; Shahid, A.A.; Khan, H.A.A. Could biorational insecticides be used in the management of aflatoxigenic *Aspergillus parasiticus* and its insect vectors in stored wheat? *PeerJ* **2016**, *4*, e1665, doi:10.7717/peerj.1665.
58. Nesvorná, M.; Gabrielová, L.; Hubert, J. Suitability of a range of *Fusarium* species to sustain populations of three stored product mite species (Acari: Astigmata). *J. Stored Prod. Res.* **2012**, *48*, 37–45, doi:10.1016/j.jspr.2011.08.006.
59. Webster, B.; Bruce, T.; Pickett, J.; Hardie, J. Volatiles functioning as host cues in a blend become nonhost cues when presented alone to the black bean aphid. *Anim. Behav.* **2010**, *79*, 451–457, doi:10.1016/j.anbehav.2009.11.028.
60. Bruce, T.J.A.; Pickett, J.A. Perception of plant volatile blends by herbivorous insects - Finding the right mix. *Phytochemistry* **2011**, *72*, 1605–1611, doi:10.1016/j.phytochem.2011.04.011.

61. Gerken, A.R.; Scully, E.D.; Campbell, J.F. Red flour beetle (Coleoptera: Tenebrionidae) response to volatile cues varies with strain and behavioral assay. *Environ. Entomol.* **2018**, *47*, 1252–1265, doi:10.1093/ee/nvy107.
62. Balakrishnan, K.; Holighaus, G.; Weißbecker, B.; Schütz, S. Electroantennographic responses of red flour beetle *Tribolium castaneum* Herbst (Coleoptera: Tenebrionidae) to volatile organic compounds. *J. Appl. Entomol.* **2017**, *141*, 477–486, doi:10.1111/jen.12366.
63. Balakrishnan, K. Olfactory responses of two coleopteran species: The stored product pest *Tribolium castaneum* and the forest pest predator *Dastarcus helophoroides*, Universität Göttingen, 2019.
64. Szendrei, Z.; Rodriguez-Saona, C. A meta-analysis of insect pest behavioral manipulation with plant volatiles. *Entomol. Exp. Appl.* **2010**, *134*, 201–210, doi:10.1111/j.1570-7458.2009.00954.x.
65. Athanassiou, C.; Bray, D.P.; Hall, D.R.; Phillips, C.; Vassilakos, T.N. Factors affecting field performance of pheromone traps for tobacco beetle, *Lasioderma serricorne*, and tobacco moth, *Ephestia elutella*. *J. Pest Sci. (2004)*. **2018**, *91*, 1381–1391, doi:10.1007/s10340-018-0987-8.
66. Sammani, A.M.P.; Dissanayaka, D.M.S.K.; Wijayaratne, L.K.W.; Morrison, W.R. Effect of pheromone blend components, sex ratio, and population size on the mating of *Cadra cautella* (Lepidoptera: Pyralidae). *J. Insect Sci.* **2020**, *20*, 30, doi:10.1093/jisesa/ieaa128.
67. Leskey, T.C.; Khrimian, A.; Weber, D.C.; Aldrich, J.C.; Short, B.D.; Lee, D.-H.; Morrison, W.R. Behavioral responses of the invasive *Halyomorpha halys* (Stål) to traps baited with stereoisomeric mixtures of 10,11-epoxy-1-bisabolen-3-ol. *J. Chem. Ecol.* **2015**, *41*, 418–429, doi:10.1007/s10886-015-0566-x.

68. Weber, D.C.; Morrison, W.R.; Khrimian, A.; Rice, K.B.; Short, B.D.; Herlihy, M. V.; Leskey, T.C.; Nielsen, A. Attractiveness of pheromone components with and without the synergist, methyl (2E,4E,6Z)-2,4,6-decatrienoate, to brown marmorated stink bug (Hemiptera: Pentatomidae). *J. Econ. Entomol.* **2020**, *113*, 712–719, doi:10.1093/jee/toz312.
69. Morrison, W.R.; Grosdidier, R.F.; Arthur, F.H.; Myers, S.W.; Domingue, M.J. Attraction, arrestment, and preference by immature *Trogoderma variabile* and *Trogoderma granarium* to food and pheromonal stimuli. *J. Pest Sci. (2004)*. **2020**, *93*, 135–147, doi:10.1007/s10340-019-01171-z.
70. Domingue, M.J.; Morrison, W.R.; Yeater, K.; Myers, S.W. Oleic acid emitted from frozen *Trogoderma* spp. larvae causes conspecific behavioral aversion. *Chemoecology* **2020**, *30*, 161–172, doi:10.1007/s00049-020-00307-3.
71. Guo, Z.; Döll, K.; Dastjerdi, R.; Karlovsky, P.; Dehne, H.W.; Altincicek, B. Effect of fungal colonization of wheat grains with *Fusarium* spp. on food choice, weight gain and mortality of meal beetle larvae (*Tenebrio molitor*). *PLoS One* **2014**, *9*, e100112, doi:10.1371/journal.pone.0100112.
72. Jian, F. Influences of stored-product insect movements on integrated pest management decisions. *Insects* **2019**, *10*, 100, doi:10.3390/insects10040100.
73. Morrison, W.R.; Larson, N.L.; Brabec, D.; Zhang, A. Methyl benzoate as a putative alternative, environmentally friendly fumigant for the control of stored-product insects. *J. Econ. Entomol.* **2019**, *112*, 2458–2468, doi:10.1093/jee/toz179.
74. Janković-Tomanić, M.; Petković, B.; Todorović, D.; Vranković, J.; Perić-Mataruga, V. Physiological and behavioral effects of the mycotoxin deoxynivalenol in *Tenebrio molitor* larvae. *J. Stored Prod. Res.* **2019**, *83*, 236–242, doi:10.1016/j.jspr.2019.07.008.

75. Wilkins, R.V.; Zhu, K.Y.; Campbell, J.F.; Morrison, W.R. Mobility and dispersal of two cosmopolitan stored-product insects are adversely affected by long-lasting insecticide netting in a life stage-dependent manner. *J. Econ. Entomol.* **2020**, *In press*, doi:<https://doi.org/10.1093/jee/toaa094>.
76. Trebels, B.; Dippel, S.; Schaaf, M.; Balakrishnan, K.; Wimmer, E.A.; Schachtner, J. Adult neurogenesis in the mushroom bodies of red four beetles (*Tribolium castaneum*, Herbst) is influenced by the olfactory environment. *Sci. Rep.* **2020**, *10*, 1090.
77. Maille, J.; Gerken, A.; Adrianos, S.; Arthur, F.; Campbell, J.; Oppert, B.; Perkin, L.; Scheff, D.; Morrison, I.W.; Scully, E. Exploiting chemosensory genomics for improved monitoring and control of stored product pests. *Insects* **2021**, *In press*.
78. Begerow, D.; Nilsson, H.; Unterseher, M.; Maier, W. Current state and perspectives of fungal DNA barcoding and rapid identification procedures. *Appl. Microbiol. Biotechnol.* **2010**, *87*, 99–108, doi:[10.1007/s00253-010-2585-4](https://doi.org/10.1007/s00253-010-2585-4).
79. Awater, S.; Fürstenau, B. The potential of host-specific volatiles from *Tribolium confusum* larval faeces for luring the ectoparasitoid *Holepyris sylvanidis*. In Proceedings of the 12th International Working Conference on Stored Product Protection (IWCSPP) in Berlin, Germany, October 7-11; 2018; pp. 139–143.
80. Bartelt, R.J.; Wicklow, D.T. Volatiles from *Fusarium verticillioides* (Sacc.) Nirenb. and their attractiveness to nitidulid beetles. *J. Agric. Food Chem.* **1999**, *47*, 2447–2454, doi:[10.1021/jf9901340](https://doi.org/10.1021/jf9901340).
81. Cui, K.; He, L.; Cui, G.; Zhang, T.; Chen, Y.; Zhang, T.; Mu, W.; Liu, F. Biological activity of trans-2-hexenal against the storage insect pest *Tribolium castaneum* (Coleoptera:

- Tenebrionidae) and mycotoxigenic storage fungi. *J. Econ. Entomol.* **2021**, *in press*, 1–9, doi:10.1093/jee/toab001.
82. Dolinski, M.G.; Loschiavo, S.R. The effect of fungi and moisture on the locomotory behavior of the rusty grain beetle, *Cryptolestes ferrugineus* (Coleoptera: Cucujidae). *Can. Entomol.* **1973**, *105*, 485–490.
83. Fürstenau, B.; Adler, C.; Schulz, H.; Hilker, M. Host habitat volatiles enhance the olfactory response of the larval parasitoid *Holepyris sylvanidis* to specifically host-associated cues. *Chem. Senses* **2016**, *41*, 611–621.
84. Germinara, G.S.; De Cristofaro, A.; Rotundo, G. Bioactivity of short-chain aliphatic ketones against adults of the granary weevil, *Sitophilus granarius* (L.). *Pest Manag. Sci.* **2012**, *68*, 371–377, doi:10.1002/ps.2272.
85. Germinara, G.S.; Conte, A.; De Cristofaro, A.; Lecce, L.; Di Palma, A.; Rotundo, G.; Del Nobile, M.A. Electrophysiological and behavioral activity of (E)-2-hexenal in the granary weevil and its application in food packaging. *J. Food Prot.* **2012**, *75*, 366–370, doi:10.4315/0362-028X.JFP-11-142.
86. Green, P.W.C. Substrate selection by *Liposcelis bostrychophila* Badonnel (Psocoptera: Liposcelididae): effects of insect extracts and biodeteriorated book-paper. *J. Stored Prod. Res.* **2005**, *41*, 445–454, doi:10.1016/j.jspr.2004.05.004.
87. Green, P.W.C. Fungal isolates involved in biodeterioration of book-paper and their effects on substrate selection by *Liposcelis bostrychophila* (Badonnel) (Psocoptera: Liposcelididae). *J. Stored Prod. Res.* **2008**, *44*, 258–263, doi:10.1016/j.jspr.2008.01.003.

88. Kao, S.-S.; Dunkel, F. V.; Harein, P.K. Behavioral response of the larger black flour beetle (Coleoptera: Tenebrionidae) to olfactory cues from food sources. *J. Econ. Entomol.* **1984**, *77*, 110–112, doi:10.1093/jee/77.1.110.
89. Ndomo-Moualeu, A.F.; Ulrichs, C.; Adler, C. Behavioral responses of *Callosobruchus maculatus* to volatile organic compounds found in the headspace of dried green pea seeds. *J. Pest Sci. (2004)*. **2016**, *89*, 107–116, doi:10.1007/s10340-015-0652-4.
90. Phelan, P.L.; Lin, H. Chemical characterization of fruit and fungal volatiles attractive to dried-fruit beetle, *Carpophilus hemipterus* (L.) (Coleoptera: Nitidulidae). *J. Chem. Ecol.* **1991**, *17*, 1253–1272, doi:10.1007/BF01402948.
91. Pierce, A.M.; Borden, J.H.; Oehlschlager, A.C. Olfactory response to beetle-produced volatiles and host-food attractants by *Oryzaephilus surinamensis* and *O. mercator*. *Can. J. Zool.* **1981**, *59*, 1980–1990, doi:10.1139/z81-270.
92. Pierce, A.M.; Borden, J.H.; Oehlschlager, A.C. Effects of age and population density on response to beetle and food volatiles by *Oryzaephilus surinamensis* and *O. mercator* (Coleoptera: Cucujidae). *Environ. Entomol.* **1983**, *12*, 1367–1374, doi:10.1093/ee/12.5.1367.
93. Pierce, A.M.; Pierce, H.D.; Oehlschlager, A.C.; Borden, J.H. Attraction of *Oryzaephilus surinamensis* (L.) and *Oryzaephilus mercator* (Fauvel) (Coleoptera: Cucujidae) to some common volatiles of food. *J. Chem. Ecol.* **1990**, *16*, 465–475, doi:10.1007/BF01021778.
94. Pierce, A.M.; Pierce, H.D.; Oehlschlager, A.C.; Borden, J.H. 1-Octen-3-ol, attractive semiochemical for foreign grain beetle, *Ahasverus advena* (Waltl) (Coleoptera: Cucujidae). *J. Chem. Ecol.* **1991**, *17*, 567–580, doi:10.1007/BF00982127.
95. Rodríguez-González, Á.; Casquero, P.A.; Suárez-Villanueva, V.; Carro-Huerga, G.; Álvarez-García, S.; Mayo-Prieto, S.; Lorenzana, A.; Cardoza, R.E.; Gutiérrez, S. Effect of

- trichodiene production by *Trichoderma harzianum* on *Acanthoscelides obtectus*. *J. Stored Prod. Res.* **2018**, 77, 231–239, doi:10.1016/j.jspr.2018.05.001.
96. Starratt, A.N.; Loschiavo, S.R. Aggregation of the confused flour beetle, *Tribolium confusum*, elicited by mycelial constituents of the fungus *Nigrospora sphaerica*. *J. Insect Physiol.* **1971**, 17, 407–414.
97. Starratt, A.N.; Loschiavo, S.R. Aggregation of the confused flour beetle, *Tribolium confusum* (Coleoptera: Tenebrionidae) elicited by fungal triglycerides. *Can. Entomol.* **1972**, 104, 757–759.
98. Steiner, S.; Erdmann, D.; Steidle, J.L.M.; Ruther, J. Host habitat assessment by a parasitoid using fungal volatiles. *Front. Zool.* **2007**, 4, 3, doi:10.1186/1742-9994-4-3.
99. Tsai, W.T.; Mason, L.J.; Woloshuk, C.P. Effect of three stored-grain fungi on the development of *Typhaea stercorea*. *J. Stored Prod. Res.* **2007**, 43, 129–133, doi:10.1016/j.jspr.2006.01.001.
100. Vanhaelen, M.; Vanhaelen-Fastré, R.; Geeraerts, J.; Wirthlin, T. Cis- and trans-octa-1,5-dien-3-ol, new attractants to the cheese mite *Tyrophagus putrescentiae* (Schrank) (Acarina, Acaridae) identified in *Trichothecium roseum* (Fungi imperfecti). *Microbios* **1978**, 23, 199–212.
101. Vanhaelen, M.; Vanhaelen-Fastré, R.; Geeraerts, J. Occurrence in mushrooms (Homobasidiomycetes) of cis- and trans-octa-1,5-dien-3-ol, attractants to the cheese mite *Tyrophagus putrescentiae* (Schrank) (Acarina, Acaridae). *Experientia* **1980**, 36, 406–407, doi:10.1007/BF01975114.
102. Herrera, J.M.; Pizzolitto, R.P.; Zunino, M.P.; Dambolena, J.S.; Zygadlo, J.A. Effect of fungal volatile organic compounds on a fungus and an insect that damage stored maize. *J. Stored Prod. Res.* **2015**, 62, 74–80, doi:10.1016/j.jspr.2015.04.006.

Chapter 2 - Grain inoculated with different growth stages of the fungus, *Aspergillus flavus*, affect the close-range foraging behavior by a primary stored product pest, *Sitophilus oryzae* (Coleoptera: Curculionidae)

Published as: Ponce, M. A., Lizarraga, S., Bruce, A., Kim, T. N., & Morrison III, W. R. (2022). Grain Inoculated with Different Growth Stages of the Fungus, *Aspergillus flavus*, Affect the Close-Range Foraging Behavior by a Primary Stored Product Pest, *Sitophilus oryzae* (Coleoptera: Curculionidae). *Environmental Entomology*, 51(5), 927-939.

<https://doi.org/10.1093/ee/nvac061>

Abstract

Although some research has investigated the interactions among stored product insects and microbes, little research has examined how specific fungal life stages affect volatile emissions in grain and linked it to the behavior of *Sitophilus oryzae*, the cosmopolitan rice weevil. Thus, our goals were to 1) isolate, culture, and identify two fungal life stages of *Aspergillus flavus*, 2) characterize the volatile emissions from grain inoculated by each fungal morphotype, and 3) understand how microbially-produced volatile organic compounds (MVOCs) from each fungal morphotype affect foraging, attraction, and preference by *S. oryzae*. We hypothesized that the headspace blends would be unique among our treatments and that this will lead to preferential mobility by *S. oryzae* among treatments. Using headspace collection coupled with GC-MS, we found the sexual life stage of *A. flavus* had the most unique emissions of MVOCs compared to the other semiochemical treatments. This translated to a higher interaction with kernels containing grain with the *A. flavus* sexual life stage, as well as a higher cumulative time spent in those zones by *S. oryzae* in a video-tracking assay in comparison to the asexual life stage. While fungal cues were important for foraging at close-range, the release-recapture assay indicated that grain volatiles were more important for attraction at longer distances. There was no significant preference between grain and MVOCs in a four-way olfactometer. Overall, this study enhances our understanding of how fungal cues affect the close and longer range foraging ecology of a primary stored product insect.

Introduction

Insects and microbes represent the two largest threats to the post-harvest supply chain exceeding \$100 US billion losses annually worldwide (Wacker 2018). With the world's population expected to exceed 9 billion people by 2050 (FAO 2018), it is imperative that agriculture increases its efficiency by roughly 70% (Ray et al. 2013). Insects and microbes can cause decreased yield after harvest through both changes in the quantity and quality of commodities. Controlling these pests after harvest represents an easier method of meeting food production targets than growing more food, because one must only prevent the loss of already existing products during the post-harvest supply chain.

Crops both before and after harvest are vulnerable to a variety of microbes that infest and damage commodities throughout the agricultural system. Key stored product pathogens after harvest include fungi belonging to *Aspergillus*, *Fusarium*, *Penicillium*, and *Alternaria* spp. (Mannaa and Kim 2017). Some strains of these fungal species naturally produce mycotoxins, which are toxins secreted by hyphae that can contaminate the food supply for animals (Magan and Olsen 2004). Different mycotoxins may be produced by different taxa and life stages, including aflatoxins, ochratoxins, fumonisins, and deoxynivalenol, among others (Sulyok et al. 2010). In agriculture, mycotoxin contamination is responsible for approximately \$1 billion USD in losses (Amaiike and Keller 2011). The presence of aflatoxin has been linked to numerous health issues in humans and livestock (Hubert et al. 2018). As a result, microbes intimately affect the post-harvest supply chain and the health of end consumers and animals.

A widespread and common fungal pathogen of stored grain is *Aspergillus flavus* Link (Eurotiales: Trichocomaceae) (Tang et al. 2018) (Supplemental Figure 1). This microbe causes significant economic losses after harvest (e.g. Magan et al. 2003) as a result of mycotoxin

contamination and associated health hazards. Contamination by *A. flavus* and other fungal species may result in complaints by consumers of off-odor or musty grain (Fleurat-Lessard 2017). *Aspergillus flavus* can be identified by its morphology. For example, asexual stages of infestation will be white and green, with visible spores, and as it vegetatively grows, old areas appear darker green. The asexual fungus produces hyphae, which is a filamentous structure that secretes enzymes to break down nutrients (Horn et al. 2009). By contrast, the sexual stages are whitish in coloration and produce spores through meiosis. It can produce spores asexually or through sexual reproduction, depending on environmental conditions. Prior work has shown that different microbial growth stages (e.g., sexual vs. asexual) of other fungal species may result in the production of different microbially-produced volatile organic compounds (MVOCs) that differ in both abundance and composition (Misztal et al. 2018). However, this has never been evaluated for *A. flavus*.

Simultaneously, post-harvest pest management is made more complicated by insect infestation. Among the most pernicious insect pests of stored bulk grains is *Sitophilus oryzae* (L.) (Coleoptera: Curculionidae), the rice weevil. This species is a cosmopolitan primary pest (Hagstrum and Subramanyam 2006) and a full generation generally requires 32 d at 17.2°C. While bin aeration can be used to help slow development of this species (Arthur et al. 2020), this often fails in warmer climates (Morrison et al. 2020a, 2021a), and the most common remedial action is fumigation with phosphine (e.g., Navarro 2006). The overuse of this fumigant has caused an increase in insecticide resistance (e.g., Konemann et al. 2017; Nayak et al. 2020). Additionally, there is increasing consumer demand for little or no insecticide inputs throughout the supply chain (Batte et al. 2007). As a result, *S. oryzae* is an economically important pest whose management requires the evaluation of alternative pest management tactics to minimize

losses while reducing insecticide use. Unfortunately, little is known about how *S. oryzae* interacts with the microbial community in grain (Supplemental Figure 2), with no published study investigating complex headspaces or pure microbial volatiles (e.g. Ponce et al. 2021).

One broad class of alternative tactics to potentially target *S. oryzae* includes semiochemical-mediated, behaviorally-based management, whereby pest or natural enemy behavior is manipulated from a distance for improved integrated pest management (e.g., Morrison et al. 2021b). This approach exploits the fact that insects have highly developed, sophisticated chemosensory systems (Sánchez-García et al. 2017), which allow them to navigate an environment filled with odors (Maille et al. 2021). In food facilities, MVOCs may be especially advantageous cues to manipulate agricultural (Beck and Vannette 2017) or stored product insects' behavior. For example, because food facilities try to eliminate any areas of microbial contamination, and are extremely dry, MVOCs may be relatively unique signals amongst a large backdrop of otherwise ubiquitous food and grain cues (Morrison et al. 2021b). In addition, it is assumed that before the advent of agriculture, stored product insects evolved to seek out and exploit caches of food by a diversity of animals, including insects and small mammals (Linsley 1944). However, with imprecise spatial memory by animals that may lose track of up to a third of caches (MacDonald 1997), fungal colonization of seeds seems to have been likely associated with caches used by current-day stored product insects. As a result, it is possible that there will be a conserved behavioral response to some fungal cues by stored product insects across taxa. However, there is a relative lack of knowledge of insect-microbe interactions in the post-harvest supply chain compared to other groups associated with plants (reviewed in Biere and Bennett 2013). Among the stored product insects, especially *S. oryzae* has been neglected in outsized proportion to its importance (e.g., Ponce et al. 2021).

While there has generally been less work done in the post-harvest supply chain, there is at least some evidence suggesting that MVOCs modulate the behavior of stored product insects (Vanhaelen et al. 1978). Some microbes produce volatiles that may attract stored product insects. For example, *Carpophilus hemipterus* L. (Coleoptera: Nitidulidae), dried fruit beetle, seek out bananas inoculated with yeast by volatiles emitted from fruit and through fungal decomposition (Phelan and Lin 1991). In a few studies, *Aspergillus flavus* was found to be the preferred egg-laying substrate for both the *Typhaea stercorea* L. (Coleoptera: Mycetophagidae), the hairy fungus beetle (Tsai et al. 2007) as well as *Amyelois transitella* Walker (Lepidoptera: Pyralidae), the navel orange worm (Beck and Higbee 2015). Furthermore, five species of stored product beetles in the family Cucujidae were attracted to specific synthetic MVOCs (Pierce et al. 1991). Ponce et al. (2021) found that five, four, and 13 stored product arthropods were significantly attracted, repelled, and unaffected by emission of MVOCs from over 10 fungal taxa. With a more comprehensive understanding of these insect-microbe interactions, we could potentially use targeted MVOCs in behaviorally-based tactics. Thus, the goal of this study was to elucidate the behavioral response of *S. oryzae* to emissions of asexual or sexual strains of *A. flavus*, specifically focusing on 1) preference, 2) close-range movement, and 3) the ability to attract from a distance. We also identified each fungal morphotype using DNA sequencing and used GC-MS to link behavioral changes to altered headspace volatile emissions from asexual and sexual *A. flavus* life stages.

Materials and Methods

Experimental Insects and Source of Grain

Beetles used in this study were obtained from stock colonies kept in the laboratory of the USDA Agricultural Research Service's (ARS) Center for Grain and Animal Health Research (CGAHR) situated in Manhattan, Kansas, USA. Colonies of *S. oryzae* (field-derived strain Hudson, Kansas in 2012) were reared on organic whole kernel wheat after tempering it to 15% grain moisture from 10.6% moisture. Colonies with 4- to 8-week-old adults were kept in 800-mL mason jars (8.5 D × 17 cm H) and stored in an environmental chamber (136VL, Percival Instruments, Perry, IA, USA) set at constant conditions (27.5°C, 60% RH, and 14:10 L:D). Whole wheat grain used for colonies and inoculations below were obtained from a local Kansas farmer and held in cold storage (~4°C) at the USDA-ARS Center for Grain and Animal Health Research (CGAHR) for ~3 years until needed for experiments.

Fungal Isolation, Culturing, and Identification

Sets of 25 (100 × 15 mm) agar petri dishes were prepared by mixing 34 g of potato dextrose agar (Neogen, Lansing, MI, USA) with 800 mL of deionized water in a 1 L glass media container. A sterile stirring magnet was placed inside the container to mix the solution for 10 min. Afterwards, the agar solution was placed in the autoclave (Model 533LS, Getinge, Wayne, NJ, USA) for 30 min to steam-sterilize the solution. Autoclave tape was placed on all glass to confirm success in the sterilization process. Subsequently, the agar solution was stirred for 20 min on a hot plate. Agar was poured into petri dishes (100 × 10 mm) within a certified biosafety cabinet (Labconco, Purifier Biosafety Cabinet, Kansas City, KS) after interior surfaces were sanitized with 70% Ethanol and 10 min of exposure to high intensity UV light. The agar solution was allowed to solidify over 24 h.

Wheat kernels from grain stores held in cold storage (e.g. 4°C) at the USDA-ARS CGAHR were placed on the potato dextrose starch agar plates with four kernels placed equidistantly from each other in the four quadrants of a plate and incubated at 30°C and 65% RH to promote fungal growth and development. After 7 d, fungi were excised, then subcultured from individual isolates onto a new plate to obtain a pure culture. The two fungal morphotypes were initially identified by close examination of their morphological characteristics such as spore shape, size, and color as well as the morphology of hyphae using a high-powered dissecting microscope (SMZ18 Nikon Inc., Tokyo, Japan). Morphotype designations M1 (e.g., morphotype 1) and M2 (eg., morphotype 2) were initially assigned to each fungal life stage through this process.

Fungal identification was confirmed with DNA isolation and the use of polymerase chain reaction (PCR). Both fungal morphotypes (the asexual stage of *A. flavus* or M1 and the sexual stage of *A. flavus* or M2) were removed from infested wheat kernels by scrapping the mycelium (in excess) from a pure culture on a Petri dish into microcentrifuge tubes with a sterilized dissecting needle. DNA was extracted using the Norgen Biotek Corp. plant/fungal DNA isolation kit (E5038, Ontario, Canada,). The ITS region of the fungal DNA was then amplified using the primer ITS4 5'-TCCTCCGCTTATTGATATGC-3' and ITS5 5'-

GGAAGTAAAAGTCGTAACAAGG-3' (White et al. 1990) for asexual *A. flavus* and 5'-AACTCCCAAACCCCTGTGAACATA-3' and 5'-TTTAACGGCGTGGCCGC-3' for sexual *A. flavus*. Each reaction consisted of 1 µl of extracted DNA, 1 µl of each primer (10 µM), 9.5 µl of nuclease-free water, and 12.5 µl of master mix containing 50 units/ml of Taq DNA polymerase (Hot Start Taq 2X Master Mix, Promega, Madison, WI) in a proprietary reaction buffer (pH 8.5). The PCR program consisted of 2 min of initial denaturation at 95°C, followed by 30 cycles of

95°C for 30 s, 55°C for 1 min, and 72°C for 1.5 min. Afterward, a final extension at 72°C for 5 min was performed, then PCR products were held chilled at 4°C. A total of 5 µl of PCR products were mixed with 2 µl of ExoSAP-it (ThermoFisher Scientific, Waltham, MA) for DNA clean-up, then placed in the thermal cycler at 37°C for 15 min, and ramped to 80°C for 15 min. Finally, the amplicons were sent for bidirectional sequencing on an ABI 3730XL instrument (Eurofins Scientific, Brussels, Belgium), and the resulting sequences were quality-filtered and aligned using Sequencher (v. 5.4.6, Gene Codes, Ann Arbor, MI). The consensus sequences were searched against NCBI's nucleotide database (nt) using the BLASTn algorithm (Altschul et al. 1997). In order to circumvent taxonomic misassignments, the consensus sequences were also checked against Michigan State's Ribosomal Database Project (RDP) that searches the UNITE Database (Wang et al. 2007). The consensus sequences were submitted to GenBank under the access numbers OK361788-OK361791.

Treatments

In the assays described below, we analyzed the behavioral response of *S. oryzae* to five different blends of semiochemicals found and introduced in wheat (Table 1). Briefly, these included no stimuli (negative control), UV-sanitized grain, clean grain from storage (unmanipulated, positive control), as well as grain from storage inoculated with fungal morphotype 1 (M1, identified as the asexual life stage of *Aspergillus flavus*) and fungal morphotype 2 (M2, identified as the sexual life stage of *A. flavus*). Fresh samples of semiochemicals were used for each day of testing for each assay. In order to prevent cross-contamination, 300 g of grain (tempered to 15% grain moisture) was initially sanitized using UV for 20 min. This procedure was done before inoculating grain with either morphotype 1 or 2. The 300 g of grain was kept in a sanitized mason jar (8.5 D × 17 cm H). To inoculate grain with the

two different morphologies, we scraped an entire isolation from a petri dish into the 300 g of grain. Each isolation was ~1 week old and completely colonized by the given morphotype. After inoculation, each treatment was placed in an environmental chamber (136VL, Percival Instruments, Perry, IA, USA) set at constant conditions (30°C, 65% RH, and 14:10 L:D). This procedure was the same for both morphologies and was done every 2 weeks to ensure fresh treatments for each experimental assay.

Four-way still-air olfactometer

To evaluate the preference among the various grain and fungal volatiles by *S. oryzae* a four-way still-air olfactometer was employed (Figure 1). The apparatus consisted of a central release chamber of glass (12.1 cm height × 9 cm diameter) with the bottom etched by acid to provide a roughened surface over which insects could walk. There were four passageways (3.5 cm long × 2.5 cm diameter) spaced at 90° angles connecting the main release chamber at ground level to four odor chambers. The odor chambers (11.5 cm height × 7 cm diameter) also had a single passageway (5.0 cm length × 1.5 cm diameter) protruding from the main chamber whose end was covered with a porous glass barrier that allowed diffusion of volatiles but prevented entry by insects in the first round of testing, and which had two equally spaced small holes in the second round of testing to ensure volatiles were not blocked. The odor and release chambers were connected with a glass tube (7.5 cm length × 2.2 diameter) attached on both sides with a PTFE (polytetrafluoroethylene) screw and septum. The final distance between the release and the odor chambers was 8.9 cm, and the apparatus was held in place by PTFE parts designed to fit the release and odor chambers. The following treatments were compared to assess preference by adding 10 g of each into one of the four odor chambers: positive control (clean, unsanitized grain), grain inoculated with morphotype 1 (asexual *Aspergillus flavus*), grain inoculated with

morphotype 2 (sexual *A. flavus*), and UV-sterilized grain (Table 1). The position of the treatments was rotated every five replicates. Insects were individually introduced into the central release chamber and given 3 min to make a choice. A choice was considered to have been made when the insect left the release chamber and traveled 2.54 cm up one of the adjoining passages. Non-responders were excluded from the final data analysis, as is common practice in behavioral research (e.g., Morrison et al., 2020b; Wilkins et al., 2020). A total of $n = 240$ replicate insects were evaluated in each round of testing. Bioassays were conducted in a BSL2 biosafety cabinet (Labconco, Kansas City, MO, USA) to prevent diffusion of ambient odors or landmarks from affecting results. Every ten replicates, all glassware, including connectors, screw caps, and septa, were washed first with methanol, then hexane, and allowed to dry before reuse. The side chosen by an individual, and time it took to make a decision were recorded.

To analyze the data from the four-way olfactometer, a generalized linear model based on a Poisson distribution and log link function was used. The response was the counts of insects choosing a particular stimulus, with date of testing used as a random blocking variable. The explanatory variable included the odor treatments from Table 1. Overdispersion was checked and found not to be a problem. Upon a significant result from the model, multiple comparisons in a generalized linear model framework were implemented using the function *ghlt* (e.g., Tukey HSD) from the package *multcomp* in R Software (Hothorn et al. 2008). R software (R Core Team, 2019) was used for this and all other tests, with $\alpha = 0.05$ except where noted.

Ethovision

To examine how grain and microbial volatiles affect the mobility and orientation of *S. oryzae* at close range, we used video-tracking coupled with Ethovision software v.14.0 (Noldus, Inc., Leesburg, VA, USA: Noldus et al. 2002; Supplemental Figure 3). This system has been

used in the past for analyzing the mobility of stored product insects (Morrison et al. 2018, 2019b; Wilkins et al. 2020). A network video camera (GigE, Basler AG, Ahrensburg, Germany) was suspended using a pole and clamp 80 cm above test arenas, and the movement of five individuals was simultaneously livestreamed to an adjacent computer. Arenas consisted of Petri dishes (VWR® Petri dishes, 100 × 15 mm) with an 85-mm filter paper (Grade 1, Whatman™, GE Healthcare, Chicago, IL) adhered to the bottom using double-sided sticky tape. A thin ring of PTFE was used to coat the vertical surface of the test arena to prevent insect escape. In Ethovision, four zones were created, including two zones encompassing each half of the petri dish (treatment zone vs. control zone), and two smaller zones placed halfway and midway on each half (treatment kernel zone, and control kernel zone) (Supplemental Figure 3; Figure 2). Smaller kernel zones were 1.16 cm diameter. Control zones lacked stimuli, while treatment zones contained a single kernel of one of the treatments listed in Table 1. Treatments were compared to a side without a treatment, and the position of treatments was randomized between replicates. A single insect was introduced into an arena and its mobility was tracked for a period of 30 min. Several variables were tracked, including the cumulative or total distance moved (cm), instantaneous velocity (cm/s), the number of times that an insect enters each kernel zone, the cumulative time spent in the kernel zone (treatment vs. control), the time it took to first enter the kernel zone (s) (treatment vs. control), and the amount of time spent on each side of test arena (treatment vs. control). A total of n = 15 replicate individuals were tested per treatment.

To analyze how grain and microbial volatiles affected the mobility and orientation of the *S. oryzae* during video-tracking, a generalized linear model based on a Gaussian distribution with a log-link function was used. In particular, separate models were used for the distance moved, and instantaneous velocity as response variables, while the explanatory variable included the

odor treatment in each test arena (Table 1). An aggregate variable was used consisting of the frequency of interaction with the kernel, latency to finding the kernel, the cumulative time spent in the each half of the petri dish and each kernel zone. A multivariate analysis of variance (MANOVA) was then performed on the aggregate response variable using the semiochemical treatments as a fixed explanatory variable. Upon a significant overall result, sequential ANOVAs for each response variable were performed. For responses with significant ANOVAs, multiple comparisons were performed with Tukey HSD. Post-hoc t-tests were performed as a contrast between the two means of treatment vs. control zone and treatment vs. control kernel zone to assess differences within a semiochemical treatment with a Bonferroni-corrected alpha-threshold for multiple testing.

Small Release-Recapture Trapping Assay

To determine whether microbial volatiles may improve capture of *S. oryzae*, a trapping assay was conducted using plastic bins (Sterilite, Townsend, MA, USA: $87.9 \times 47.6 \times 32.1$ cm, L:W:H; Supplemental Figure 4A and 4B). The bottom surface of the plastic bin was roughened with sandpaper to allow for easy movement by insects, but a ring of polytetrafluoroethylene (PTFE) was placed at a height of 7.6 cm from the bottom on the vertical surfaces to prevent insects from escaping. A total of 20 *S. oryzae* were released in one corner of the bin. Diagonally across from the release point, a commercially-available pitfall trap (Dome Trap™, Trécé, Inc., Adair, OK, USA) was added. The pitfall trap consisted of two connectable pieces. The bottom half of the trap was rough along the ascending edges to create traction for the rice weevils, but a ring of PTFE was added halfway down on the vertical surface of the central well to prevent captured insects from escaping. The traps were baited with 10 g of one of the treatments in Table 1. The bins were located in a $5 \times 6 \times 2$ m L:W:H walk-in environmental chamber (Percival

Instruments, Dallas County, IA, USA) under constant conditions (25°C, 65% RH, 14:10 L:D). On a given round of release, two replicates of each treatment were performed simultaneously, and further replicates were accumulated over time. In total, there were $n = 16$ replicates for each treatment. The released *S. oryzae* were given 24 h to respond to the stimuli. The traps were then collected and the number of insects in each trap were recorded.

Large Release-Recapture Trapping Assay

Because there was a concern with saturation of the small bin with the volatile stimuli, a large release-recapture assay was conducted to measure long-distance attraction to each semiochemical treatment in a larger space (Supplemental Figure 4C). A $5 \times 6 \times 2$ m L:W:H walk-in environmental chamber (Percival Instruments, Dallas County, IA, USA) under constant conditions (25°C, 65% RH, 14:10 L:D) was used for this experiment. The chamber floor was covered with white paper that was taped down to easily locate *S. oryzae* and allow free movement. A strip of PTFE was also placed 25 cm above the floor of the chamber to discourage insects from climbing on the walls. The same pitfall traps (as above) (Dome Trap™, Trécé, Inc., Adair, OK, USA) were baited with 10 g of one of the treatments in Table 1 and placed equidistantly around the perimeter of the chamber. After each round of release, trap position for each treatment was rotated to reduce any positional bias. In each replicate, all five semiochemical treatments were used and 100 *S. oryzae* were released per replicate, with a total of $n = 10$ replicates. The released *S. oryzae* were given 24 h to respond to the stimuli. The traps were then collected and the number of insects in each trap were recorded.

To evaluate whether microbial volatiles may affect attraction to traps in the small and large release-recapture assays, generalized linear model based on a Poisson distribution above

were used with number of *S. oryzae* recaptured as the response variable and the explanatory variable consisting of the full range of treatments listed in Table 1.

Headspace Volatile Collection

To analyze how microbial inoculation changes the volatile blends emitted by grain, headspace collection using a push-only setup was used (Supplemental Figure 5). A total of 20 g of each of the odor treatments above (Table 1) was placed into a screw top headspace chamber (500 mL capacity; 10.2 × 12.7 cm D: H). Central air was scrubbed of background volatiles using an activated carbon filter, and flow was split into four lines and regulated using flow meters (Volatile Collection Systems, Gainesville, FL, USA). Chemically inert PTFE tubes were used to connect each piece of the system. A drip tip borosilicate glass tube packed with 20 mg of absorbent Poropaq-Q™ (Volatile Collection Systems, Gainesville, FL, USA) was added to the output point of the headspace chamber to adsorb volatiles with a stainless-steel screen (No. 316), borosilicate glass wool, and PTFE compression seal bracketing the adsorbent material. Volatiles were collected for 3 h, then volatiles were eluted with 150 µl of dichloromethane into 2-ml GC vials with 200-µl inserts (Phenomenex, Torrance, CA, USA) with the solvent pushed through by inert N₂ gas. After extraction, 1 µl of tetradecane (190.5 ng at 99% purity, MilliporeSigma, Burlington, MA) was added to each of the labeled vials using a microsyringe (7000 Series Modified Microliter™ Syringe, Hamilton Company, Inc., Reno, NV, USA) and was used as an internal standard for quantification of compounds. Between uses, all volatile collection traps were washed with 700 µl of dichloromethane in triplicate. All volatile collection vials were sealed with PTFE tape and stored at -4°C until GC-MS analysis, using standard methodology (Morrison et al. 2016b). Between each replicate, all glassware was washed first with methanol,

then hexane, and baked in a convection drying oven for 5 min prior to reuse. There was a total of $n = 7$ replicates per treatment.

Gas chromatography coupled with mass spectrometry

All headspace sample extracts were run on an Agilent 7890B gas chromatograph (GC) equipped with an Agilent Durabond HP-5 column (30 m length, 0.250 mm diameter and 0.25 μ m film thickness) with He as the carrier gas at a constant 5 mL/min flow and 39 cm/s velocity, which was coupled with an Agilent 5997B mass spectrometer (MS) single quadrupole detector. The compounds were separated by injecting 1 μ L of each sample at an inlet temperature of at 250°C under split mode into the machine at a 15:1 ratio. The oven program began at 60°C for 1 min followed by 10°C/min ramping to 300°C over 26.5 min, and subsequently held for 4 min at 300°C. After a solvent delay of 4 min, mass ranges between 50 and 550 atomic mass units were scanned. Preliminary identification of compounds from representative chromatograms were obtained by comparing sample spectral data with the NIST 17 library, by GC retention index (Adams, 2009) and calculated Kovats indices for compounds (Girard, 1996).

To characterize the presence of headspace volatiles from the extracts, raw peak areas were extracted from the gas chromatograms using MSD ChemStation v2.00 software (Agilent Technologies, Inc., Santa Clara, CA, USA). The emissions rate was calculated on a ng per gram of grain weight and per hour basis by using the ratio of the peak area for tetradecane to each headspace volatile. Background volatiles found in the negative control without grain were discarded from the other samples, since these represent transient background volatiles in the general vicinity of headspace collection but are not informative of differences among the treatments. In addition, volatiles only identified in a single sample were discarded, because they are not informative of similarities between groups of treatments. Pairwise Bray-Curtis

similarities were calculated between each headspace sample, and non-metric multi-dimensional scaling (NMDS) was used to visualize the differences in volatile emissions among treatments. A total of $n = 1,000$ permutations were used for the ordination procedure. Stress values for the NMDS procedure were < 0.1 , indicating that good interpretation was possible. An analysis of similarity (ANOSIM) was used to determine significant differences for headspace volatiles among odor treatment categories. ANOSIM calculates an R statistic that can vary from 1 to -1, with values above zero interpreted as greater dissimilarity between treatments than within and values below zero interpreted as greater dissimilarity within treatments than between treatments. A total of $n = 1,000$ permutations were performed for the test. For all multivariate statistics, the R Package *vegan* was used (Oksanen et al. 2019). To assess differences in the complexity of the headspace blends (e.g., number of compounds per treatment), pairwise chi-square tests were performed among treatments with the null hypothesis of an equal number of compounds identified from each treatment. To determine differences in overall quantitative emissions of volatiles, pairwise t-tests among means were performed using a Bonferroni-correction to the P-value.

Results

Four-way olfactometer assay

There were no significant differences between the four semiochemical stimuli ($\chi^2 = 2.9$; $df = 3$; $P = 0.39$; Figure 3A) when tested in the four-way olfactometer with fused porous glass barriers. *Sitophilus oryzae* chose each side 22 ± 2 – $27 \pm 3\%$ (means $\pm$ SE) of the time, but there was no significant preference among the positive control, UV, M1, and M2 sides. The side that *S. oryzae* also had chosen had no significant effect on the time to decision ($\chi^2 = 2.65$; $df = 3$; $P = 0.45$). In each case, it took *S. oryzae* an average of 60 ± 8 – 76 ± 7 s to choose a stimulus.

Additionally, there were also no significant differences between the semiochemical stimuli ($\chi^2 = 5.97$; $df = 3$; $P = 0.11$; Figure 3B) when tested in the four-way olfactometer with fused porous glass barriers containing holes. *Sitophilus oryzae* chose each side $18 \pm 5 - 35 \pm 6\%$ (means $\pm$ SE) of the time, but there was no significant preference among the positive control, negative control, UV, and M1 sides. The side that *S. oryzae* also had chosen had no significant effect on the time to decision ($\chi^2 = 6.87$; $df = 3$; $P = 0.07$). In each case, it took *S. oryzae* an average of $44 \pm 5 - 72 \pm 13$ s to choose a stimulus.

Ethovision Assay

The semiochemical treatments did not significantly affect the instantaneous velocity and the distance moved of *S. oryzae* (MANOVA: Approx. $F = 1.5$; $df = 4, 69$; $P > 0.22$; Figure 4A, 4B). The mean ($\pm$ SE) distance moved by *S. oryzae* ranged between 200 (± 28) cm when exposed to the positive control to 317 (± 44) cm in the negative control. By contrast, the semiochemical treatments significantly increased the frequency with which *S. oryzae* entered the kernel zones (MANOVA: Approx. $F = 5.2$; $df = 4, 69$; $P < 0.01$; Figure 5). However, the frequency of entrances to the control kernel zone, which lacked stimuli, were not significantly different among the treatments (ANOVA: $F = 1.5$; $df = 4, 69$; $P = 0.29$), but frequency of entrances by *S. oryzae* were significantly affected in the treatment kernel zone by the stimuli present ($F = 5.0$; $df = 4, 69$; $P < 0.01$). In particular, there were almost 2-fold and greater than 3-fold more entries into the treatment kernel zone for M2 compared to the negative control and positive control, respectively (Tukey HSD).

The semiochemical treatments did not significantly affect the cumulative duration that *S. oryzae* spent on each half of the overall test arena (MANOVA: Approx. $F = 1.7$; $df = 4, 69$; $P = 0.16$; Figure 6A). Individuals spent an average of 732 – 964 s and 821 – 947 s on halves of the

arena with stimuli and without stimuli, respectively. By contrast, the semiochemical treatments significantly affected the time spent in the smaller kernel zones (MANOVA: Approx. $F = 32.9$; $df = 4, 69$; $P < 0.0001$; Figure 6B). In particular, the semiochemical treatments significantly affected the time spent in the kernel zones with stimuli ($F = 32.3$; $df = 4, 69$; $P < 0.0001$), but not the kernel zones lacking stimuli ($F = 1.43$; $df = 4, 69$; $P = 0.23$). In the kernel zones, *S. oryzae* spent 12-fold more time in the M2 treatment than the corresponding treatment zone for the negative control, and 4–8-fold more time in the M2 treatment than in the kernel zones for the other semiochemical treatments (Tukey HSD, Figure 6B).

Release-Recapture Assays

In the small release recapture assay, traps baited with one of the four semiochemical treatments showed a significantly increased mean percent recapture of *S. oryzae* compared to the negative control ($\chi^2 = 62.2$; $df = 4$; $P < 0.0001$; Figure 7A). In particular, traps baited with UV-sterilized grain, the positive control, grain incubated with M1 or grain incubated with M2 had 19–23% greater captures than the negative control. Similarly, in the large release-recapture assay, semiochemical lure significantly affected recapture of *S. oryzae* ($\chi^2 = 10.1$; $df = 4$; $P < 0.05$; Figure 7B), with 6–7-fold greater recapture in traps baited with one of the four semiochemical stimuli compared to the negative control lacking a stimulus. The four semiochemical treatments were not significantly different among one another (Tukey HSD, Figure 7).

Headspace Assay

Overall, 35 compounds were tentatively identified in headspace. The headspace blends were significantly more complex for positive control ($\chi^2 = 4.00$; $df = 1$; $P < 0.05$), asexual *A. flavus* (M1)-inoculated ($\chi^2 = 9.80$; $df = 1$; $P < 0.001$), and sexual *A. flavus* (M2)-inoculated grain ($\chi^2 =$

14.3; $df = 1$; $P < 0.001$) compared to UV-sterilized grain (Table 2). A total of 12 compounds were present in the UV-sterilized grain, with a single compound, 1,4-diethyl-benzene comprising 89% of the total emissions (Table 2). The positive control with untreated grain, by contrast, had almost 2-fold more compounds (e.g. 22), but with similar main components; together, 1,4- and 1,2-diethyl-benzene comprised 50% of the headspace emissions. The fungal-inoculated grain exhibited even more complex emission patterns, but mostly among the minor components. For example, asexual *A. flavus* (M1)-inoculated grain contained 22 compounds, with 1,4-diethyl-benzene and 1,2-diethyl-benzene comprising 87.3% of total emissions, whereas 20 compounds comprised the remaining 13%. Likewise, sexual *A. flavus* (M2)-inoculated grain contained 22 compounds, with just three compounds (e.g. 1,4-diethyl-benzene; 1,2-diethyl-benzene; (Z)-9-octadecenamide; Table 2) comprising 77% of emissions. Interestingly, fungal-inoculated grain also emitted quantitatively more volatiles. For instance, asexual *A. flavus* (M1)-inoculated grain emitted a 5–6-fold greater amount of volatiles than UV-sterilized ($t = 17.0$; $df = 12$; $P < 0.0001$) and the positive control grain ($t = 16.9$; $df = 12$; $P < 0.0001$). Likewise, sexual *A. flavus* (M2)-inoculated grain emitted an 6–7-fold greater amount of volatiles than UV-sterilized ($t = 30.3$; $df = 12$; $P < 0.0001$) and the positive control grain ($t = 31.1$; $df = 12$; $P < 0.0001$). Overall, while there was significant overlap in the composition of the treatments, they were significantly different from each other ($R = 0.200$; $Perm = 1000$; $P < 0.001$; $Stress < 0.10$; Figure 8), suggesting that inoculation with fungi affected composition of volatile emissions from the grain.

Discussion

For the first time in stored products, we specifically manipulated the fungal community present on the grain to determine how it affects the behavioral response by the primary pest, *S.*

oryzae. Our tests explicitly tested different aspects of behavior, including preference (e.g., 4-way olfactometer), close-range foraging decisions (Ethovision), and long-distance attraction (release-recapture assays), contributing to a fuller understand of the behavioral response by *S. oryzae*. Overall, we have shown that manipulating the fungal community resulted in altered emissions of headspace volatiles, and shifting fungal cues affected the foraging decisions of *S. oryzae* when in close proximity to grain inoculated with the sexual strain of *A. flavus* but not at a distance. At close proximity, this suggests that the behavioral response by *S. oryzae* may be prompted by either gustatory or odorant receptors, and future work should attempt to identify which are the primary modes of chemoreception. It appears MVOCs may not be as important for long-distance foraging to *S. oryzae* as they are to other insects (Davis et al. 2013). In fact, traps baited with cultures of yeast (e.g., *Aureobasidium pullulans*) attracted 39 other species of insects from seven orders and captured 481% more insects than non-baited traps outside in agricultural landscape (Davis and Landolt 2013). In particular, our work adds to the evidence that stored product insects use fungal cues at close-range to inform foraging decisions. For instance in other postharvest weevils, the fungi *Fusarium verticillioides* has shown repellency against *Sitophilus zeamais* (Motschulsky) (Coleoptera: Cucurionidae) (Usseglio et al. 2017) whereas the fungal volatiles, 1-octanol and 2-methyl-1-butanol increased egg-laying by *Sitophilus granarius* (L.) (Coleoptera: Curculionidae) (Niewiada et al. 2005). Van Winkle et al., (2021) incubated grain for various periods after tempering to specific grain moistures and found grain with moderate fungal colonization was more attractive to the primary pest, lesser grain borer, *Rhyzopertha dominica* (F.) (Coleoptera: Bostrichidae) than to the red flour beetle, *Tribolium castaneum* (Herbst) (Coleoptera: Tenebrionidae). Selitskaya et al., (2014) tested the behavioral response of *S. oryzae* to seven different species of *Fusarium*. Those authors found that species comprising weak

pathogens of grain were attractive to *S. oryzae*, while those that were strong pathogens were repellent to weevils. However, they did not specifically manipulate the fungal community nor did they characterize the volatiles associated with each fungal species.

Interestingly, we found through the Ethovision trials that, at close range, fungal cues by particular growth stages of fungi in grain could affect foraging decisions. In particular, *S. oryzae* was more likely to approach and stay in close proximity to grain kernels with MVOCs from the sexual life stage of *A. flavus* but not the asexual life stage-inoculated grain. This indicates that the fungal cues from the sexual life stage of *A. flavus* had an arresting effect on the behavior of *S. oryzae*. Arrestment is a behavioral process that involves retention at a resource of interest (Blaauw et al. 2017), generally through increased klinotaxis. This increased arrestment may be for one of two reasons, namely 1) the presence of the sexual stage correlates with improved food quality or oviposition opportunities for *S. oryzae* compared to the asexual sexual stage or no fungus (mentioned as a hypothesis in Ponce et al. 2021), or 2) it may be because the presence of the sexual stage of *A. flavus* is a surrogate indicator for some other abiotic factor or suite of factors that may result in increased survivorship or fecundity by *S. oryzae*. For example, in support of the second hypothesis, it is known that some fungi may alter the microclimate in grain masses in ways that benefit stored product insects, including *S. oryzae* (M. Ponce, unpublished data). However, the presence of fungal cues from sexual *A. flavus* did not significantly alter the overall distance moved or velocity of *S. oryzae*; grain volatiles were the more important determinant for these overall measures of movement. Our study corresponds with prior research that found close-range repellency by *S. oryzae* for volatiles emitted by *Fusarium* spp. (Selitskaya et al. 2014) as well stored product insects showing behavioral changes to grain with moderate incubation with complex fungal communities (Van Winkle et al. 2021). Our study suggests that

once *S. oryzae* reaches a food source, food quality such as microbial infestation may become more important in controlling foraging decisions at a local scale than other types of factors.

In the release-recapture assay, we used commercially available pitfall traps to assess long-distance attraction to MVOCs and grain. *Sitophilus oryzae* did not show elevated long-distance attraction to grain inoculated with the asexual or sexual life stage of *A. flavus* compared to the positive control or UV-sanitized grain. When initially using the smaller release-recapture assays, it was possible that volatile emissions from traps saturated the small release arenas; a larger version of the test was employed. However, the results from the smoke and odor tests indicated the bins likely were not saturated. Further, results from the larger assay indicated the same results, namely that the presence of grain was more important than whether grain had been inoculated with either fungal growth stage for recapture of *S. oryzae*. Both the small and large release-recapture assay were likely a successful test of long-distance attraction to grain odors relative to the size of the insect considering that *S. oryzae* is only 2 mm long and cannot fly, while traps were placed 879 and 3000 mm away. Indeed, prior research has found that *S. oryzae* are attracted to a range of grain odors, including maltol, vanillin, and valeraldehyde (Phillips et al. 1993). As a consequence, the volatiles produced by the sexual life stage of *A. flavus*-inoculated grain may be necessary but not sufficient without additional long-range stimuli for use in behaviorally-based management methods. This is because behaviorally-based management methods require attractive long-range stimuli for successful implementation at food facilities. For example, the stimuli for attract-and-kill and push-pull tactics often need to attract pest populations from a longer distance away than the immediate vicinity of where the commodity is being protected (e.g., Cox, 2004; Morrison et al., 2016, 2019a), and be able to retain the pest until it can be removed from the foraging population (Morrison et al. 2021b). For

example, Wilkins et al., (2021) placed attract-and-kill based interception traps on the perimeter of food facilities and baited them with combined pheromone and kairomone lure. Nonetheless, from a distance, grain volatiles appear to be the primary semiochemical used to drive *S. oryzae* foraging decisions.

The goal of the four-way olfactometer assay was to assess preference by *S. oryzae* to four different semiochemical treatments, including our positive control (unaltered grain), UV-sterilized grain, asexual (M1) and sexual *A. flavus* (M2)-inoculated grain. We found that *S. oryzae* did not prefer either fungal growth stage more than the positive control grain or UV-sanitized grain. Each odor source was preferred equally by *S. oryzae*. The olfactometer contained crystalized glass semi-permeable barriers, which was intended to prevent dispersal of insects, but may have hindered volatile diffusion over the short periods used here for testing *S. oryzae*. As a result, a follow-up version of this experiment was performed, wherein two small holes were pierced in each of the glass barriers, but again there was no significant preference among the negative control (no grain), positive control (unaltered grain), UV-sterilized grain, and asexual (M1) *A. flavus*-inoculated grain. There are four possible alternative hypotheses for this behavior. It could be that the glass olfactometer elicits a thigmotactic response by *S. oryzae* that disrupts its olfaction. Domingue et al. (2021) demonstrated the thigmotactic arrestment of the stored product quarantine threat, khapra beetle, *Trogoderma granarium* Everts (Coleoptera: Dermestidae) when it came into contact with control netting (even without insecticide), which disrupted orientation to semiochemicals. A second hypothesis is that *S. oryzae* may have needed air movement in order to exhibit a preference to the semiochemical treatments, which would not have been provided in the still-air olfactometer. Prior work has shown that response to semiochemicals and traps can be improved by stored product insects when air movement is present (Sajeewani et al.

2020; Campbell 2012; Jian 2019), which is more realistic for food facilities where air flow and eddies are common. However, this appears unlikely, since many *S. oryzae* chose a stimulus quickly. A third explanation is that the time provided in the trial may have insufficient for diffusion of volatiles. This also appears highly unlikely given the supplementary data showing adequate diffusion of volatiles (see Supplementary Materials). Finally, it could be that gustatory receptors are involved and close proximity is required for *S. oryzae* to differentiate among the signals, as in the Ethovision trial. This latter hypothesis appears to be the most likely given the whole of the dataset.

We confirmed through smoke and odor tests that volatiles diffused sufficiently in the assay, but did not saturate arenas (Supplemental Methods and Results). For example, we observed smoke exiting Ethovision arenas, and never forming a uniform, saturated layer of volatiles in the arenas. While observers were able to smell grain on the far side of the Ethovision arenas after 15 and 30 min, this was only for about 80% of arenas. This suggests volatiles do diffuse, but do not saturate the arenas. In the four-way olfactometer, it took grain volatiles longer to diffuse than smoke volatiles, likely due to their lower emission rates. In the release-recapture bins, we were able to detect smoke volatiles after about 24 s, but could not detect grain volatiles after 10 min or 24 h. However, we did detect grain odors directly outside of the pitfall trap, suggesting that grain odors do diffuse, but do not saturate the small bins. In each assay, it appears likely that a volatile gradient was formed to which *S. oryzae* could react to behaviorally. The most common grain headspace volatiles found in our study were diethyl benzenes across all grain treatments regardless of fungal presence. Various benzene compounds have commonly been isolated from headspace emitted by both whole wheat and milled grain in prior work (Maeda et al. 2008). The key differences among the treatments seem to be more in the presence

and complexity of minor components, evidenced by the increased complexity of the fungal-inoculated grain. For instance, over half the total tentatively identified compounds were found only in the fungal-inoculated grain. Among these, key volatiles associated with fungi in prior studies included, but were not limited to, 3-octanone, 1-octen-3-ol, butanal, limonene, butyl citrate, octane, and many others (Borjesson et al. 1992; Ponce et al. 2021; reviewed in Jelen and Wasowicz 1998). It is possible that stored product insects such as *S. oryzae* respond to more complex emissions at close range, especially considering that increased complexity of volatile blends may result in elevated attraction across a range of other insects (Szendrei and Rodriguez-Saona 2010). At low concentrations, other stored product insects such as *T. castaneum* exhibited the strongest antennal responses to (Z)-3-hexen-1-ol, hexenal, 2-heptanone, 3-octen-2-one, and ethyl hexanoate (Balakrishnan et al. 2017). Van Winkle et al. (2021) found that 3-methyl-1-butanol, 2-methyl-1-butanol, and octane were the most common fungal volatiles produced after grain was incubated and tempered for extended periods to higher grain moisture, while Sinha et al. (1987) found 3-methyl-1-butanol, 1-octen-3-ol, and 3-octanone were the most common in experimental grain bins. These and related volatiles may provide potential targets for use in behaviorally-based management tactics.

We have found that the asexual stage (M1) and sexual stage (M2) of *A. flavus* produce different MVOCs. Misztal et al., (2018) found that when developing fungi go through different stages of cellular respiration (e.g., different life stage of microbes), this had a significant effect on not only the amount of MVOCs produced but also their identity. Additional research suggests that *Tuber* spp., a symbiotic fungus found in plant roots, releases specific volatiles during its different life stages in order to attract or repel insects (Splivallo et al. 2011). *Leiodes cinnamomea* (L.) (Coleoptera: Leiodidae) is a pest that is only attracted to unripe truffles from a

distance, indicating that volatile cues are only produced by unripe truffles (Splivallo et al. 2011). Similarly, we analyzed how different life stages of *A. flavus* (asexual vs. sexual) can modulate the behavior of *S. oryzae*. We saw that grain inoculated with the sexual *A. flavus* (M2) affected the close-range foraging decisions of *S. oryzae*, while the asexual *A. flavus* (M1) did not. While prior work characterized grain sources that were likely not homogenous communities of fungi (Van Winkle et al. 2021), we determined here that M1 was primarily asexual *A. flavus*, while M2 was primarily sexual *A. flavus*.

Overall, our in-depth behavioral analysis has revealed that *S. oryzae* likely uses grain volatiles at a distance but may prefer to use other cues of host quality at close-range. Our study also demonstrated that postharvest grain constitutes a complex ecosystem of fungal stages, each of which is emitting a unique blend of volatiles that may have contrasting behavioral effects. In the future, there may be several research avenues that would provide important additional information. For example, future work should assess antennal response via gas chromatography coupled with electroantennographic detection (GC-EAD) to understand which volatiles identified here are actually detected by *S. oryzae*. This may also help narrow the possible behaviorally-active components of the headspace blends to those that are being detected by the insect. Furthermore, the life stages of other fungi during testing (e.g., sexual vs. asexual) may impact volatile emissions (Misztal et al. 2018), but whether these changes are primarily detected by gustatory or odorant receptors of *S. oryzae* should be specifically tested. Differing temperatures during insect orientation and response may influence plume reach of volatiles as well as chemosensory and neurophysiological processes in the insect (Maille et al. 2021) and so may air movement (Sajeewani et al. 2020), so in the future this should be explicitly considered. Finally, specific behaviorally-active volatiles should be individually tested in order to determine

whether they could be used in behaviorally-based management strategies. Overall, this study has substantively enhanced our understanding of how MVOCs affect the behavior of *S. oryzae*. Further research into this area on related stored product species and specific headspace compounds will undoubtedly help us improve management, while ensuring that our planet can feed its growing population.

References

- Amaike, S, and N.P. Keller. 2011. *Aspergillus flavus*. Annu. Rev. Phytopathol. 49: 107–133.
<https://doi.org/10.1146/annurev-phyto-072910-095221>
- Ayerst, G. 1969. The effects of moisture and temperature on growth and spore germination in some fungi. J. Stored Prod. Res. 5: 127–141.
- Balakrishnan, K., G. Holighaus, B. Weißbecker, and S. Schütz. 2017. Electroantennographic responses of red flour beetle *Tribolium castaneum* Herbst (Coleoptera: Tenebrionidae) to volatile organic compounds. J. Appl. Entomol. 141: 477–486. <https://doi.org/10.1111/jen.12366>
- Barney, R.J., B.D. Price, J.D. Sedlacek, and M. Siddiqui. 1995. Fungal species composition and abundance on stored corn as influenced by several management practices and maize weevil (Coleoptera: Curculionidae). Crop Prot. 14: 159–164. [https://doi.org/10.1016/0261-2194\(95\)92871-J](https://doi.org/10.1016/0261-2194(95)92871-J)
- Batte, M.T., N.H. Hooker, T.C. Haab, J. Beaverson. 2007. Putting their money where their mouths are: Consumer willingness to pay for multi-ingredient, processed organic food products. Food Policy 32: 145–159. <https://doi.org/10.1016/j.foodpol.2006.05.003>
- Beck, J.J., and B.S. Higbee. 2015. Plant- or fungal-produced conophthorin as an important component of host plant volatile-based attractants for agricultural lepidopteran insect pests. ACS. Symp. Ser. 1204:111–127. <https://doi.org/10.1021/bk-2015-1204.ch009>
- Beck, J.J., R.L. Vannette. 2017. Harnessing insect–microbe chemical communications to control insect pests of agricultural systems. J. Agric. Food Chem. 65: 23–28.
<https://doi.org/10.1021/acs.jafc.6b04298>.

- Blaauw, B.R., W.R. Morrison III, C. Mathews, T.C. Leskey, and A.L. Nielsen. 2017. Measuring host plant selection and retention of *Halyomorpha halys* by a trap crop. *Entomol. Exp. Appl.* 163: 197–208. <https://doi.org/10.1111/eea.12571>
- Borjesson, T., U. Stollman, and J. Schnurer. 1992. Volatile metabolites produced by six fungal species compared with other indicators of fungal growth on cereal grains. *Appl. Environ. Microbiol.* 58: 2599–2605. <https://doi.org/10.1128/aem.58.8.2599-2605.1992>
- Campos, M., and T.W. Phillips. 2014. Attract-and-kill and other pheromone-based methods to suppress populations of the Indianmeal moth (Lepidoptera: Pyralidae). *J. Econ. Entomol.* 107: 473–480. <https://doi.org/10.1603/ec13451>
- Cox, P.D. 2004. Potential for using semiochemicals to protect stored products from insect infestation. *J. Stored Prod. Res.* 40: 1–25. [https://doi.org/10.1016/S0022-474X\(02\)00078-4](https://doi.org/10.1016/S0022-474X(02)00078-4)
- Davis, T.S., T.L. Crippen, R.W. Hofstetter, and J.K. Tomberlin. 2013. Microbial volatile emissions as insect semiochemicals. *J. Chem. Ecol.* 39: 840–859. <https://doi.org/10.1007/s10886-013-0306-z>
- Davis, T.S., and P.J. Landolt. 2013. A survey of insect assemblages responding to volatiles from a ubiquitous fungus in an agricultural landscape. *J. Chem. Ecol.* 39: 860–868. <https://doi.org/10.1007/s10886-013-0278-z>
- Dilkin, P., C.A. Mallmann, C.A.A. De Almeida, et al 2002. Production of fumonisins by strains of *Fusarium moniliforme* according to temperature, moisture and growth period. *Brazilian J. Microbiol.* 33: 111–118. <https://doi.org/10.1590/S1517-83822002000200003>
- FAO. 2018. The State of Food Security and Nutrition in the World. United Nations, Food and Agriculture Organization, Rome, Italy

- Fleurat-Lessard, F. 2017. Integrated management of the risks of stored grain spoilage by seedborne fungi and contamination by storage mould mycotoxins – An update. *J. Stored Prod. Res.* 71: 22–40. <https://doi.org/10.1016/j.jspr.2016.10.002>
- Gerken, A.R., E.D. Scully, and J.F. Campbell. 2018. Red flour beetle (Coleoptera: Tenebrionidae) response to volatile cues varies with strain and behavioral assay. *Environ. Entomol.* 47: 1252–1265. <https://doi.org/10.1093/ee/nvy107>
- Hagstrum, D.W., and B. Subramanyam. 2006. *Fundamentals of Stored-Product Entomology*. AACC International, St. Paul.
- Horn, B.W., G.G. Moore, and I. Carbone. 2009. Sexual reproduction in *Aspergillus flavus*. *Mycologia* 101: 423–429. <https://doi.org/10.3852/09-011>
- Hubert, J., V. Stejskal, C.G. Athanassiou, and J.E. Throne. 2018. Health hazards associated with arthropod infestation of stored products. *Annu Rev Entomol* 63: 553–573. <https://doi.org/10.1146/annurev-ento-020117-043218>
- Jelen, H.H., and E. Wasowicz. 1998. Volatile fungal metabolites and their relation to the spoilage of agricultural commodities. *Food Rev. Int.* 14: 391–426.
- Khan, Z.R., C.A.O. Midega, J.O. Pittchar, A.W. Murage, M.A. Birkett, T.J.A. Bruce, and J.A. Pickett. 2014. Achieving food security for one million sub-Saharan African poor through push-pull innovation by 2020. *Philos. Trans. R. Soc. B Biol. Sci.* 369: 20120284. <https://doi.org/10.1098/rstb.2012.0284>
- Konemann, C.E., Z. Hubhachen, G.P. Opit, S. Gautam, and N.S. Bajracharya. 2017. Phosphine resistance in *Cryptolestes ferrugineus* (Coleoptera: Laemophloeidae) collected from grain storage facilities in Oklahoma, USA. *J. Econ. Entomol.* 110: 1377–1383. <https://doi.org/10.1093/jee/tox101>

- Lahouar, A, S. Marin, A. Crespo-Sempere, S. Said, V. Sanchis. 2016. Efectos de la temperatura, la actividad de agua y el tiempo de incubación en el crecimiento fúngico y la producción de aflatoxina B1 por aislados toxicogénicos de *Aspergillus flavus* en sorgo. Rev. Argent. Microbiol. 48:78–85. <https://doi.org/10.1016/j.ram.2015.10.001>
- Linsley, E.G. 1944. Natural sources, habitats, and reservoirs of insects associated with stored food products. Hilgardia 16: 185–224. <https://doi.org/10.3733/hilg.v16n04p185>
- Liu, Z., C. Ning, M. Yuan, S. Yang, X. Wei, M. Xiao, X. Fu, C. Zhu, H. Mou. 2020. High-level expression of a thermophilic and acidophilic β -mannanase from *Aspergillus kawachii* IFO 4308 with significant potential in mannoooligosaccharide preparation. Bioresour. Technol. 295: 122257. <https://doi.org/10.1016/j.biortech.2019.122257>
- MacDonald, I.M.V. 1997. Field experiments on duration and precision of grey and red squirrel spatial memory. Anim. Behav. 54: 879–891. <https://doi.org/10.1006/anbe.1996.0528>
- Maeda, T., J.H. Kim, Y. Ubukata, N. Morita. 2008. Analysis of volatile compounds in polished-graded wheat flours using headspace sorptive extraction. Eur. Food Res. Technol. 227: 1233–1241. <https://doi.org/10.1007/s00217-008-0841-y>
- Magan, N., R. Hope, V. Cairns, and D. Aldred. 2003. Post-harvest fungal ecology: Impact of fungal growth and mycotoxin accumulation in stored grain. Eur. J. Plant Pathol. 109: 723–730. <https://doi.org/10.1023/A:1026082425177>
- Magan, N., and M. Olsen. 2004. Mycotoxins in Food: Detection and Control. CRC Press: Boston, 488 pp.
- Maille, J., A. Gerken, S. Adrianos, et al 2022. Exploiting chemosensory genomics for improved monitoring and control of stored product pests. Insects, in press.

- Mannaa, M., K.D. Kim. 2017. Influence of temperature and water activity on deleterious fungi and mycotoxin production during grain storage. *Mycobiology* 45: 240–254.
<https://doi.org/10.5941/MYCO.2017.45.4.240>
- Misztal, P.K., D.S. Lymperopoulou, R.I. Adams, et al 2018. Emission factors of microbial volatile organic compounds from environmental bacteria and fungi. *Environ. Sci. Technol.* 52: 8272–8282. <https://doi.org/10.1021/acs.est.8b00806>
- Morrison, III W.R., F.H. Arthur, C.G. Athanassiou, E. Lampiri, L.T. Wilson, Y. Yang, and J. Wang. 2021a. Modeling of *Sitophilus oryzae* (L.) (Coleoptera: Curculionidae) based on historical weather data indicates aeration is effective for management of wheat stored in Greece. *Comput Electron. Agric.* 197: 106926.
- Morrison, III W.R., F.H. Arthur, L.T. Wilson, Y. Yang, J. Wang, and C.G. Athanassiou. 2020a. Aeration to manage insects in wheat stored in the Balkan peninsula: Computer simulations using historical weather data. *Agronomy* 10: 1927. <https://doi.org/10.3390/agronomy10121927>
- Morrison, III W.R., B.R. Blauw, B.D. Short, A.L. Nielsen, J.C. Bergh, G. Krawczyk, Y.-L. Park, B. Butler, A. Khimian, and T.C. Leskey. 2019a. Successful management of *Halyomorpha halys* (Hemiptera: Pentatomidae) in commercial apple orchards with an attract-and-kill strategy. *Pest Manag Sci* 75: 104–114. <https://doi.org/10.1002/ps.5156>
- Morrison, III W.R., R.F. Groshodier, F.H. Arthur, S.W. Myers, and M.J. Domingue. 2020b. Attraction, arrestment, and preference by immature *Trogoderma variabile* and *Trogoderma granarium* to food and pheromonal stimuli. *J Pest Sci* 93: 135–147. <https://doi.org/10.1007/s10340-019-01171-z>

- Morrison, III W.R., N.L. Larson, D. Brabec, A. Zhang. 2019b. Methyl benzoate as a putative alternative, environmentally friendly fumigant for the control of stored product insects. *J. Econ. Entomol.* 112: 2458–2468. <https://doi.org/10.1093/jee/toz179>
- Morrison, III W.R., D.H. Lee, B.D. Short, A. Khrimian, and T.C. Leskey. 2016. Establishing the behavioral basis for an attract-and-kill strategy to manage the invasive *Halyomorpha halys* in apple orchards. *J. Pest. Sci.* 89:81–96. <https://doi.org/10.1007/s10340-015-0679-6>
- Morrison, III W.R., E.D. Scully, and J.F. Campbell. 2021b. Towards developing behaviorally-based integrated pest management programs for stored product insects. *Pest. Manag. Sci.* 77: 2667–2682.
- Morrison, III W.R., R.V. Wilkins, A.R. Gerken, D.S. Scheff, K.Y. Zhu, F.H. Arthur, and J.F. Campbell. 2018. Mobility of adult *Tribolium castaneum* (Coleoptera: Tenebrionidae) and *Rhyzopertha dominica* (Coleoptera: Bostrichidae) after exposure to long-lasting insecticide-incorporated netting. *J. Econ. Entomol.* 111: 2443–2453. <https://doi.org/10.1093/jee/toy173>
- Navarro, S. 2006. New global challenges to the use of gaseous treatments in stored products. 9th Int Work Conf Stored Prod Prot 1: 495–509.
- Nayak, M.K., G.J. Daglish, T.W. Phillips, and P.R. Ebert. 2020. Resistance to the fumigant phosphine and its management in insect pests of stored products: A global perspective. *Annu. Rev. Entomol.* 65: 333–350.
- Niewiada, A., J. Nawrot, J. Szafranek, B. Szafranek. 2005. Some factors affecting egg-laying of the granary weevil (*Sitophilus granarius* L.). *J. Stored Prod. Res.* 41: 544–555. <https://doi.org/10.1016/j.jspr.2004.11.001>

- Phelan, P.L., and H. Lin. 1991. Chemical characterization of fruit and fungal volatiles attractive to dried-fruit beetle, *Carpophilus hemipterus* (L.) (Coleoptera: Nitidulidae). J. Chem. Ecol. 17: 1253–1272. <https://doi.org/10.1007/BF01402948>
- Phillips, T.W., X.-L. Jiang, W.E. Burkholder, J.K. Phillips, and H.Q. Tran. 1993. Behavioral responses to food volatiles by two species of stored-product coleoptera, *Sitophilus oryzae* (Curculionidae) and *Tribolium castaneum* (Tenebrionidae). J. Chem. Ecol. 19: 723–734. <https://doi.org/10.1007/BF00985004>
- Pierce, A.M., H.D. Pierce, J.H. Borden, and A.C. Oehlschlager. 1991. Fungal volatiles: semiochemicals for stored-product beetles (Coleoptera: Cucujidae). J. Chem. Ecol. 17: 581–597.
- Ponce, M.A., T.N. Kim, and W.R. Morrison III. 2021. A systematic review of the behavioral responses by stored-product arthropods to individual or blends of microbially produced volatile cues. Insects 12: 391. <https://doi.org/10.3390/insects12050391>
- Ray, D.K., N.D. Mueller, P.C. West, and J.A. Foley. 2013. Yield trends are insufficient to double global crop production by 2050. PLoS One 8: e66428.
- Sánchez-García, F.J., V. Machado, J. Galián, D. Gallego. 2017. Application of the eco-field and general theory of resources to bark beetles: Beyond the niche construction theory. Biosemiotics 10: 57–73. <https://doi.org/10.1007/s12304-016-9283-2>
- Selitskaya, O.G., O.P. Gavrilova, A.V. Schenikova, I.V. Shamshev, and T.Y. Gagkaeva. 2014. The effect of toxin-producing *Fusarium* fungi on behavior of the rice weevil *Sitophilus oryzae* (Coleoptera, Dryophthoridae). Entomol. Rev. 94: 820–825. <https://doi.org/10.1134/S0013873814060037>
- Sinha, R. 1987. Role of odor volatiles in the stored-grain ecosystem. Agric Canada Res Stn 1:3–13

- Sinha, S., W. Watorek, and S. Karr. 1987. Primary structure of human neutrophil elastase. *Proc. Natl. Acad. Sci. USA* 84: 2228–2232. <https://doi.org/10.1073/pnas.84.8.2228>
- Splivallo, R., S. Ottonello, A. Mello, and P. Karlovsky. 2011. Truffle volatiles: From chemical ecology to aroma biosynthesis. *New. Phytol.* 189: 688–699. <https://doi.org/10.1111/j.1469-8137.2010.03523.x>
- Sulyok, M., R. Krska, and R. Schuhmacher. 2010. Application of an LC-MS/MS based multi-mycotoxin method for the semi-quantitative determination of mycotoxins occurring in different types of food infected by moulds. *Food Chem.* 119: 408–416. <https://doi.org/10.1016/j.foodchem.2009.07.042>
- Szendrei, Z., and C. Rodriguez-Saona. 2010. A meta-analysis of insect pest behavioral manipulation with plant volatiles. *Entomol Exp Appl* 134: 201–210. <https://doi.org/10.1111/j.1570-7458.2009.00954.x>
- Tang, X, Y.L. Shao, Y.J. Tang, and W.W. Zhou. 2018. Antifungal activity of essential oil compounds (geraniol and citral) and inhibitory mechanisms on grain pathogens (*Aspergillus flavus* and *Aspergillus ochraceus*). *Molecules* 23: 2108. <https://doi.org/10.3390/molecules23092108>
- Tsai, W.T., L.J. Mason, and C.P. Woloshuk. 2007. Effect of three stored-grain fungi on the development of *Typhaea stercorea*. *J. Stored Prod. Res.* 43: 129–133. <https://doi.org/10.1016/j.jspr.2006.01.001>
- Usseglio, V.L., R.P. Pizzolitto, C. Rodriguez, M.P. Zunino, J.A. Zygadlo, V.A. Areco, J.S. Dambolena. 2017. Volatile organic compounds from the interaction between *Fusarium verticillioides* and maize kernels as a natural repellents of *Sitophilus zeamais*. *J. Stored Prod. Res.* 73: 109–114. <https://doi.org/10.1016/j.jspr.2017.08.001>

- Van Winkle, T., M.A. Ponce, H.E. Quellhorst, A. Bruce, C.E. Albin, T.N. Kim, K.Y. Zhu, and W.R. Morrison III. 2021. Microbial volatile organic compounds mediate attraction by a primary but not a secondary stored product insect pest in wheat. *J. Chem. Ecol.* 48: 27–40.
- Vanhaelen, M., R. Vanhaelen-Fastré, J. Geeraerts, T. Wirthlin. 1978. Cis- and trans-octa-1,5-dien-3-ol, new attractants to the cheese mite *Tyrophagus putrescentiae* (Schränk) (Acarina, Acaridae) identified in *Trichothecium roseum* (Fungi imperfecti). *Microbios* 23: 199–212
- Wacker, F. 2018. Food waste and food losses – Importance of international partnerships and research. In: 12th International Working Conference on Stored Product Protection, 7–11 October 2018. Julius-Kuhn Institut, Berlin, Germany
- Wilkins, R.V., J.F. Campbell, K.Y. Zhu, L.A. Starkus, T. McKay, and W.R. Morrison III. 2021. Long-lasting insecticide-incorporated netting and interception traps at pilot-scale warehouses and commercial facilities prevents infestation by stored product beetles. *Front. Sustain. Food Syst.* 4: 561820
- Wilkins, R.V., K.Y. Zhu, J.F. Campbell, W.R. Morrison III. 2020. Mobility and dispersal of two cosmopolitan stored-product insects are adversely affected by long-lasting insecticide netting in a life stage dependent manner. *J. Econ. Entomol.* 113:1768–1779.
<https://doi.org/10.1093/jee/toaa094>

Table 2-1. List of semiochemical treatments and their amounts used in each assay during experiments at the USDA-ARS Center for Grain and Animal Health Research in Manhattan, KS from Jun-Sep 2020.

Abbreviations	Semiochemical Treatment	Amount in Assay					Function	Source
		Olfactometer	Ethovision	Release-Recapture	Headspace Collection	DNA Identification		
-Ctrl	Negative Control	Empty	Empty	Empty	Empty	Empty		Unbaited control
M1	Morphotype 1 (identified as asexual <i>Aspergillus flavus</i>)	10 g	1 kernel	10 g	20 g	10 g	Food & one microbial stimulus	300 g wheat kernels inoculated with a petri dish containing a pure culture morphotype 1 and incubated for 7 d
M2	Morphotype 2 (identified as sexual <i>Aspergillus flavus</i>)	10 g	1 kernel	10 g	20 g	10 g	Food & microbial stimulus	300 g wheat kernels inoculated with a petri dish containing a pure culture of morphotype 2 and incubated for 7 d
UV	Sanitized grain	10 g	1 kernel	10 g	20 g	10 g	Food stimulus without microbes	300 g of wheat kernels sanitized with UV light for 10 min in pan
+Ctrl	Positive control (unsanitized)	10 g	1 kernel	10 g	20 g	10 g	Food stimulus with ambient microbes	Wheat kernels directly from wheat source in laboratory

Table 2-2. Mean emission rates (ng · g⁻¹ · h⁻¹) and percent contribution to total emissions of identified compounds in headspace from different grain treatments. Fungal-inoculated grain showed significantly higher and more complex emissions compared to UV-sterilized or untreated grain (n=7 replicates per treatment).

Compound	Retention Time	UV-Sterilized Grain				Positive Control Grain				Asexual <i>A. flavus</i> (M1)-Inoculated Grain				Sexual <i>A. flavus</i> (M2)-Inoculated Grain			
		Mean	±	SE	% of Total	Mean	±	SE	% of Total	Mean	±	SE	% of Total	Mean	±	SE	% of Total
Pivalaldehyde, semicarbazone	4.735	0.0	±	0.0	0.0	0.1	±	0.1	0.8	0	±	0	0.0	0	±	0	0.0
2-Butenal	4.788	0.0	±	0.0	0.0	0.3	±	0.1	2.4	0	±	0	0.0	0	±	0	0.0
2,4-dimethyl-1-heptene	4.792	0.0	±	0.0	0.0	0	±	0	0	0.8	±	0.2	1.2	0.1	±	0.1	0.1
2-Pentanone, 4-hydroxy-4-methyl	4.802	0.2	±	0.2	1.4	0.7	±	0.5	5.6	0.2	±	0.2	0.3	0.5	±	0.3	0.6
Benzene, propyl	6.292	0.0	±	0.0	0.0	0.1	±	0.1	0.8	0.4	±	0.3	0.6	0.3	±	0.1	0.4
1-Octen-3-ol	6.516	0.0	±	0.0	0.0	0	±	0	0	0.5	±	0.3	0.8	3.5	±	2	4.1
Butanal	6.525	0.0	±	0.0	0.0	0	±	0	0	0.6	±	0.4	0.9	0	±	0	0.0
4,5-Dichloro-1,3-dioxolan-2-one	6.628	0.0	±	0.0	0.0	0.2	±	0.1	1.6	0	±	0	0.0	0	±	0	0.0
3-Octanone	6.648	0.0	±	0.0	0.0	0	±	0	0	1.8	±	0.3	2.7	6.2	±	3.6	7.3
Decane	6.823	0.0	±	0.0	0.0	0.2	±	0.2	1.6	0.2	±	0.2	0.3	0	±	0	0.0
Mesitylene	6.869	0.1	±	0.1	0.6	0.2	±	0.2	1.6	0.2	±	0.2	0.3	1.3	±	1.1	1.5
Benzene, 1,2,4-trimethyl-	7.319	0.0	±	0.0	0.0	0.2	±	0.2	1.6	0.1	±	0.1	0.2	0	±	0	0.0
D-Limonene	7.364	0.1	±	0.1	0.7	0.1	±	0.1	0.8	0	±	0	0.0	0.3	±	0.2	0.4
Benzeneethanol, beta-ethyl	7.541	0.0	±	0.0	0.0	0	±	0	0	0	±	0	0.0	0.2	±	0.1	0.2
Benzene, 1,4-diethyl	7.659	10.1	±	10.1	88.9	4.8	±	4.8	38.4	26.1	±	18.9	39.4	18.6	±	###	22.0
Benzene, 1,2-diethyl	7.759	0.1	±	0.1	1.3	1.4	±	0.6	11.2	31.7	±	22.5	47.9	37.4	±	###	44.3
1,3,8-p-Menthatriene	7.86	0.0	±	0.0	0.0	0	±	0	0	0.5	±	0.3	0.8	0.4	±	0.2	0.5

4-dichloromethyl-2[[2-[1-methyl-2-pyrrolidinyl]ethyl]amino-6-trichloromethylpyrimidine Benzene, (2-methyl-1-propenyl)-	8.271	0.0	±	0.0	0.0	0.2	±	0.1	1.6	0.1	±	0.1	0.2	0.9	±	0.8	1.1
	8.279	0.0	±	0.0	0.0	0.6	±	0.4	4.8	0.2	±	0.2	0.3	0	±	0	0.0
1-Phenyl-1-butene	8.282	0.0	±	0.0	0.0	0	±	0	0	0.2	±	0.1	0.3	0.1	±	0.1	0.1
Linalool	8.314	0.2	±	0.2	1.6	0.1	±	0.1	0.8	0	±	0	0.0	0.5	±	0.3	0.6
Nonanal	8.37	0.1	±	0.1	1.3	0.7	±	0.3	5.6	0.4	±	0.2	0.6	0.7	±	0.6	0.8
2-Thiophenecarboxylic acid, 5-nonyl-	9.044	0.0	±	0.0	0.0	0.2	±	0.1	1.6	0	±	0	0.0	0.4	±	0.4	0.5
Dichloroacetaldehyde	9.735	0.0	±	0.0	0.0	0.1	±	0.1	0.8	0	±	0	0.0	0	±	0	0.0
Linalyl acetate	10.558	0.0	±	0.0	0.0	0	±	0	0	0	±	0	0.0	0.2	±	0.2	0.2
beta-Ocimene	10.561	0.2	±	0.2	1.4	0.1	±	0.1	0.8	0	±	0	0.0	0	±	0	0.0
2-Thiophenecarboxylic acid	10.59	0.1	±	0.1	0.8	0.8	±	0.5	6.4	0.7	±	0.3	1.1	2.6	±	2.5	3.1
1-Pent-3-ynylcyclopenta-1,3-diene	10.653	0.1	±	0.1	0.8	0.2	±	0.1	1.6	0.3	±	0.2	0.5	0.3	±	0.2	0.4
1,5,6,7-Tetramethylbicyclo[3.2.0]hepta-2,6-diene	10.822	0.0	±	0.0	0.0	0	±	0	0	0.1	±	0.1	0.2	0.4	±	0.2	0.5
Ethanone, 1-(4-ethylphenyl)	11.108	0.0	±	0.0	0.0	0	±	0	0	0.5	±	0.4	0.8	0.6	±	0.3	0.7
Butyl citrate	21.812	0.0	±	0.0	0.0	0	±	0	0	0.1	±	0.1	0.2	0	±	0	0.0
1-methyl-4-phenyl-5-thioxo-1,2,4-triazolidin-3-one	23.957	0.1	±	0.1	0.7	0.6	±	0.3	4.8	0	±	0	0.0	0	±	0	0.0
9-Octadecenamide, (Z-)	26.311	0.1	±	0.1	0.5	0.6	±	0.3	4.8	0.5	±	0.3	0.8	9	±	8.6	10.7
Total		11.4	±	0.2	100.0	12.5	±	0.3	100	66.2	±	1.4	100	84.5	±	0.7	100

Figure 2-1. Experimental 4-way still-air olfactometer assay: 10 g of each treatment in odor source chambers, single *Sitophilus oryzae* placed in central release chamber. 2-minute decision time, defined as walking 2.54 cm toward an arm.

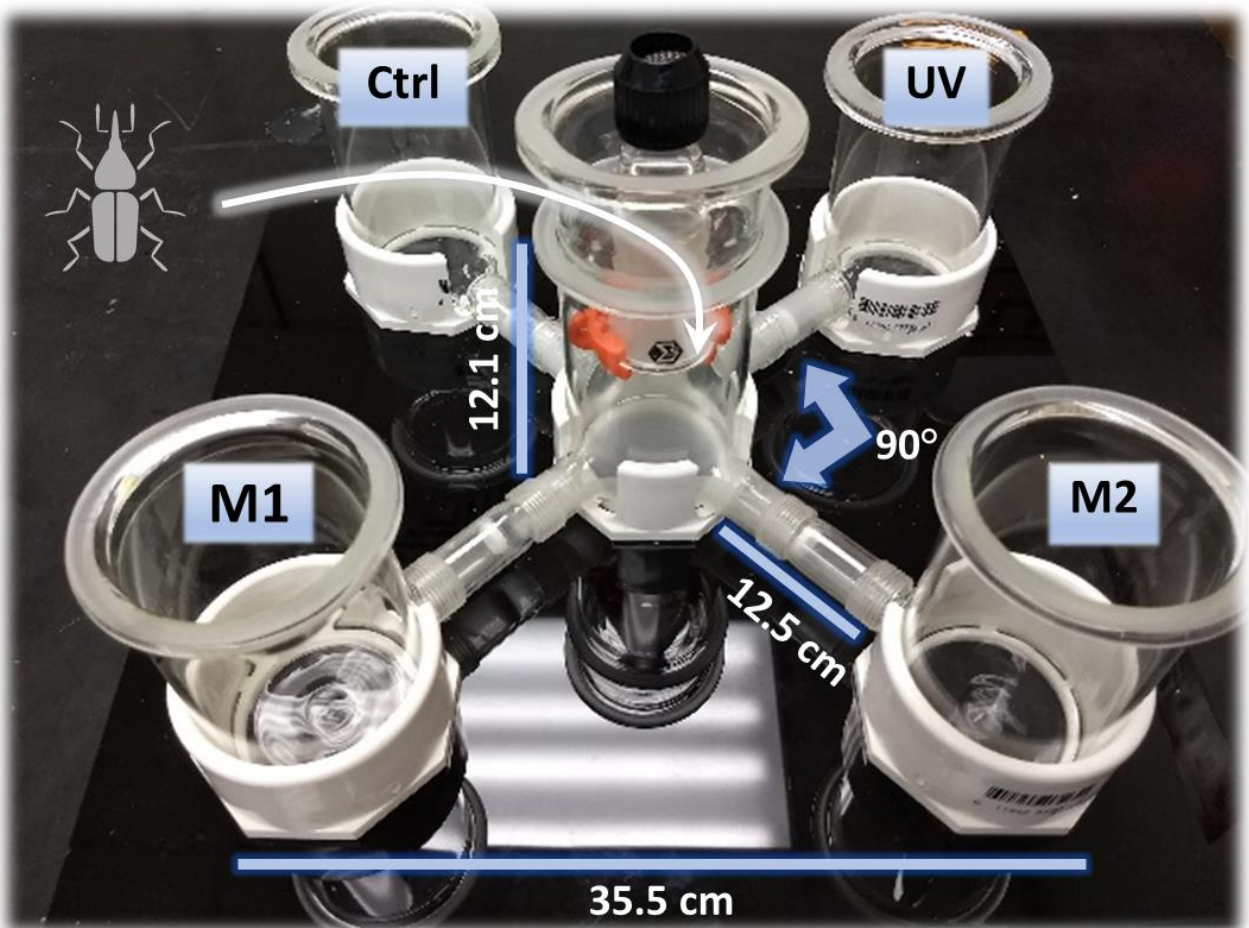


Figure 2-2. Schematic of test arenas for video-tracking assay with insects placed in the center. Ethovision software used for tracking. Treatment areas (blue) and control areas (grey) illustrated.

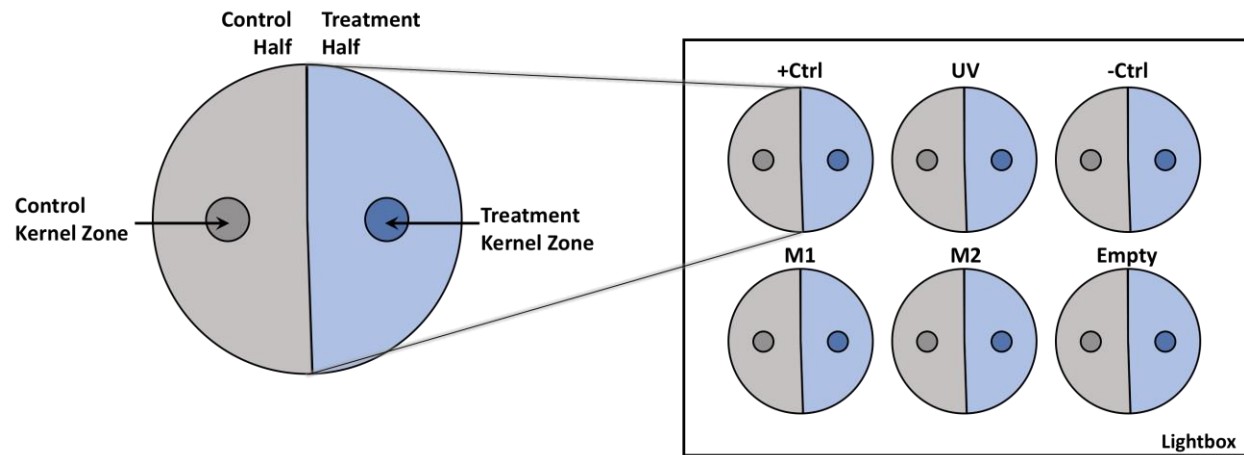


Figure 2-3. Percent of *S. oryzae* choosing different semiochemical treatments in olfactometer assay with porous glass barriers or holes. No significant differences among treatments observed.

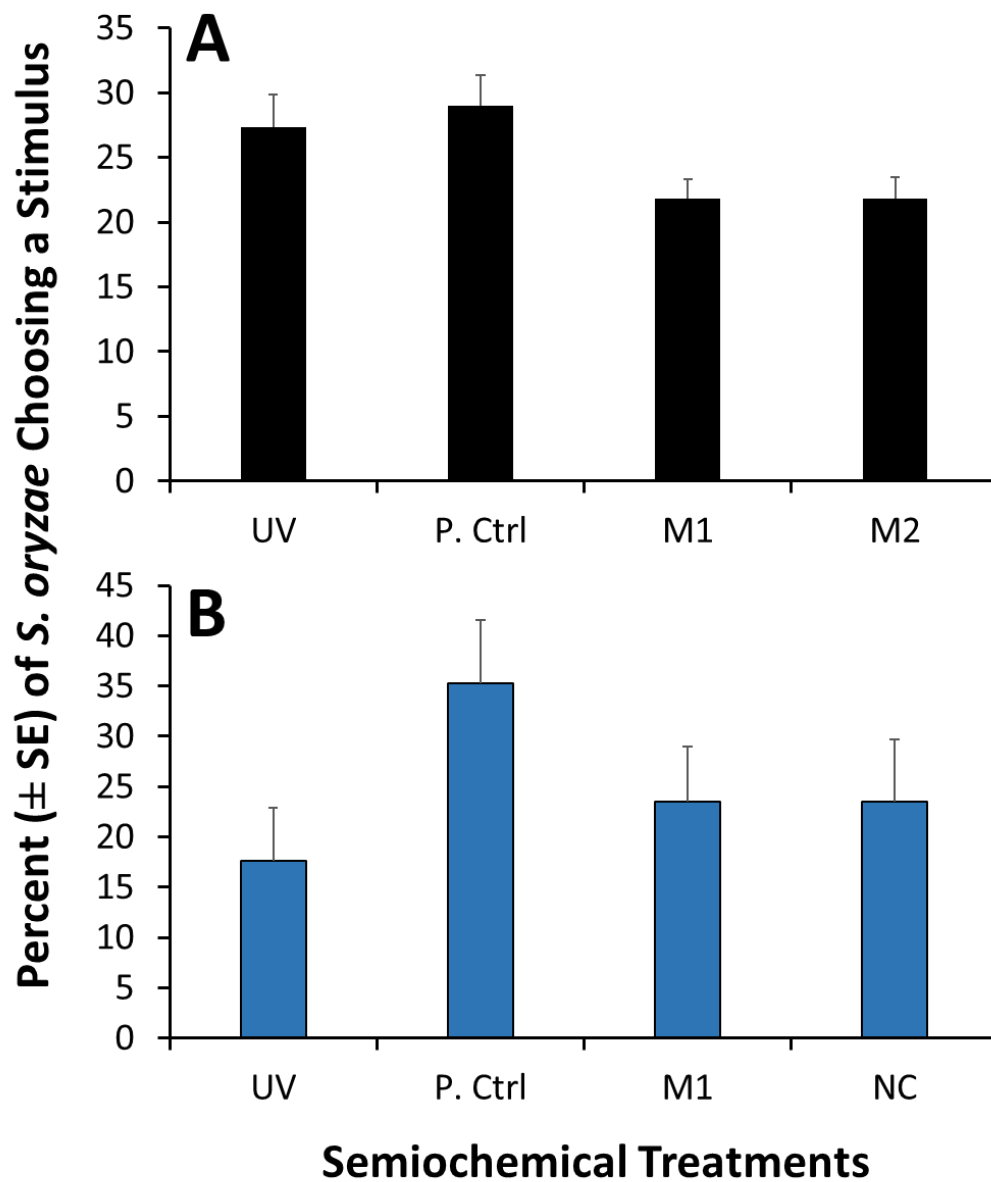


Figure 2-4. Movement parameters of *S. oryzae* in Ethovision assay. Semiochemical treatments did not significantly affect velocity or distance moved.

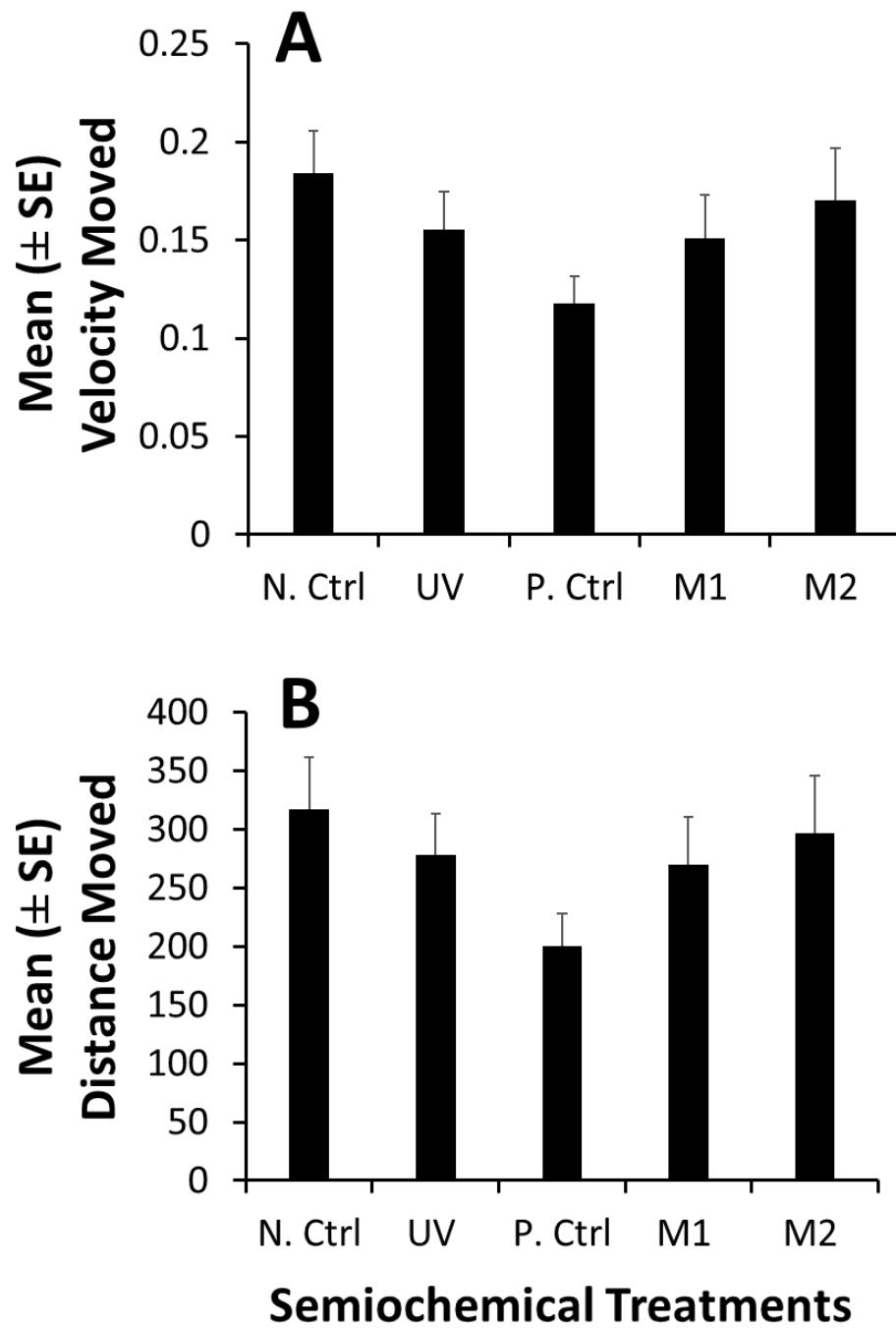


Figure 2-5. Mean frequency of *S. oryzae* entering different kernel zones in Ethovision assay. *S. oryzae* entered M2 treatment zone significantly more compared to other treatments.

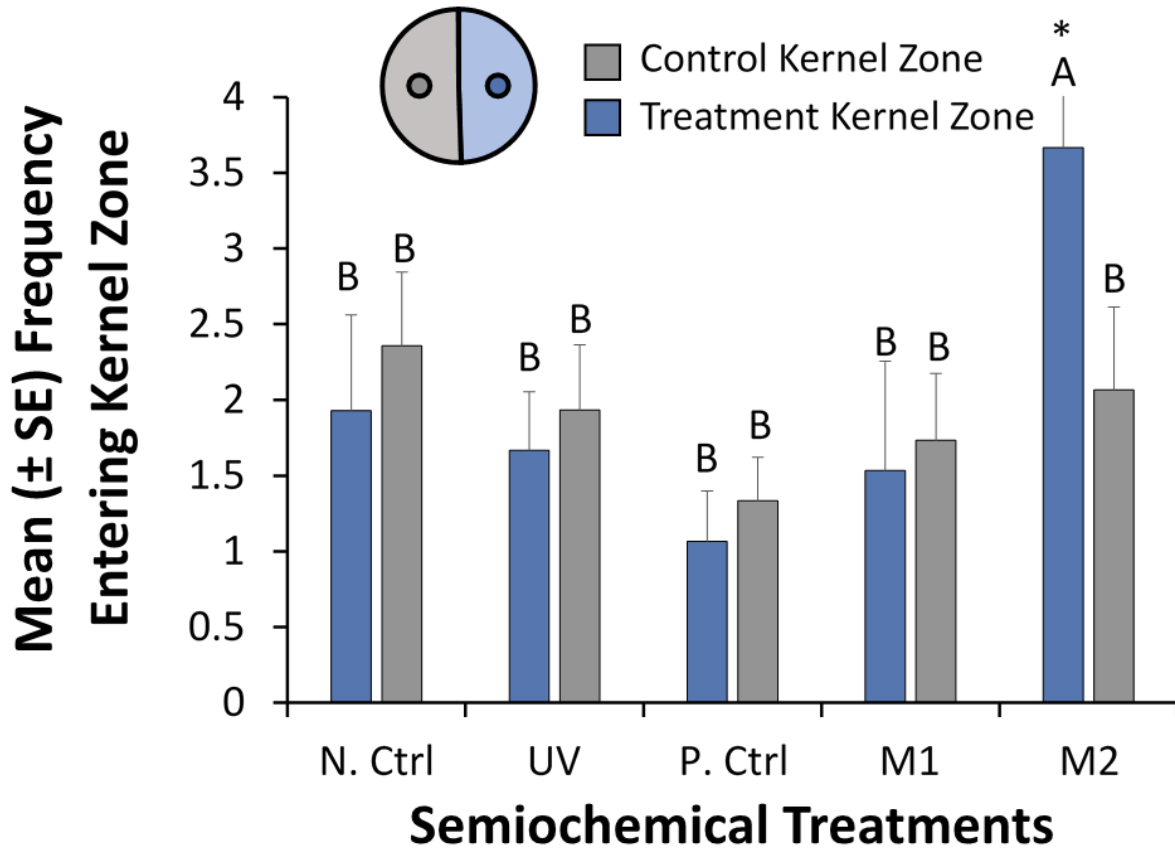


Figure 2-6. Cumulative duration of *S. oryzae* in treatment and control zones in Ethovision assay. M2 treatment resulted in significantly longer duration in kernel zone compared to other treatments.

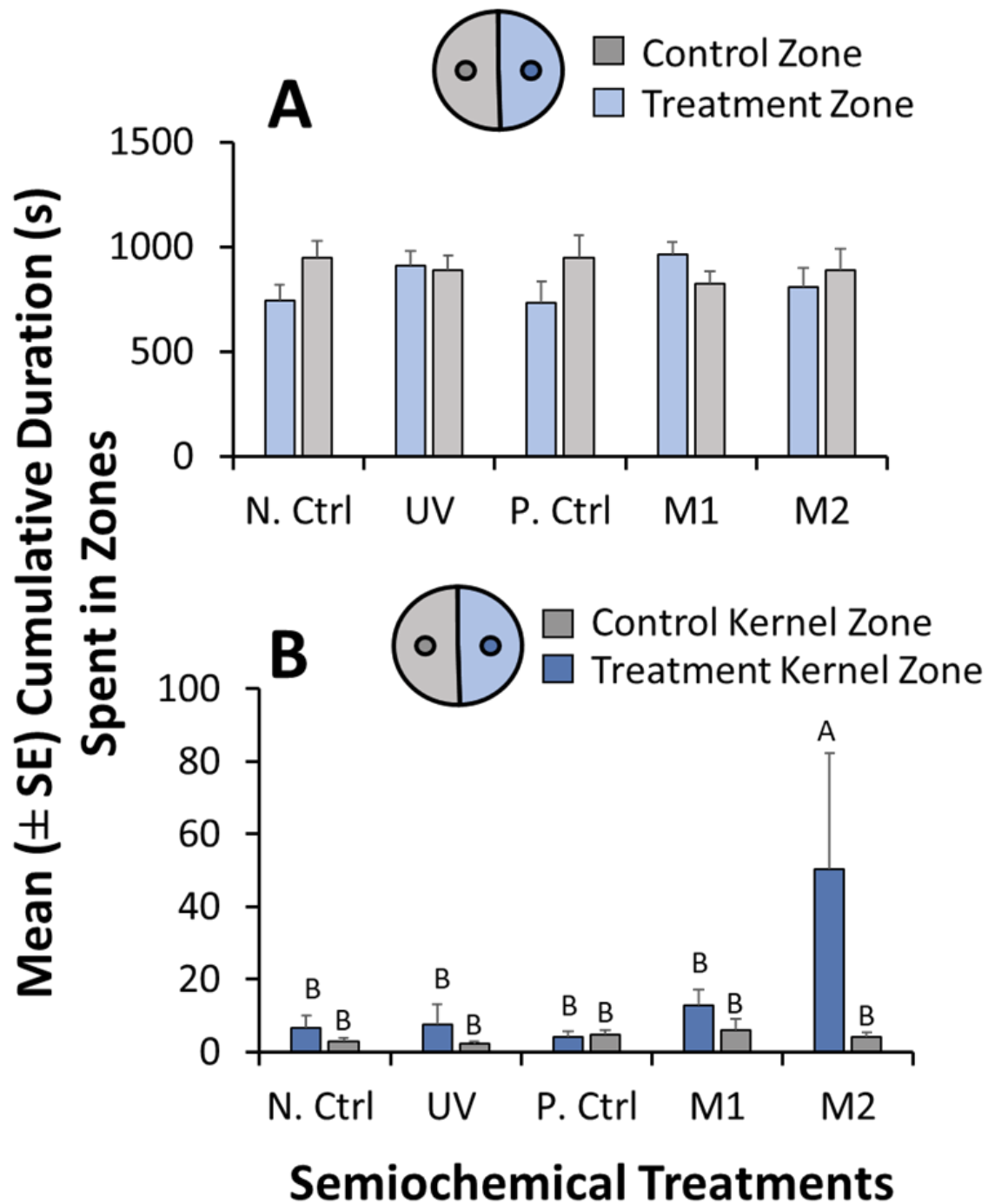


Figure 2-7. Percent of *S. oryzae* recaptured in release-recapture assay using pitfall traps. Grain traps had significantly higher captures compared to negative control, but MVOCs did not affect capture.

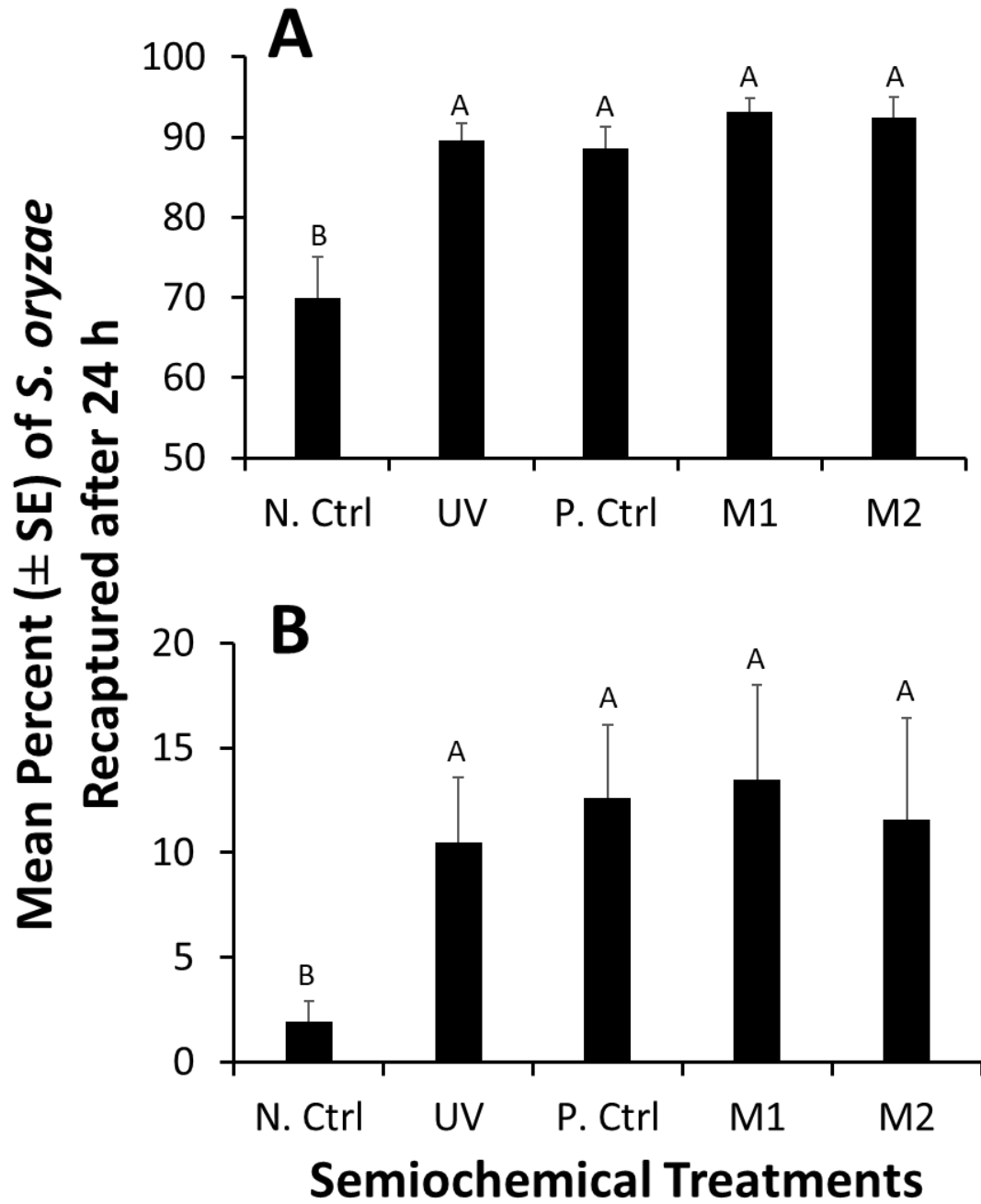
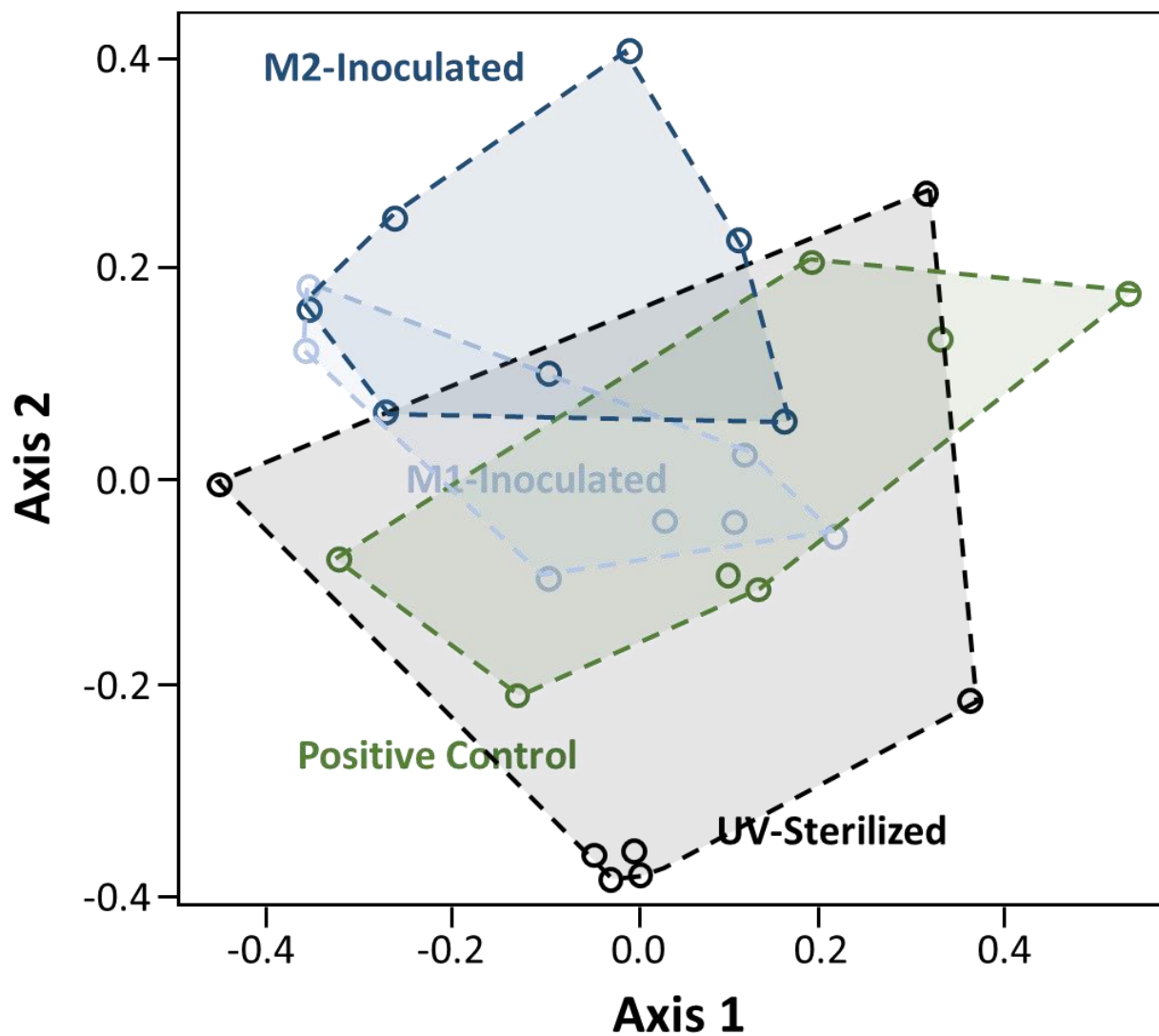


Figure 2-8. Non-metric multidimensional scaling ordination of headspace samples based on compound emissions. Treatments showed significant differences from each other.



Chapter 3 - Attraction, mobility, and preference by *Lasioderma serricorne* (F.) (Coleoptera: Ptinidae) to microbially-mediated volatile emissions by two species of fungi in stored grain

Published as: Ponce, M.A., Sierra, P., Maille, J.M. *et al.* Attraction, mobility, and preference by *Lasioderma serricorne* (Coleoptera: Ptinidae) to microbially-mediated volatile emissions by two species of fungi in stored grain. *Sci Rep* **13**, 6176 (2023).

<https://doi.org/10.1038/s41598-023-32973-y>

Abstract

Little research has linked how specific fungal species affect the behavior of the secondary stored product pest, *Lasioderma serricorne*. Enhanced knowledge about attraction to microbially-produced volatile organic compounds (MVOCs) may be used to manipulate insect behavior. Our goals were to 1) isolate, and culture two fungal morphotypes, 2) characterize the volatile emissions from grain inoculated by each fungal morphotype (*A. flavus* or *F. verticilloides*) compared to uninoculated and sanitized grain, and 3) understand how MVOCs from each morphotype affects mobility, attraction, and preference by *L. serricorne*. Headspace collection revealed that the *Fusarium*- and *A. flavus*-inoculated grain produced significantly different volatiles compared to sanitized grain or the positive control. Changes in MVOC emissions affected close-range foraging during an Ethovision assay, with a greater frequency of entering and spending time in a small zone with kernels inoculated with *A. flavus* compared to other treatments. In the release-recapture assay, MVOCs were found to be attractive to *L. serricorne* at a longer distances in commercial pitfall traps. While there was no preference shown among semiochemical stimuli in a still-air, four-way olfactometer, it is possible that methodological limitations prevented robust interpretation from this assay. Overall, our study suggests that MVOCs are important for close- and long-range orientation of *L. serricorne* during foraging, and that MVOCs may have the potential for inclusion in behaviorally-based tactics for this species.

Introduction

Insects inflict over \$100 billion in economic losses to commodities after harvest (Wacker 2018), while microbes may endanger the safety of human through the production of mycotoxins (Magan et al. 2003). Insects and microbes may interact in a variety of ways at food facilities, compounding damage and threatening food security. Although insects and microbes may be found together in food facilities, they are often not discussed together even though research research has shown that microbes can alter the grain in environment in ways that are beneficial to stored product insects (Ponce *et al.*, 2021). For example, the presence of microbes increased progeny production of *Sitophilus oryzae* (L.) (Coleoptera: Curculionidae) and simultaneously elevated the humidity and temperature of the grain mass, thereby creating favorable growth conditions for themselves and *S. oryzae*. *Fusarium verticillioides* likewise was found to increase the progeny of *Sitophilus zeamais* (Coleoptera: Curculionidae) (Usseglio et al. 2020).

In addition to altering the abiotic environment, microbes produce microbial volatile organic compounds (MVOCs, hereafter), which may act as insect pheromones or food kairomones that mediate key behaviors including attraction, repulsion, mating, foraging, dispersal, and aggregation (Davis et al., 2013). In a recent metanalysis, Ponce et al., (2021) found that insect behavioral responses (e.g., attraction, repellency, or net neutrality) to MVOCs are complex and can vary by microbial taxa and community composition, abiotic environment, insect species, life history (internal-infesting or external-infesting stored product insects). Van Winkle et al., (2021) found that tempering grain to different moisture levels and incubating for 9–28 d at 30°C significantly changed the blend of volatiles and microbial communities, which showed attraction at moderate levels to *Rhyzopertha dominica* (F.) (Coleoptera: Bostrichidae),

but not the secondary pest, *Tribolium castaneum* (Herbst) (Coleoptera: Tenebrionidae). Lizarraga 2020 and Ponce et al. 2022 found that MVOCs affected the close-range foraging of an internal-infesting pest, *S. oryzae*, after inoculation of grain with different life stages (e.g., sexual vs. asexual) of *A. flavus*. An important knowledge gap that needs to be addressed is how and whether fungal MVOCs impact behavior of external-infesting stored product pests.

One important external-infesting pest is the cigarette beetle, *Lasioderma serricorne* (F.) (Coleoptera: Ptinidae). This is a cosmopolitan pest globally in and around grain storage and food processing facilities that has the unique ability to detoxify plant secondary metabolites, including nicotine and caffeine, through interactions with its midgut symbionts (Edde, 2019). This species is highly mobile, able to disperse readily by flight during most of the year in Mediterranean climates (Papadopoulou and Buchelos, 2002) and possesses strong walking and climbing capabilities (Campbell et al., 2002). Prior work in Japan isolated 17 species of molds and 5 species of yeasts from the cuticles of *L. serricorne*, including *Aspergillus*, *Arthrinium*, *Eurotium*, *Fusarium*, and *Penicillium* (Kawakami and Takahashi, 2002). However, the ecological role of these microbes and how they affect *L. serricorne* behavior is poorly understood.

Pathogens that colonize raw grain and finished products also threaten commodities and can have significant negative impacts on both human and animal health (Miller, 1995; Mannaa and Kim, 2017, Hubert et al., 2018). In addition, off-odors in raw and finished commodities have also been linked to microbial contamination (Inamdar et al. 2020). Common fungal species that contaminate grain and commodities post-harvest typically include those from the genera *Aspergillus*, *Fusarium*, *Penicillium*, and *Alternaria*, among others. A specific pathogen that is especially widespread in stored grains is *Aspergillus flavus* (Tang et al., 2018), which causes significant economic losses after harvest (Magan et al., 2003), and is known to produce the

carcinogenic secondary metabolite, aflatoxin (Amaiike and Keller 2011). Although much progress has been made in reducing aflatoxin contamination, it remains a sporadic problem for corn stored in the southern United States and it is routinely detected in stored commodities at markets in developing countries (Bumbangi et al., 2016). Another common fungal contaminant of raw grain is *Fusarium graminearum*, which may attack wheat either pre- or post-harvest (Savi et al., 2014). This fungus has been implicated in the production of the trichothecene class of mycotoxins, which includes deoxynivalenol, 3-acetyldeoxynivalenol, and 15-acetyldeoxynivalenol. While *F. graminearum* and *A. flavus* can both produce spores, the former primarily grows filamentously, while the latter spreads through the production and dispersal of spores. Morphologically, *A. flavus* is often appears yellowish or green in pigment, while *F. graminearum* often appears white (Figure 1). Despite the commonality of these fungal pathogens in post-harvest environments, little work has been done to investigate headspace volatiles of grain contaminated by either microbe and the behavioral impacts on stored product insects.

One alternative suite of tactics in IPM to combat stored product pests are semiochemical-mediated, behaviorally-based tactics (reviewed recently in Morrison et al. 2021) that use volatile cues to manipulate behavior in ways that are conducive to protecting commodities from insect colonization. Effective attractants and/or repellents are required for the success of these IPM tactics. One class of stimuli that may be broadly attractive to stored product insects are MVOCs, partially because of the evolutionary history of these taxa that involved using animal caches of food before the advent of agriculture. One particular application of MVOCs may be in attract-and-kill, whereby insects are attracted to a spatially-circumscribed area by a long-range attractant treated with an insecticide and then removed from the foraging population (e.g., Morrison et al.

2016, 2019b; Gregg et al. 2018). Recent work has found that attract-and-kill based interception traps placed on the perimeter of food facilities were successful at preventing progeny production when they included long-lasting insecticide netting, capturing over 3,000 individuals in weekly 48-h deployments in two years (Wilkins et al., 2021). However, a challenge in deploying this tactic at food facilities is competing with existing volatile stimuli, including nearby food resources, insects emitting aggregation or sex pheromones, and sites of spillage. Nonetheless, the incorporation of MVOCs into lures for attract-and-kill tactics may make these volatile sources unique among the backdrop of odors at food facilities and support delivering improved and unique IPM tools in the food environment to stakeholders.

Thus, this study aims to 1) isolate, and culture two fungal morphotypes (*Aspergillus flavus* and *Fusarium* spp. cultured from the cuticle of *L. serricorne*), 2) characterize the volatile emissions from grain inoculated by the fungal morphotypes compared to uninoculated and sanitized grain, and 3) understand how MVOCs from the morphotypes affected the mobility, attraction, and preference by *L. serricorne*. We hypothesized that headspace blends will be unique among our fungal treatments, and that this will lead to differences in preference and mobility by *L. serricorne*.

Materials and Methods

Source Insects

Adult cigarette beetles, *Lasioderma serricorne* (F.) (Coleoptera: Ptinidae), were used from insect colonies that were originally collected in 2012 from a rice mill in Arkansas and continuously maintained on a diet of 95% bleached wheat flour and 5% brewer's yeast with oats sprinkled on top and a moistened, crumpled towel added. Colonies were kept at the United States Department of Agriculture Agricultural Research Service's (USDA-ARS) Center for Grain and

Animal Health Research facility in Manhattan, KS. Insects were routinely subcultured by sieving (No. 30 sieve, 594×594 μm mesh, W.S. Tyler Co., Cleveland, OH, USA) 150 mixed-sex *L. serricorne* adults from diet mixtures, transferring them into new mason jars (950-ml capacity) filled two-thirds of the way to the top with diet, and then placed inside environmental chambers under constant conditions at 27.5°C, 65% RH, and 14:10 L:D photoperiod. Individuals used in bioassays described below were never tested more than once.

Fungal Morphotype Culturing

To initially isolate the two fungal morphotypes, *L. serricorne* were allowed to disperse on agar. A total of 32 g of potato dextrose agar (Merck, Darmstadt, Germany) was mixed with 900 ml of deionized water in a 1000-mL glass media bottle with a magnetic stirring rod placed inside the bottle. The agar solution was autoclaved (533LS, Getinge, Rochester, NY, USA) for 30 min and then stirred on a hot plate to cool down for 20 min. Before pouring the potato agar solution into petri dishes (100 $\times$ 15), the biosafety cabinet (75 $\times$ 73 $\times$ 95 cm L:H:W, #302381101, Labconco, Kansas City, MO, USA) was sanitized with 70% ethanol and exposed to UV light for 10 min. A total of 36 petri dishes with potato dextrose agar solution were left in the biosafety cabinet to solidify overnight.

A single *L. serricorne* was introduced into to a single PDA dish and microbial growth was visualized after 3 and 5 d. Each PDA plate was sealed with parafilm, and stored in an environmental chamber under constant conditions of 30°C, 60% RH, and 14:10 L:D photoperiod. Transfer of *L. serricorne* from containers to agar was performed inside the biosafety cabinet to prevent contamination. Pictures of the agar dishes and corresponding microbial growth were acquired using a DSLR camera (EOS 7D Mark II, Canon, Tokyo, Japan) mounted to 3D imaging StackShot (CogniSys, Inc., Traverse City, MI, USA) equipped with a

dual flash (MT-26EX-RT, Canon, Tokyo, Japan). Light was diffused using a partially cut occluded plastic jar (15.2 × 7.6 cm D:H) making a total of 12 replicates. From the fungal isolation, two fungal morphotypes were selected by close examination of microbial characteristics such as spore shape, size, and color. This was used to generate the two primary morphotypes for the rest of the assays in the experiments below, and to generate sequences for the morphotypes.

Treatments

To study how MVOCs affected *L. serricorne* behavior, the two morphotypes identified above were introduced to grain and compared to uninoculated and UV-sanitized grain in various behavioral assays. The uninoculated grain treatment (e.g., P. ctrl hereafter) consisted of 10 g of hard winter wheat at a 10.6% moisture content taken directly from cold storage (Table 1). UV-sanitized grain was as above but treated with ultraviolet radiation for 10 min in the biosafety cabinet (75 × 73 × 95 cm L:H:W, #302381101, Labconco, Kansas City, MO, USA) to surface sterilize. Grain inoculated by specific fungal species included *Fusarium*-inoculated grain (e.g. *F. verticillioides*, determined by sequencing, see below: FS, hereafter) and *Aspergillus flavus*-inoculated grain (AF, hereafter). In order to develop uniform, inoculated cultures on grain, our process was modelled on mushroom farming techniques with boiling (Cotter 2014). To prepare the *Fusarium*-inoculated grain, 600 g of grain was transferred to a stainless-steel pot filled with water and placed on a hot plate at 100°C. Once boiling for 15 min, the water was drained and the grain was evenly spread out on sterile wipes (38.1 × 42.5 cm, 3 ply, Tech wipes, Skilcraft, NIB, Alexandria, VA) and dried inside a laminar flow hood (ca. 3 h). Afterwards, grain was evenly divided (~300 g) and placed in 2 separate autoclaved mason jars (950-mL capacity). A single hole was pierced through each lid and lined with a cotton ball. The jars were then sealed with

aluminum foil and were autoclaved (533LS, Getinge, Rochester, NY, USA) for 30 min. To inoculate with *Fusarium* spp. or *A. flavus*, a 3-inch agar plug of fungi was placed into each jar containing the grain. The plug had been colonized and growing without contamination in a petri dish for 7 d-10 d in an environmental chamber set to 30°C, 60% RH, and 14:10 L:D photoperiod. Jars with freshly prepared *Fusarium*-inoculated grain sat at room temperature for roughly 10 d-15 d or until the fungi evenly covered as much the grain as possible. Grain was never used more than once for each replicate of every trial in each assay experiment to prevent cross contamination.

Microbial Morphotype Sequencing

To identify microbes associated with the cuticle of *L. serricorne*, fungal morphotypes were isolated from cuticles and cultured for the purpose of sequencing. Pure isolations from the two morphotypes were made by excising a 1 × 1 cm agar plug of fungi and subculturing it onto a new potato dextrose agar dish, sealed with parafilm to obtain a pure culture. DNA was extracted from pure cultures after 7 d of growth in an environmental chamber set to 30°C, 60% RH, and 14:10 L:D photoperiod using the Quick-DNA Fecal/Soil Microbe Miniprep Kit (D6010, Zymo Research Corp, Irvine, CA, USA). Concentration of DNA and quality were assessed using the Take 3 Assay on a microplate reader (Gen5™, BioTek Instruments, Winooski, VT, USA) before performing the PCR.

Polymerase chain reaction (PCR) was used to amplify the internal transcribed spacer (ITS) region for both morphologically identified fungi using primer sets: ITS4 5'-TCCTCCGCTTATTGATATGC-3' and ITS5 5'-GGAAGTAAAAGTCGTAACAAGG-3' (White et al. 1990). For the morphologically identified *Fusarium* sample, the translation elongation factor 1 alpha (TEF1 α) region and the RNA polymerase II subunit (RBP2) region were also

amplified to facilitate species levels identification. The following primer pairs were used for TEF1 α and RBP1, respectively: EF1 (5'-ATGGGTAAGGARGACAAGAC-3')/EF2 (5'-GGARGTACCAGTSATCATGTT-3') and RBP2-5F2 (5'-GGGGWGAYCAGAAGAAGGC-3') / RPB2-7cR (5'-CCCATRGCTTGYTTRCCCAT-3') (O'Donnell et al. 1998; O'Donnell et al. 2007; LeBlanc et al. 2015). Each reaction contained 1.0 μ L extracted DNA, 1.0 μ L of each primer (10 μ M), 9.5 μ L of nuclease free water, and 12.5 μ L of master mix containing 50 units/mL of Taq DNA polymerase master mix (Hot Start Taq 2X Master Mix, Promega, Madison, WI, USA). Briefly, the PCR program consisted of 2 min of initial denaturation at 95°C, followed by 30 cycles of 95°C for 30 s, 55°C for 1 min, and 72°C for 1.5 min, and a final extension at 72°C for 5 min (S1000 Thermal Cycler, Bio-Rad, Hercules, CA, USA). PCR products were treated with ExoSAP-it prior to sequencing following the manufacturer's protocol (ThermoFisher Scientific, Waltham, MA, USA) Amplicons were sequenced bidirectionally on an ABI 3730XL instrument (Eurofins Scientific, Brussels, Belgium), and the resulting sequences were quality-filtered and aligned using Geneious Prime 2021.0.3 (Biomatters Ltd, Auckland, New Zealand). The consensus sequences were searched against NCBI's nucleotide database (nt) using the BLASTn algorithm (Altschul et al. 1997). In order to circumvent taxonomic misassignments, the ITS consensus sequences were also checked against the UNITE Database using the Ribosomal Database Project Classifier algorithm (Wang et al. 2007). The consensus sequence for the ITS sequence of *A. flavus* was submitted to GenBank under OM490684 while the ITS, TEF1 α , and RBP2 sequences from *F. verticillioides* were deposited under OM460744, OM542207, and OM542208.

4-Way Olfactometer

In order to assess preference by *L. serricorne* among the stimuli, a still-air four-way olfactometer was used. This device (Sigma Scientific, LLC., Micanopy, FL, USA) consisted of a cylindrical central release glass chamber (9.0 × 12.1 cm D: H) with exit ports (2.5 × 3.5 cm D:L) at each cardinal direction connecting the central chamber to four identical glass chambers (7.0 × 11.5 cm D:H) where 10 g of each odor source was placed (Table 1). The bottom of the release arena was acid-etched to provide a surface over which insects could easily crawl. Each of the four outer chambers were spaced at a 90° angle from each other. Inert septa buffered and sealed each component. A removable glass lid with a hole was used to cover the top of the main central glass chamber to allow for airflow, but also prevent insects from exiting. A single *L. serricorne* was released into the middle of the central arena for each bioassay and given a maximum of 4 min to make a decision to choose one of the stimuli. A choice was considered to have been made when the individual traveled 2.54 cm into the adjoining 1-way exit port that branched off to the odor chamber. After every 5 replicates, the entire olfactometer was rotated 90° to control for positional effects. After every 15 replicates, the whole apparatus was washed first with methanol followed by hexane, and a new, independent set of each treatment was used, with the grain replaced. The time to decision and choice of odor treatment were recorded and an individual insect was never tested more than once. Non-responsive (NR) insects were also recorded, but excluded from the final statistical analysis. The assay was conducted in a biosafety cabinet to maintain a constant flow of air and odor. A total of n = 200 replicate individuals were tested.

Movement Assay

A movement assay was used to assess if fungal inoculated grain impacted close-range foraging behavior of *L. serricorne*. This was accomplished through the use of video-tracking

with a network camera (GigE Network Camera, Basler AG, Germany), suspended using a pole and clamp 80 cm above the test arenas, coupled with Ethovision Software (Noldus Inc., Leesburg, VA, USA). The test arenas consisted of five petri dishes (100 × 15 mm) with 85 mm diameter filter paper adhered to the bottom of the petri dish with double sided tape, on an artist's lightbox, each containing a single *L. serricorne* and a single treatment kernel (Table 1). Each test arena was halved vertically, with the left half designated for the control half (e.g., no kernel) and the right half for the treatment half. Embedded within each half of the arenas were 1.16 cm diameter control kernel zones or treatment kernel zones, located midway and halfway on each respective half (Supplemental Figure 1). These were coded as hidden zones in Ethovision and the smaller treatment kernel zones were where the grain treatments were placed, consisting of a single kernel of each treatment listed in Table 1. A single *L. serricorne* was released at the midpoint in the center of each arena. Trials lasted for a period of 30 min. The Ethovision software automatically measured several variables including the total distance moved, the cumulative duration of time spent in each half/kernel zone, the frequency of entry to each half/kernel zone, and latency to entering the half/kernel zone. The location of each treatment was randomized between each replicate to control any positional bias. There was a total of n = 20 replicates per treatment. No petri dish, filter paper, or adult was ever used more than once.

5.1 Release-Recapture Assay

A trapping assay was utilized to determine how microbial fungal volatiles impact the capture by *L. serricorne* in monitoring traps at a distance. In a large, walk-in environmental chamber (5 × 6 × 2 m, L:W:H), plastic bins (86.3 × 30.5 × 39.4 cm L:H:W) were set up to perform the experiment under constant environmental conditions (28°C, 59% RH, 14:10 L:D). A ring of polytetrafluoroethylene (Millipore Sigma, Burlington, MA, USA) around the top and bottom of

the plastic bins was added to prevent insects from climbing out of the bins. The bottom of the bins was roughened with sandpaper to provide traction. The traps consisted of commercially-available pitfall traps (Storgard, Trece, Adair, OK, USA) with two connectible pieces (Doud and Phillips 2020; Doud et al. 2021), containing a central well where the treatments were added as bait. Traps were baited with treatments in Table 1 and placed in one corner inside the bins. A total of 20 adult, mixed-sex *L. serricorne* were released in the corner opposite from where the trap was placed. The positions of traps were randomized in a different corner inside the bin to reduce any positional bias between each replicate. The insects were given 24 h to respond to stimuli. Afterwards, traps were collected, and the number of insects recaptured was recorded. A total of n = 8 replicate releases were performed per treatment.

MVOC Headspace Collection

To determine how microbial colonization impacted volatile emissions from grain, headspace collections were performed on fungal-inoculated, uninoculated, and UV-treated grain. The headspace volatile collection apparatus consisted of four 500-ml glass chambers, each with an air inlet and outlet. Central air was first purified, then regulated with a flow meter to 1 L/m, after which it entered the headspace chamber via PTFE tubing and was collected onto a volatile collection trap (VCT). Each glass chamber was secured during headspace volatile collection with a plastic lid and a PTFE-faced butyl septum. After each use, chambers were washed with methanol first followed by hexane inside a laminar fume hood. The VCT consisted of an angled drip-tip collection point borosilicate glass tube with a mesh (Stainless Steel #316 screen), packed with 20 mg of PoraPak-Q™ chemical absorbent held in place with a borosilicate glass wool plug, and followed by a PTFE Teflon™ compression seal, which was used to collect and concentrate MVOCs over 3 h periods from 20 g of each treatment (Table 1). Background

volatiles were also collected from empty glass chambers as a negative control. Volatiles were eluted from VCTs inside the fume hood with 150 μ l of dichloromethane, which was pushed through with N₂ gas into labeled 2 mL GC vials with 150- μ l glass inserts with PTFE polymer feet and magnetic seal caps. Using a microsyringe (2- μ l capacity, Hamilton Co., Reno, NV, USA), 1 μ l of internal standard (190.5 ng tetradecane) was added to each sample. Between each use, the syringe was washed with 2 μ l of dichloromethane in triplicate to avoid cross contamination between samples. All headspace samples were sealed with Teflon tape and stored at -13°C until GC-MS analysis below. For reuse of VCT, traps were washed in triplicate with 700 μ l of dichloromethane, which was pushed through with N₂ gas.

MVOC GC-MS Analysis

All headspace collection sample extracts were run on an Agilent 7890B gas chromatograph (GC) equipped with an Agilent Durabond HP-5 column (30 m length, 0.250 mm diameter and 0.25 μ m film thickness) with He as the carrier gas at a constant 1.2 mL/min flow and 40 cm/s velocity. This was coupled with a single-quadrupole Agilent 5997B mass spectrometer (MS). The compounds were separated by auto-injecting 1 μ l of each sample under split mode into the GC-MS at room temperature (approximately 23°C). The flow was split in a 15:1 ratio with a split flow rate of 18 ml/min. The GC program consisted of 40°C for 1 min followed by 10°C/min increases to 300°C and then held for 26.5 min. After a solvent delay of 3 min, mass ranges between 50 and 550 atomic mass units were scanned. Compounds were tentatively identified by comparison of spectral data with those from the NIST 17 library and by GC retention index (Adams 2007). Using the ratio of the peak area for the internal standard to the peak area for the other compounds in the headspace, the emission rates of samples was normalized in ng of volatile per g of grain, per μ l of solvent, and per h of collection were calculated.

Statistical Analysis

In order to analyze the quantitative data from the vectoring assay, the mean greyscale value was used as the response variable in a linear model. Semiochemical treatment (Table 1) was included as a fixed, explanatory variable. Residuals were inspected to validate assumptions of normality and homoscedasticity. If these assumptions were not met, log -transformed data were used, which then satisfied both assumptions. Upon a significant result of the model, Tukey HSD was used for multiple comparisons and implemented using the function *ghlt* from the package *multcomp* in R Software. R software (R Core Team 2019) was used for this and all other tests, with $\alpha = 0.05$ except where noted.

To analyze the data from the four-way olfactometer, a Chi-squared test was performed with a Bonferroni adjustment to the α -threshold. The null hypothesis assumed that there was an equal probability that an insect would choose one of the four sides.

To analyze how grain and microbial volatiles affect the mobility and foraging of *L. serricorne* during video-tracking, a generalized linear model based on a Gaussian distribution with a log-link function was used. In particular, separate models were used for the distance moved, and instantaneous velocity as response variables, while the explanatory variable included the odor treatment included in each test arena (e.g. from Table 1). To detect behavioral differences in this assay, the frequency of entering each zone, cumulative duration spent in each zone, and latency to finding each zone were each analyzed using a multivariate analysis of variance (MANOVA) with the semiochemical treatments as a fixed explanatory variable. Upon a significant overall result, sequential ANOVAs were performed for each variable. For responses with significant ANOVAs, multiple comparisons were performed with Tukey HSD. Post-hoc t-tests were performed as a contrast between the two means of treatment vs. control zone and

treatment vs. control kernel zone to assess differences within a semiochemical treatment with a Bonferroni-corrected alpha-threshold for multiple testing.

To analyze the data from the release-recapture assay, a generalized linear model based on a Poisson distribution and log link function was used. The response was the number of *L. serricorne* recaptured in traps for a particular stimulus. The explanatory variable included the odor treatments from Table 1. Overdispersion was not found to be a problem. Upon a significant result from the model, multiple comparisons in a generalized linear model framework were implemented using the function *ghlt* (e.g. Tukey HSD) from the package *multcomp* in R Software.

To characterize the change in headspace volatiles with inoculation of fungi in grain, raw peak areas were extracted from the gas chromatograms using MSD ChemStation v2.00 software (Agilent Technologies, Inc., Santa Clara, CA, USA). The emissions rate was calculated on a ng per gram of grain weight and per hour basis by using the ratio of the peak area for tetradecane to each headspace volatile. Background volatiles found in the negative control without grain were discarded from the other samples, since these represent transient background volatiles. Pairwise Bray-Curtis similarities were calculated between each headspace sample, and non-metric multi-dimensional scaling (NMDS) was used to visualize the differences in volatile profiles among treatments. A total of $n = 1,000$ permutations were used for the ordination procedure. Stress values for the NMDS procedure were <0.1 , indicating that good interpretation was possible. An analysis of similarity (ANOSIM) was used to determine significant differences for headspace volatile profiles among odor treatment categories. ANOSIM calculates an R statistic that can vary from 1 to -1, with values above zero interpreted as greater dissimilarity between treatments than within and values below zero interpreted as greater dissimilarity within treatments than

between treatments. A total of $n = 1,000$ permutations were performed for the test. For all multivariate statistics, the R Package *vegan* was used (Oksanen et al. 2019). To assess differences in the complexity of the headspace blends (e.g. number of compounds per treatment), pairwise chi-square tests were performed among treatments with the null hypothesis of an equal number of compounds identified from each treatment. To determine differences in overall quantitative emissions of volatiles, pairwise t-tests among means were performed using a Bonferroni-correction to the P-value.

Results

Fungal Morphotype Sequencing

After running a blastn search against the nt database, the top ten matches for the ITS consensus sequences from AF morphotype were all with isolates of *Aspergillus*, with nine of them *A. flavus* and one of them *A. niger* with 99% sequence identity and 97–98% query coverage ($E \approx 0$). Among the top 10 matches for the ITS sequence of the FS morphotype, 90% were to *Fusarium verticillioides*, while one match was to *Fusarium annulatum*. There was 99% sequence identity with the matches over 43% query coverage ($E \approx 0$) for the FS morphotype.

Four-Way Olfactometer

The presence of the four stimuli did not significantly affect the percentage of adult *L. serricorne* choosing a treatment side in the four-way olfactometer ($X^2 = 3.53$; $df = 3$; $P = 0.32$; Figure 3). Overall, *L. serricorne* chose UV-sanitized grain 30% of the time, while on average, they chose *Fusarium*-inoculated grain 21% of the time.

The semiochemical treatments in the four-way olfactometer had no significant effect on the time to decision by adult *L. serricorne* (ANOVA: $F = 0.22$; $df = 3,174$; $P = 0.88$). On

average, decisions were made in 88–96 s by *L. serricorne* for the *Fusarium*-inoculated grain and *Aspergillus flavus*-inoculated grain, respectively.

Movement Assay Overall Measures

The semiochemical treatments significantly affected the distance moved by adult *L. serricorne* over the 30-min observation periods (ANOVA: $F = 541$; $df = 4, 86$; $P < 0.0001$).

Adult *L. serricorne* moved 2–4-fold less when exposed to a grain kernel of one of the four semiochemical treatments compared to the negative control (Tukey HSD, Figure 4). Adults moved numerically the least when exposed to *A. flavus*-inoculated grain kernels.

While velocity moved by *L. serricorne* showed the same numerical pattern in response to semiochemical treatments, it did not differ significantly (ANOVA: $F = 1.28$; $df = 4, 86$; $P = 0.28$). Overall, *L. serricorne* velocity was numerically decreased by 2–3-fold when exposed to a grain kernel of one of the four semiochemical treatments compared to the negative control (Figure 4). The mean velocity ranged from 0.12–0.37 cm s⁻¹ for the *Aspergillus flavus*-inoculated grain and negative control, respectively.

Movement Assay in Kernel Zones

The semiochemical treatments significantly affected close-range foraging by altering the frequency by which an adult *L. serricorne* entered each kernel zone (MANOVA: $F = 11.8$; $df = 4, 86$; $P < 0.0001$; Figure 5). The semiochemical treatments significantly affected the frequency by which an adult *L. serricorne* entered the control kernel zone, which lacked stimuli (ANOVA: $F = 3.81$; $df = 4, 86$; $P < 0.01$). Adult *L. serricorne* were 2–3-fold less likely to enter the control kernel zone in the *A. flavus*-inoculated grain and *Fusarium*-inoculated grain arenas compared to the negative control or UV-sanitized grain arenas (Figure 5). Furthermore, the frequency of adult *L. serricorne* entering treatment kernel zones was significantly affected by the semiochemical

treatment present (ANOVA: $F = 6.01$; $df = 4, 86$; $P < 0.001$). There were 2–3-fold more entries by adult *L. serricorne* to the treatment kernel zone containing the *Aspergillus flavus*-inoculated grain than to treatment kernel zones with uninoculated grain or no grain at all (Tukey HSD, Figure 5). Importantly, *L. serricorne* entered treatment kernel zones with *A. flavus*-inoculated grain significantly more often than the associated control kernel zones (Post-hoc: $t = 2.50$; $df = 19$; $P < 0.05$; Figure 5).

The semiochemical treatment significantly affected the cumulative duration spent in each kernel zone or arena half by adult *L. serricorne* (MANOVA: $F = 51.9$; $df = 4, 86$; $P < 0.0001$). Particularly, the presence of the semiochemical treatments significantly affected the duration spent in both the control kernel zone (ANOVA: $F = 3.19$; $df = 4, 86$; $P < 0.05$) and treatment kernel zone (ANOVA: $F = 50.9$; $df = 4, 86$; $P < 0.0001$). The mean ($\pm$ SE) cumulative duration spent in the control kernel zone was short and ranged from $3 (\pm 1.1)$ – $8 (\pm 3.0)$ s over the 30-min period. By contrast, *L. serricorne* spent 6–17-fold more time in the treatment kernel zone containing *Fusarium*-inoculated grain and *Aspergillus flavus*-inoculated grain, respectively, compared to the treatment kernel zone in the negative control. Adult *L. serricorne* spent the most time in the treatment kernel zone for UV-sanitized grain and *A. flavus*-inoculated grain, which was approximately 40 ± 28 and 119 ± 73 s, respectively. Importantly, *L. serricorne* spent significantly more time in the treatment kernel zone than the control kernel zone for *A. flavus*-inoculated grain (Post-hoc: $t = 2.14$; $df = 19$; $P < 0.05$).

The semiochemical treatments significantly affected the latency with which adult *L. serricorne* found each kernel zone or arena half (MANOVA: $F = 44.7$; $df = 4, 86$; $P < 0.0001$). For example, the semiochemical treatments significantly affected the latency to finding and entering the control kernel zone (ANOVA: $F = 7.86$; $df = 4, 86$; $P < 0.0001$) as well as the

treatment kernel zone (ANOVA: $F = 3.63$; $df = 4, 86$; $P < 0.01$). Adult *L. serricorne* were 2-3-fold faster in finding *Fusarium*-inoculated grain control kernel zones than ones for UV-sanitized grain or uninoculated grain (Figure 5). All semiochemical treatments in treatment kernel zones had a similar latency to being found and entered by *L. serricorne* except *A. flavus*-inoculated grain, which resulted in 1.5-fold higher latency than the other treatments.

Movement Assay in Arena Halves

The frequency by which an adult *L. serricorne* entered the control half varied significantly among the semiochemical treatments (ANOVA: $F = 5.21$; $df = 4, 86$; $P < 0.001$). Adults entered control halves rarely, on average between 2 and 5 times over the 30 min period, corresponding to the *Fusarium*-inoculated grain and UV-sanitized grain treatment respectively (Figure 6). Moreover, the frequency of entering the treatment half by an adult *L. serricorne* was significantly affected by the semiochemical treatment (ANOVA: $F = 6.30$; $df = 4, 86$; $P < 0.001$). There were each about 2-fold less frequent entries by adult *L. serricorne* to treatment halves with *A. flavus*-inoculated and *Fusarium*-inoculated grain compared to the negative control. Importantly, each semiochemical treatment in the treatment half resulted in significantly more entries than the corresponding control half (Post-hoc paired t-test, Bonferroni correction; Figure 6).

By contrast, the semiochemical treatments did not significantly affect the cumulative duration spent in both control half (ANOVA: $F = 1.12$; $df = 4, 86$; $P = 0.35$) nor in the treatment half (ANOVA: $F = 0.81$; $df = 4, 86$; $P = 0.52$). Adult *L. serricorne* spent a range of 519–875 s in the control halves, on average, associated with the UV-sanitized and negative control treatment, respectively. Adults spent an average duration ranging from 584–902 s in the treatment halves, which corresponded to the uninoculated- and *A. flavus*-inoculated grain, respectively.

Importantly, the cumulative duration spent in the *A. flavus*-inoculated treatment half was significantly more than the control half (Post-hoc: $t = 2.01$; $df = 19$; $P < 0.05$), as was the cumulative duration spent in the UV-sanitized treatment half compared to the control half ($t = 2.56$; $df = 19$; $P < 0.01$).

The semiochemical treatments significantly affected the latency to entering the control half (ANOVA: $F = 28.1$; $df = 4,86$; $P < 0.0001$) and treatment half (ANOVA: $F = 13.7$; $df = 4,86$; $P < 0.0001$) of the arenas. It took *L. serricornis* adults 4–13-fold longer to find control halves associated with UV-sanitized, uninoculated-, AF-inoculated grain, and *Fusarium*-inoculated grain semiochemical treatments compared to the negative control (Figure 6). By contrast, *L. serricornis* were 3-4-fold faster in finding the treatment halves associated with *Fusarium*-inoculated and *A. flavus*-inoculated grain compared to the negative control. Numerically, *L. serricornis* adults were over 3-fold faster in finding UV-sanitized treatment halves compared to control halves, while adults were 2-fold faster in finding *A. flavus*-inoculated treatment halves compared to control halves.

Release-Recapture Assay

In total, the percent of adults recaptured out of 800 was 41%, ranging between 26–51%. The attractant in the trap significantly affected recapture of *L. serricornis* in commercially-available pitfall traps (ANOVA: $F = 7.11$; $df = 4,35$; $P < 0.001$; Figure 7). In fact, the greatest recapture was in traps baited with UV-sanitized, uninoculated-, and *A. flavus*-inoculated grain, which each captured approximately 2-fold more *L. serricornis* than the negative control. Traps baited with *Fusarium*-inoculated grain captured an intermediate percentage of *L. serricornis*.

Headspace Volatiles Collected from Inoculated Grain

In total, 44 compounds were tentatively identified from the semiochemical treatments (Table 2). The uninoculated grain had the fewest number of compounds (e.g. just 15), while the UV-sanitized grain, *A. flavus*-inoculated grain, and *Fusarium*-inoculated grain each had 23, 26, and 25 compounds in its headspace, respectively (Table 2). There was some overlap, but significant differentiation among treatments in volatile composition (ANOSIM: $R = 0.305$; Perm = 1000; $P < 0.0001$; Figure 8). Many of the compounds occurring in the *A. flavus*- and *Fusarium*-inoculated grain were unique compared to the uninoculated- or UV-sanitized grain (Table 2), including 2-(1-propenyl)-6-methylphenol-butanoic acid, cyclopropanecarboxylic acid, and octane for *A. flavus*-inoculated grain and 2-epi- α -cadrene, (1*R*,4*S*,5*S*)-1,8-dimethyl-4-(prop-1-en-2-yl)spiro[4.5]dec-7-ene, and 4-ethyl-1,2-dimethoxy-benzene for *Fusarium*-inoculated grain. Finally, there was significantly higher total emissions by *Fusarium*-inoculated grain compared to the uninoculated grain ($t = 47.7$; $df = 86$; $P < 0.0001$), UV-sanitized grain ($t = 42.9$; $df = 86$; $P < 0.0001$), and *A. flavus*-inoculated grain ($t = 47.1$; $df = 86$; $P < 0.0001$), with 5-, 4-, and 5-fold higher emissions, respectively (Figure 9).

Discussion

Overall, this is the first study to examine in an in-depth manner how specific fungal species affect the behavior of adult *L. serricorne*, particularly how MVOCs from fungi may be used as foraging cues to manipulate insect attraction, mobility, and preference in the post-harvest environment. We included multiple behavioral assays (e.g., four-way olfactometer for preference, video-tracking to evaluate close-range foraging decisions, and a release-recapture assay to determine long-distance attraction) all to ultimately characterize how *L. serricorne* responds to MVOCs and interacts with microbes. Here, we show that inoculating grain with

known plant pathogens has direct implications on the MVOCs produced, and in turn, this affects the behavior of an external-infesting stored product pest, *L. serricorne*. We found that this is true at both close-range and at distance. Together, this presents a convincing case for the importance of insect-microbe interactions on the foraging ecology of *L. serricorne*. Below, we go over each of the main results in turn.

We found close-range foraging by *L. serricorne* is affected by grain and MVOCs. Altogether, the volatile emissions from inoculated grain by the fungal morphotype significantly affects the foraging decisions by *L. serricorne* both when in close-proximity and at a distance to the inoculated grain as compared to uninoculated and sanitized grain. In fact, this supports prior work showing that there are a variety of ecological and behavioral responses by insects to volatile emissions from microbes (Davis et al., 2013). At close-range, *L. serricorne* used volatiles from *Aspergillus flavus*-inoculated grain for foraging decisions, and this translated to some differentiation in the volatile composition from the *A. flavus* treatment. By contrast, a internal-infesting pest, *S. oryzae*, oriented to MVOCs from *Fusarium*-inoculated grain at close-range, but preferred to use only grain volatiles at a distance (Lizarraga 2020; Ponce et al. 2022). In other agricultural systems, when the ubiquitous yeast-like fungus *Aureobasidium pullulans* was used in baited traps, it captured 481% more insects than unbaited traps (Davis and Landolt, 2013), suggesting the generally important role of MVOCs to insects in agroecosystems. Interestingly, our results also showed that grain without microbes (i.e UV-sanitized grain) was also an attractive source for *L. serricorne* at close-range and a distance. In particular, our work showed that the presence of UV-sanitized and *A. flavus*-inoculated grain increased the cumulative duration spent in and the frequency of entering each of those kernel zones. Once *L. serricorne* entered the UV-sanitized or microbial-inoculated kernel zones, their movement was

arrested. In the experiment, mixed-sex adults were used because of difficulty distinguishing sexes as adults. However, males and females may be under different selection pressures. For example, males may be more motivated to disperse, and as such may seek advantages conferred from microbial partners to use intact grain that they may not otherwise have access to (mentioned as a hypothesis in Ponce et al. 2021). By contrast, females may be under selective pressure to find optimal oviposition locations that would be free from microbial contamination for progeny. Follow-up work could find support for this hypothesis by sexing *L. serricorne* when they are pupae, then repeating the experiment to determine if there are differences in response to these two stimuli that break down along sex-based lines. In prior work, the fungal volatile, 1-octen-3-ol was a strong attractant for *Ahaversus advena* (Waltl) (Coleoptera: Silvanidae) (Pierce et al., 1991) supporting the contention that stored product insects use MVOCs in foraging decisions. Overall, our work confirms that there is some selectivity in which fungal volatiles elicit a behavioral response by the insect, and that these volatiles may be used both at close and long range by *L. serricorne*.

Movement is an important variable undergirding fundamental life processes of insects. Here, we found that adult *L. serricorne* moved 2–4-fold less and their velocity was numerically decreased by 2–3-fold when exposed to a grain kernel of one of the four semiochemical treatments compared to the negative control. This suggests that one of the key processes happening at close range in response to the grain is arrestment, demonstrated by slower and less movement of *L. serricorne* in response to the semiochemicals. Prior work has documented the sublethal effects of insecticide, dispersal, and foraging using distance moved and velocity as surrogates of movement (Guedes et al., 2017) that can be mediated by good and poor sanitation (Morrison et al., 2019). However, prior work has rarely used velocity or distance moved and

Ethovision to evaluate response to MVOCs from grain by insects. Nevertheless, Lizarraga (2020) found a numeric decrease in the distance moved and velocity when *S. oryzae* were exposed to the uninoculated grain, but this was not significantly different from the negative control or any other treatment. McFarlane (2018) used Ethovision and found *T. castaneum* most often spent time in a wind tunnel adjacent to an empty arena instead of one with a stimulus containing *Aspergillus tubingensis*. Most other work with Ethovision has been on the sublethal effects of insecticides (Guedes et al. 2008, 2011; Morrison et al. 2018; Arthur and Morrison 2020; Wilkins et al. 2020) demonstrating an overlooked tool in understanding the effect of MVOCs on the behavioral and chemical ecology of stored product pests.

While prior studies have directly assessed response to fungal volatiles (Pierce et al. 1981, 1989, 1991; Nout and Bartelt 1998), our study links inoculation of grain by specific microbes with changes in MVOC headspace profiles and determined the implications for *L. serricorne* behavior. We found a total of 44 tentatively identified, but distinct principal compounds in the headspace emissions from clean and inoculated grain. There were unique cues in the fungal-inoculated grain, but fungal identity, and by consequence, compound identity appears to matter for behavioral response by *L. serricorne*. *Fusarium*-inoculated grain was significantly different than the other treatments, yet it did not elicit the same change in behavior as the *A. flavus*-inoculated grain, which showed more overlap with the other treatments. Thus, minor compounds uniquely emitted by or emitted in unique ratios by *A. flavus*, but not present in the other treatments (Table 2), may be important for behavioral response of *L. serricorne*. For example, both 5-methyl-2-(1-methylethyl)-1-hexanol and 2-(1-propenyl)-6-methylphenol-butanoic acid were only present in *A. flavus*-inoculated grain and not in any other treatment. Interestingly, Ponce et al., 2021 found that 3-octanone, 1-octen-3-ol, octane, 3-methyl-1-butanol and many

others were the most commonly tested MVOCs for behavioral effects against stored-product arthropods. Similarly, Sinha (1990) found 3-methyl-1-butanol, 1-octen-3-ol, and 3-octanone were the most common compounds isolated in experimental grain bins, which supports some of the headspace volatiles emitted from grain in our experiment. In addition, many of the compounds occurring in the *A. flavus*- and *Fusarium*-inoculated grain were unique compared to the uninoculated- or UV-sanitized grain (Table 2), including 2-(1-propenyl)-6-methylphenol-butanoic acid, cyclopropanecarboxylic acid, and octane for *A. flavus*-inoculated grain and 2-epi- α -cadrene, a long-chain alkene, and 4-ethyl-1,2-dimethoxy-benzene for *Fusarium*-inoculated grain. Overall, the total emission rate for volatiles produced by *Fusarium*-inoculated grain is 3–5-fold several magnitudes higher, than the uninoculated grain. While overall volatile emissions among fungal-inoculated grain were similar, the identity of the volatiles varied. This may lead to the differences observed insect behavior. In the future, complex blends of MVOCs emitted by common fungi should be evaluated against stored product insects using gas chromatography coupled with electroantennography (GC-EAD) in order to determine which volatiles are perceived by individuals. Further investigation of the MVOCs on foraging behavior of stored product insects may lead to promising candidate compounds that are behaviorally-active to manipulate pest populations in management tactics at food facilities (Phoonan et al. 2014; Morrison et al. 2021).

No preference was found by *L. serricorne* among the semiochemical stimuli including UV-sanitized grain, uninoculated grain, *Fusarium verticilloides*-inoculated grain, *Aspergillus flavus*-inoculated grain and the negative control using the four-way olfactometer. This could be because the still-air assay did not approximate real-world conditions, as a result of a few factors. For example, there may have been a thigmotactic reaction to the glass substrate, which is not

commonly found in the environment of *L. serricorne*. Other stored product pests have been documented to exhibit thigmotactic responses to unusual textures in prior work (Domingue et al. 2021). Finally, it is possible that while some of the treatments have attractant or arresting properties as individual components, complex blends admixing in the headspace result in no preference among each other. Performance of this assay was done in the biosafety cabinet which generated a barrier of air flow to prevent contamination in the main lab by *A. flavus* and *Fusarium*-inoculated grain. This airflow could have potentially altered the natural diffusion of volatiles or could have even admixed the volatile cues, making it difficult for the *L. serricorne* to make a choice via odorant cued response (e.g., Lizarraga 2020; Ponce et al. 2022). Prior work found that the invasive stored product dermestid *Trogoderma granarium* Everts (Coleoptera: Dermestidae) showed attraction to pheromonal and kairomonal stimuli that were not perfectly congruous with the results from work in related preference assays for the same stimuli (Morrison et al., 2020). Gerken et al., (2018) found that even with the same species (e.g., *T. castaneum*), one could obtain different results using multiple behavioral assays, and suggested that one must carefully select the assays and consider the actual variables being tested. However, the lack of a positive response in only one out of the multiple assays in this study suggests that there is a relatively robust response to MVOCs by *L. serricorne*.

In the future, research should be prioritized to 1) address which compounds identified in Table 2 are actually detected by *L. serricorne* through GC-EAD, 2) follow-up on GC-EAD results with behavioral tests to determine whether the compounds are attractive, repellent, or neutral, and 3) formulate new lures with the most attractive compounds to improve monitoring and management programs. This will help narrow the behaviorally-active components of the headspace blends to those that are being detected by the insect. Overall, this study has enhanced

our understanding of how microbially-produced volatiles organic compounds from postharvest fungal grain pathogens affect the behavior of *L. serricorne*. Increasingly, it is apparently essential to manage microbes and insects simultaneously in the postharvest agricultural supply to ensure that our planet can feed its growing population.

References

- Adams RP (2007) Identification of Essential Oil Components by Gas Chromatography/Mass Spectrometry. Allured Publishing Corporation, Carol Stream, IL
- Altschul SF, Madden TL, Schäffer AA, et al (1997) Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucleic Acids Res* 25:3389–3402.
<https://doi.org/10.1080/14620316.2005.11511891>
- Amaike S, Keller NP (2011) *Aspergillus flavus*. *Annu Rev Phytopathol* 49:107–133.
<https://doi.org/10.1146/annurev-phyto-072910-095221>
- Arthur F, Morrison WR (2020) Methodology for assessing progeny production and grain damage on commodities treated with insecticides. *Agronomy* 10: 804.
<https://doi.org/10.3390/agronomy10060804>
- Bumbangi NF, Muma JB, Choongo K, et al (2016) Occurrence and factors associated with aflatoxin contamination of raw peanuts from Lusaka district's markets, Zambia. *Food Control* 68:291–296. <https://doi.org/10.1016/j.foodcont.2016.04.004>
- Campbell JF, Mullen MA, Dowdy AK (2002) Monitoring stored-product pests in food processing plants with pheromone trapping, contour mapping, and mark-recapture. *J Econ Entomol* 95:1089–101.
<https://doi.org/10.1603/0022-0493-95.5.1089>
- Cotter T (2014) Organic Mushroom Farming and Mycoremediation: Simple to Advanced and Experimental Techniques for Indoor and Outdoor Cultivation. White River Junction: Chelsea Green Publishing, pp 363.
- Davis TS, Crippen TL, Hofstetter RW, Tomberlin JK (2013) Microbial volatile emissions as insect semiochemicals. *J Chem Ecol* 39:840–859. <https://doi.org/10.1007/s10886-013-0306-z>

- Davis TS, Landolt PJ (2013) A survey of insect assemblages responding to volatiles from a ubiquitous fungus in an agricultural landscape. *J Chem Ecol* 39:860–868. <https://doi.org/10.1007/s10886-013-0278-z>
- Domingue MJ, Scheff DS, Arthur FH, Myers SW (2021) Sublethal exposure of *Trogoderma granarium* Everts (Coleoptera: Dermestidae) to insecticide-treated netting alters thigmotactic arrestment and olfactory-mediated anemotaxis. *Pestic Biochem Physiol* 171:104742. <https://doi.org/10.1016/j.pestbp.2020.104742>
- Doud CW, Cuperus GW, Kenkel P, et al (2021) Trapping *Tribolium castaneum* (Coleoptera: Tenebrionidae) and other beetles in flourmills: Evaluating fumigation efficacy and estimating population density. *Insects* 12: 144.
- Doud CW, Phillips TW (2020) Responses of red flour beetle adults, *Tribolium castaneum* (Coleoptera: Tenebrionidae), and other stored product beetles to different pheromone trap designs. *Insects* 11: 733.
- Edde PA (2019) Biology, Ecology, and Control of *Lasioderma serricorne* (F.) (Coleoptera: Anobiidae): A Review. *J Econ Entomol* 112:1011–1031. <https://doi.org/10.1093/jee/toy428>
- Gerken AR, Scully ED, Campbell JF (2018) Red flour beetle (Coleoptera: Tenebrionidae) response to volatile cues varies with strain and behavioral assay. *Environ Entomol* 47:1252–1265. <https://doi.org/10.1093/ee/nvy107>
- Gregg, PC, Del Socorro, AP, Landolt, PJ (2018) Advances in attract-and-kill for agricultural pests: Beyond pheromones. *Annu Rev Entomol* 63: 453–470.
- Guedes RNC, Campbell J., Arthur FH, et al (2008) Acute lethal and behavioral sublethal responses of two stored-product psocids to surface insecticides. *Pest Manag Sci* 64:1314–1322

- Guedes RNC, Guedes NMP, Rosi-Denadai CA (2011) Sub-lethal effects of insecticides on stored-product insects: Current knowledge and future needs. *Stewart Postharvest Rev* 7:1–5.
<https://doi.org/10.2212/spr.2011.3.5>
- Guedes RNC, Walse SS, Throne JE (2017) Sublethal exposure, insecticide resistance, and community stress. *Curr Opin Insect Sci* 21:47–53. <https://doi.org/10.1016/j.cois.2017.04.010>
- Hubert J, Stejskal V, Athanassiou CG, Throne JE (2018) Health hazards associated with arthropod infestation of stored products. *Annu Rev Entomol* 63:553–573. <https://doi.org/10.1146/annurev-ento-020117-043218>
- Inamdar AA, Morath S, Bennett JW (2020) Fungal volatile organic compounds: more than just a funky smell? *Annu Rev Microbiol* 74:101–116. <https://doi.org/10.1146/annurev-micro-012420-080428>
- Kawakami, Y. IS, H. Takahashi (2002) Some fungi isolated from the cigarette beetles, *Lasioderma serricornis* Fabricius (Coleoptera: Anobiidae) in Japan. *Med Entomol Zool* 249–256
- LeBlanc N, Kinkel LL, Kistler HC (2015) Soil fungal communities respond to grassland plant community richness and soil edaphics. *Microb Ecol* 70: 188–195.
<https://doi.org/10.1007/s00248-014-0531-1>
- Lizarraga S (2020) Elucidating the behavioral response of a pestiferous stored product insect to microbially-mediated volatile emissions in grain. Biology Department, Kalamazoo College, Kalamazoo, MI
- Magan N, Hope R, Cairns V, Aldred D (2003) Post-harvest fungal ecology: Impact of fungal growth and mycotoxin accumulation in stored grain. *Eur J Plant Pathol* 109:723–730.
<https://doi.org/10.1023/A:1026082425177>
- Maille J, Gerken A, Adrianos S, et al (2021) Exploiting chemosensory genomics for improved monitoring and control of stored product pests. *Insects*, In press:

- Mannaa M, Kim KD (2017) Influence of temperature and water activity on deleterious fungi and mycotoxin production during grain storage. *Mycobiology* 45:240–254.
<https://doi.org/10.5941/MYCO.2017.45.4.240>
- McFarlane D (2018) The role of fungi and their associated volatiles in the ecology of *Tribolium castaneum*. Dissertation, School of Biological Sciences, The University of Queensland.
- Miller JD (1995) Fungi and mycotoxins in grains: implication for stored product research. *J Stored Prod Res* 31:1–16
- Morrison WR, Bruce A, Wilkins R V., et al (2019a) Sanitation improves stored product insect pest management. *Insects* 10: 77. <https://doi.org/10.3390/insects10030077>
- Morrison WR, Grosdidier RF, Arthur FH, et al (2020) Attraction, arrestment, and preference by immature *Trogoderma variabile* and *Trogoderma granarium* to food and pheromonal stimuli. *J Pest Sci* (2004) 93:135–147. <https://doi.org/10.1007/s10340-019-01171-z>
- Morrison WR, Larson NL, Brabec D, Zhang A (2019b) Methyl benzoate as a putative alternative, environmentally friendly fumigant for the control of stored product insects. *J Econ Entomol* 112:2458–2468. <https://doi.org/10.1093/jee/toz179>
- Morrison WR, Lee DH, Short BD, et al (2016) Establishing the behavioral basis for an attract-and-kill strategy to manage the invasive *Halyomorpha halys* in apple orchards. *J Pest Sci* (2004) 89:81–96. <https://doi.org/10.1007/s10340-015-0679-6>
- Morrison WR, Scully ED, Campbell JF (2021) Towards developing areawide semiochemical-mediated, behaviorally-based integrated pest management programs for stored product insects. *Pest Manag Sci* 77:2667–2682. <https://doi.org/10.1002/ps.6289>
- Morrison WR, Wilkins R V, Gerken AR, et al (2018) Mobility of adult *Tribolium castaneum* (Coleoptera: Tenebrionidae) and *Rhyzopertha dominica* (Coleoptera: Bostrichidae) after

- exposure to long-lasting insecticide-incorporated netting. *J Econ Entomol* 111:2443–2453.
<https://doi.org/10.1093/jee/toy173>
- Nout MJR, Bartelt RJ (1998) Attraction of a flying nitidulid (*Carpophilus humeralis*) to volatiles produced by yeasts grown on sweet corn and a corn-based medium. *J Chem Ecol* 24:1217–1239.
<https://doi.org/10.1023/A:1022451020013>
- O'Donnell K, Kistler HC, Cigelnik E, Ploetz RC (1998) Multiple evolutionary origins of the fungus causing Panama disease of banana: Concordant evidence from nuclear and mitochondrial gene genealogies. *Proc Natl Acad Sci USA* 95: 2044–2049.
- O'Donnell K, Sarver BA, Brandt M et al (2007) Phylogenetic diversity and microsphere array-based genotyping of human pathogenic fusaria, including isolates from the multistate contact lens-associated U.S. keratitis outbreaks of 2005 and 2006. *J Clin Microbiol* 45: 2235–2248.
- Oksanen AJ, Blanchet FG, Friendly M, et al (2019) Vegan: Community Ecology Package. <https://cran.r-project.org>, <https://github.com/vegandevs/vegan>
- Papadopoulou SC, Buchelos CT (2002) Definition of the flight period of *Lasioderma serricorne* (F.) in stored tobacco. *Anzeiger fur Schädlingskd* 81–83. <https://doi.org/10.1034/j.1399-5448.2002.02019.x>
- Phoonan W, Deowanish S, Chavasiri W (2014) Food attractant from mulberry leaf tea and its main volatile compounds for the biocontrol of *Lasioderma serricorne* F. (Coleoptera: Anobiidae). *J Stored Prod Res* 59:299–305. <https://doi.org/10.1016/j.jspr.2014.09.004>
- Pierce AM, Borden JH, Oehlschlager AC (1981) Olfactory response to beetle-produced volatiles and host-food attractants by *Oryzaephilus surinamensis* and *O. mercator*. *Can J Zool* 59:1980–1990.
<https://doi.org/10.1139/z81-270>

- Pierce AM, Pierce HD, Borden JH, Oehlschlager AC (1989) Production dynamics of Cucujolide pheromones and identification of 1-octen-3-ol as a new aggregation pheromone for *Oryzaephilus surinamensis* and *O. mercator* (Coleoptera: Cucujidae). *Environ Entomol* 18:747–755.
<https://doi.org/10.1093/ee/18.5.747>
- Pierce AM, Pierce HD, Borden JH, Oehlschlager AC (1991) Fungal volatiles: semiochemicals for stored-product beetles (Coleoptera: Cucujidae). *J Chem Ecol* 17:581–597
- Ponce MA, Kim TN, Morrison WR (2021) A systematic review of the behavioral responses by stored-product arthropods to individual or blends of microbially produced volatile cues. *Insects* 12:391.
<https://doi.org/10.3390/insects12050391>
- Ponce MA, Lizarraga S, Bruce A, et al (2022) Linking changes in headspace volatiles from grain inoculated with different growth stages of the fungus, *Aspergillus flavus*, to the foraging behavioral response by a primary stored product pest, *Sitophilus oryzae* (Coleoptera: Curculionidae). *Chemoecology* in press:
- R Core Team (2019) R: A language and environment for statistical computing. <https://www.r-project.org/>
- Savi GD, Piacentini KC, Tibola CS, Scussel VM (2014) Mycoflora and deoxynivalenol in whole wheat grains (*Triticum aestivum* L.) from Southern Brazil. *Food Addit Contam Part B* 7:232–237.
<https://doi.org/10.1080/19393210.2014.898337>
- Sinha R. (1987) Role of odor volatiles in the stored-grain ecosystem. *Agric Canada Res Stn* 3–13
- Tang X, Shao YL, Tang YJ, Zhou WW (2018) Antifungal activity of essential oil compounds (geraniol and citral) and inhibitory mechanisms on grain pathogens (*Aspergillus flavus* and *Aspergillus ochraceus*). *Molecules* 23:2108. <https://doi.org/10.3390/molecules23092108>

- Usseglio VL, Dambolena JS, Martinez MJ, Zunino MP (2020) The role of fumonisins in the biological interaction between *Fusarium verticillioides* and *Sitophilus zeamais*. *J Chem Ecol* 46:1059–1068. <https://doi.org/10.1007/s10886-020-01220-3>
- Van Winkle T, Ponce M, Quellhorst H, et al Microbial volatile organic compounds mediate attraction by a primary but not a secondary stored-product insect pest in wheat. Kalamazoo College, Kalamazoo, MI
- Wang Q, Garrity GM, Tiedje JM, Cole JR (2007) Naïve Bayesian classifier for rapid assignment of rRNA sequences into the new bacterial taxonomy. *Appl Environ Microbiol* 73:5261–5267. <https://doi.org/10.1128/AEM.00062-07>
- White TJ, Bruns T, Lee S, Taylor JW (1990) Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In: Innis MA, Gelfand DH, Sninsky JJ, White TJ (eds) *PCR Protocols: A Guide to Methods and Applications*. Academic Press, New York, pp 315–322
- Wilkins R V., Campbell JF, Zhu KY, et al (2021) Long-lasting insecticide-incorporated netting and interception traps at pilot-scale warehouses and commercial facilities prevents infestation by stored product beetles. *Front Sustain Food Syst* 4:561820. <https://doi.org/10.3389/fsufs.2020.561820>
- Wilkins R V., Zhu KY, Campbell JF, Morrison WR (2020) Mobility and dispersal of two cosmopolitan stored-product insects are adversely affected by long-lasting insecticide netting in a life stage-dependent manner. *J Econ Entomol* 113:1768–1779. <https://doi.org/10.1093/jee/toaa094>

Table 3-1. List of semiochemical treatments and the amounts used in each assay during the study.

Abbrev.	Treatments	Amount in Assay			
		4-way Olfact.	Ethovision	Headspace	Release- Recapture
Neg. Ctrl	Negative Control	0	0	0	0
P. Ctrl	Positive Control (uninoculated)	-	1 kernel	20 g	10 g
AF-grain	Aspergillus flavus-inoculated	10 g	1 kernel	20 g	10 g
FS-grain	Fusarium spp.-inoculated	10 g	1 kernel	20 g	10 g
UV-grain	UV-surface sanitized for 10 min	10 g	1 kernel	20 g	10 g

Table 3-2. Summary of headspace volatiles emitted (in ng h⁻¹ g⁻¹ grain) from grain that was uninoculated (P. ctrl), surface sanitized with UV-radiation for 10-min (UV-grain), or inoculated with *A. flavus* or *Fusarium* spp. for periods of 3 hours using a push headspace collection systems. There were n=7 replicates per treatment and mean emission rates per compound are portrayed.

Compound	Ret Time	P. Ctrl				UV-Grain				<i>A. flavus</i> -Grain				<i>Fusarium</i> -Grain			
		Mean	±	SE	% of Total	Mean	±	SE	% of Total	Mean	±	SE	% of Total	Mean	±	SE	% of Total
4-Ethylbenzamide	4.257	0	±	0.00	0	0.00	±	0.00	0	0.00	±	0.00	0	0.17	±	0.11	6
cis-1-Butyl-2-methylcyclopropane	4.376	0.10	±	0.06	18	0.03	±	0.01	4	0.04	±	0.02	6	0.01	±	0.02	0
3-Octene, (E)	4.38	0.00	±	0.00	0	0.02	±	0.01	3	0.02	±	0.02	3	0.00	±	0.00	0
4-Heptanone	4.454	0.01	±	0.01	2	0.02	±	0.01	2	0.01	±	0.01	2	0.03	±	0.04	1
Octane	4.464	0.00	±	0.00	0	0.00	±	0.00	0	0.02	±	0.03	3	0.05	±	0.07	2
2,4-Dimethyl-1-heptene	4.907	0.01	±	0.01	3	0.05	±	0.02	7	0.02	±	0.02	4	0.03	±	0.03	1
2-Pentanone, 4-hydroxy-4-methyl	4.916	0.03	±	0.02	6	0.05	±	0.04	6	0.03	±	0.04	5	0.00	±	0.00	0
3-Hexanone, 2-methyl	5.26	0.00	±	0.00	0	0.00	±	0.00	0	0.00	±	0.00	0	0.03	±	0.04	1
Pyrazole-1-methanol	5.633	0.00	±	0.00	0	0.03	±	0.02	3	0.01	±	0.01	1	0.00	±	0.00	0
Mesitylene	6.618	0.00	±	0.00	0	0.00	±	0.00	0	0.00	±	0.00	0	0.06	±	0.07	2

Pentane, 2,2,3,3-tetramethyl	7.094	0.03	±	0.01	6	0.06	±	0.02	8	0.02	±	0.02	4	0.04	±	0.03	1
Octane, 3,4,5,6-tetramethyl	7.152	0.04	±	0.01	7	0.03	±	0.01	4	0.05	±	0.03	9	0.13	±	0.13	4
Hexanoic acid, 2-ethyl-, methyl ester	7.569	0.00	±	0.00	0	0.00	±	0.00	0	0.00	±	0.00	0	0.12	±	0.06	4
Ether, hexyl pentyl	7.885	0.00	±	0.00	0	0.01	±	0.01	1	0.01	±	0.01	1	0.00	±	0.00	0
3-Hydroxy-3-methylvaleric acid	7.892	0.01	±	0.01	2	0.00	±	0.00	0	0.00	±	0.00	0	0.06	±	0.05	2
Benzene, 1,4-diethyl	7.894	0.00	±	0.00	0	0.01	±	0.01	2	0.00	±	0.00	0	0.00	±	0.00	0
Phenol, 3,4,5-trimethyl	8.134	0.00	±	0.00	0	0.01	±	0.01	2	0.01	±	0.01	1	0.15	±	0.05	5
1R,2c,3t,4t-Tetramethyl-cyclohexane	8.138	0.02	±	0.01	3	0.01	±	0.01	1	0.00	±	0.00	0	0.00	±	0.00	0
Ethanol, 2-(octyloxy)	8.14	0.01	±	0.01	1	0.00	±	0.00	0	0.01	±	0.01	1	0.00	±	0.00	0
1-Undecene, 7-methyl	8.2	0.00	±	0.00	0	0.01	±	0.01	1	0.02	±	0.01	3	0.00	±	0.00	0
1-Dodecanol	8.206	0.00	±	0.00	0	0.01	±	0.01	1	0.01	±	0.01	2	0.00	±	0.00	0
Undecane	8.407	0.04	±	0.03	7	0.03	±	0.03	4	0.02	±	0.03	3	0.01	±	0.01	0
Nonanal	8.505	0.06	±	0.04	11	0.06	±	0.05	7	0.03	±	0.03	5	0.00	±	0.00	0
Cyclopropanecarboxylic acid	8.515	0.00	±	0.00	0	0.00	±	0.00	0	0.02	±	0.03	3	0.25	±	0.26	8
5-Methylthiopyridin-2-ol	9.171	0.04	±	0.03	8	0.03	±	0.02	4	0.00	±	0.00	0	0.03	±	0.04	1

3-Trimethylsilyloxyhex-4-yne	9.176	0.00	±	0.00	0	0.00	±	0.00	0	0.03	±	0.04	6	0.06	±	0.07	2
1-Hexanol, 5-methyl-2-(1-methylethyl)	10.538	0.00	±	0.00	0	0.00	±	0.00	0	0.00	±	0.01	1	0.00	±	0.00	0
4-Phenyl-2-butanol	10.649	0.00	±	0.00	0	0.03	±	0.03	3	0.00	±	0.00	0	0.00	±	0.00	0
Benzene, 1,3-bis(1,1-dimethylethyl)-	10.784	0.09	±	0.03	16	0.16	±	0.05	20	0.13	±	0.04	22	0.11	±	0.06	4
Ethanone, 1-(4-ethylphenyl)-	10.961	0.00	±	0.00	0	0.00	±	0.00	0	0.00	±	0.00	0	0.00	±	0.00	0
Butanoic acid, 2-(1-Propenyl)-6-methylphenol -	11.039	0.00	±	0.00	0	0.00	±	0.00	0	0.02	±	0.02	4	0.00	±	0.00	0
m-Ethylacetophenone -	11.247	0.00	±	0.00	0	0.08	±	0.07	10	0.00	±	0.00	0	0.00	±	0.00	0
(S-(Propoxythiocarbonyl)thiohydroxylamine-	11.417	0.00	±	0.00	0	0.00	±	0.00	0	0.01	±	0.01	1	0.02	±	0.03	1
1-Ethyl-2,2,6-trimethylcyclohexane -	11.539	0.00	±	0.00	1	0.00	±	0.00	1	0.00	±	0.00	0	0.00	±	0.00	0
2,3,6-Trimethylhept-3-en-1-ol -	11.544	0.00	±	0.00	0	0.00	±	0.00	0	0.01	±	0.01	2	0.00	±	0.00	0
Ketone, methyl 2,2,3-trimethylcyclopentyl	11.664	0.00	±	0.00	0	0.00	±	0.00	1	0.01	±	0.01	1	0.00	±	0.00	0
Dodecane, 4-methyl-	11.67	0.02	±	0.01	3	0.00	±	0.00	0	0.01	±	0.01	2	0.00	±	0.00	0
Benzene, 4-ethyl-1,2-dimethoxy-	11.678	0.00	±	0.00	0	0.00	±	0.00	0	0.00	±	0.00	0	0.49	±	0.31	16

.alpha.-Cubebene	13.217	0.00	±	0.00	0	0.00	±	0.00	0	0.00	±	0.00	0	0.08	±	0.05	3
Germacrene D	13.371	0.00	±	0.00	0	0.00	±	0.00	0	0.00	±	0.00	0	0.16	±	0.08	5
(1R,4S,5S)-1,8-Dimethyl-4-(prop-1-en-2-yl)spiro[4.5]dec-7-ene	13.816	0.00	±	0.00	0	0.00	±	0.00	0	0.00	±	0.00	0	0.40	±	0.15	13
Di-epi-.alpha.-cedrene	15.892	0.00	±	0.00	0	0.00	±	0.00	0	0.00	±	0.00	0	0.35	±	0.17	12
Benzimidazole, 2-ethyl-1-propyl-	18.422	0.00	±	0.00	0	0.00	±	0.00	0	0.00	±	0.00	0	0.03	±	0.02	1
Hexanedioic acid, bis(2-ethylhexyl) ester	23.112	0.04	±	0.04	7	0.03	±	0.03	4	0.03	±	0.05	5	0.11	±	0.12	4
Total Emissions (ng per h per g grain)		0.6		0.01	100	0.8		0.01	100	0.58		0.01	100	3.0		0.05	100

Figure 3-1. Habitus images of morphotype 1 (left, *Aspergillus flavus*-inoculated grain) and morphotype 2 (right, *Fusarium verticillioides*-inoculated grain) at 10× magnification (SMZ-18, Nikon Inc., Tokyo, Japan) on grain after inoculation procedure.

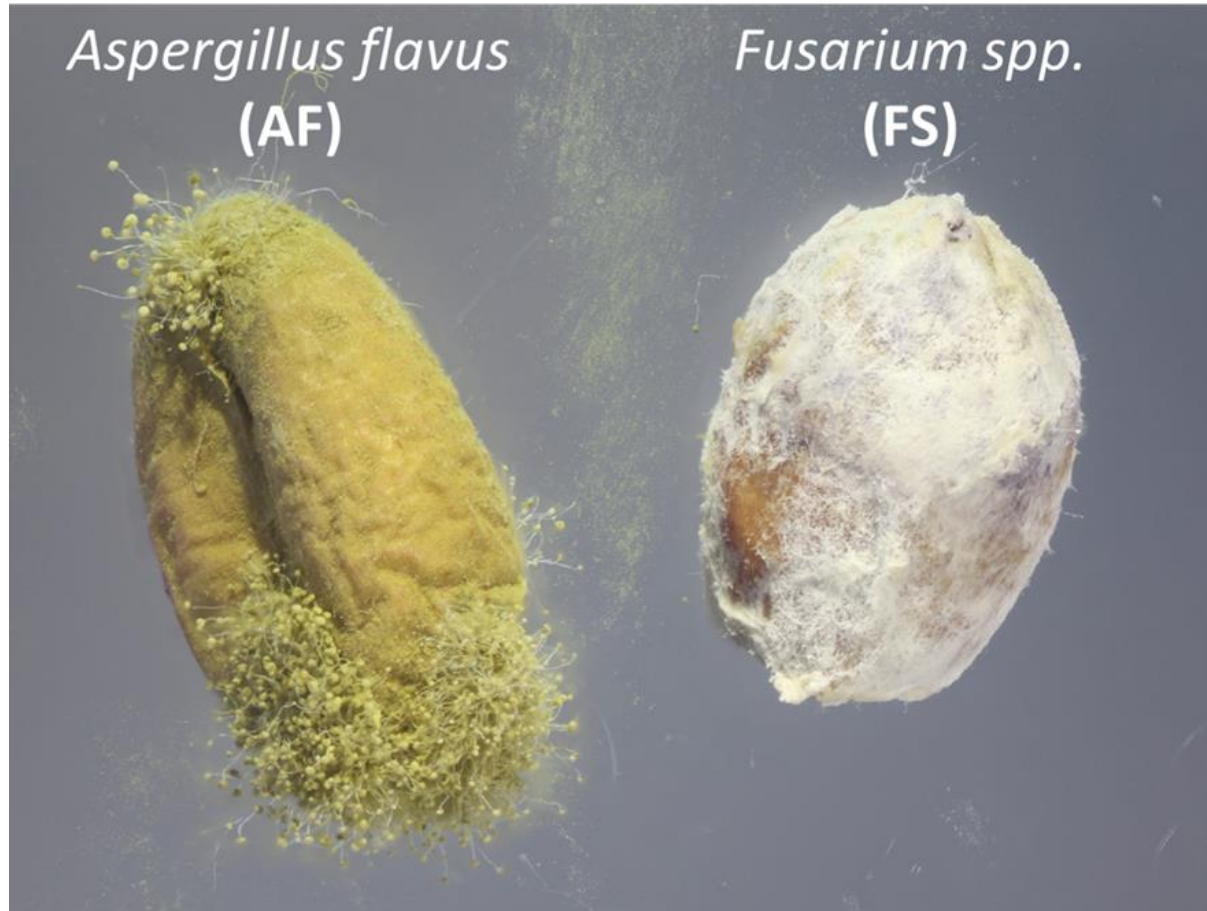


Figure 3-2. 3D Habitus images of *Aspergillus flavus* at 10× magnification (SMZ-18, Nikon Inc., Tokyo, Japan) used to inoculate grain in assays. *A. flavus* is characterized by its green, filamentous morphology and visible spores.

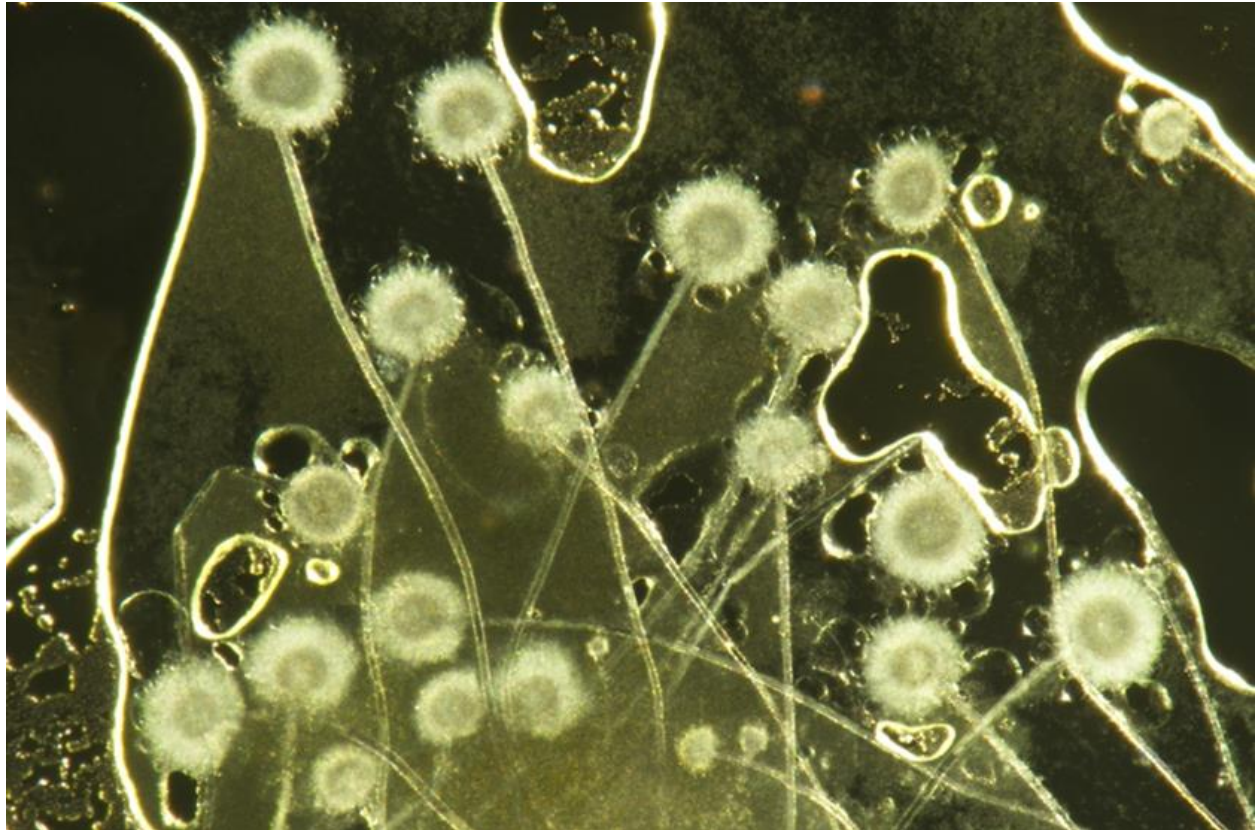


Figure 3-3. Preference shown in the four-way, still-air olfactometer using the mean percentage of *L. serricorne* adults choosing a stimulus ($\pm$ SE) after given a 4-min opportunity (top panel) and their mean time to decision ($\pm$ SE, s; bottom panel) based on the semiochemical treatment to which they were exposed in the laboratory at the USDA-ARS Center for Grain and Animal Health Research in Manhattan, KS. A total of $n = 200$ adults replicate were tested in this assay. Bars with shared letters are not significantly different from each other (Tukey HSD, $\alpha = 0.05$). Abbreviations: Neg. Ctrl – negative control; UV-grain – UV-sanitized grain; P. Ctrl – uninoculated grain; FS-grain – *Fusarium verticilloides*-inoculated grain; AF-grain – *A. flavus*-inoculated grain.

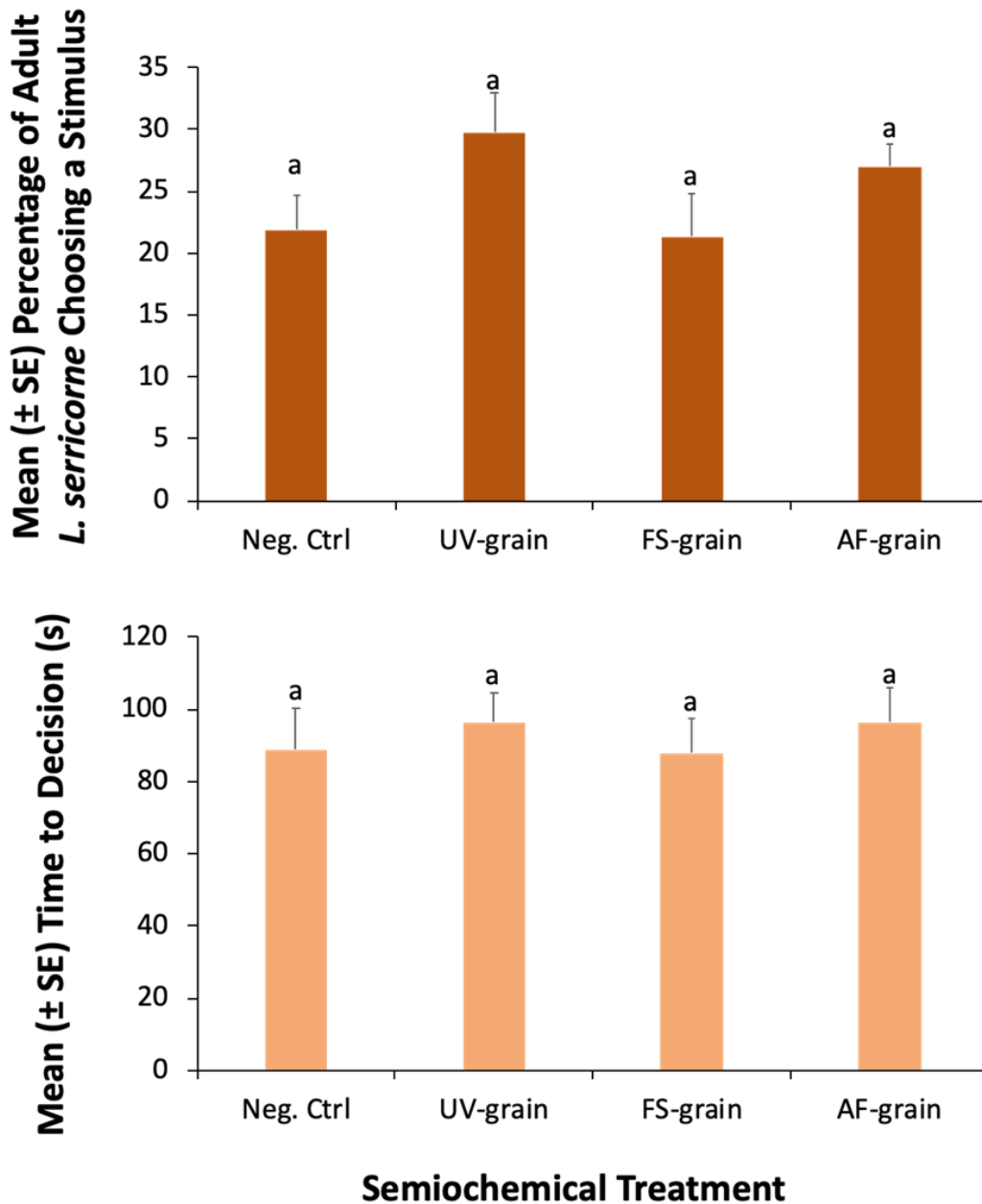


Figure 3-4. Metrics from the movement assay assessing the effects of MVOCs on mean distance moved ($\pm$ SE, top panel) and the mean instantaneous velocity ($\pm$ SE, bottom panel) by adult *L. serricorne* over a period of 30 min using a network camera coupled with Ethovision in the laboratory at the USDA-ARS Center for Grain and Animal Health Research in Manhattan, KS. There were a total of $n = 20$ replicates per treatment, translating to 50 hours of data in this assay. Bars with shared letters are not significantly different from each other (Tukey HSD, $\alpha = 0.05$). Abbreviations: Neg. Ctrl – negative control; UV-grain – UV-sanitized grain; P. Ctrl – uninoculated grain; FS-grain – *Fusarium verticilloides*-inoculated grain; AF-grain – *A. flavus*-inoculated grain.

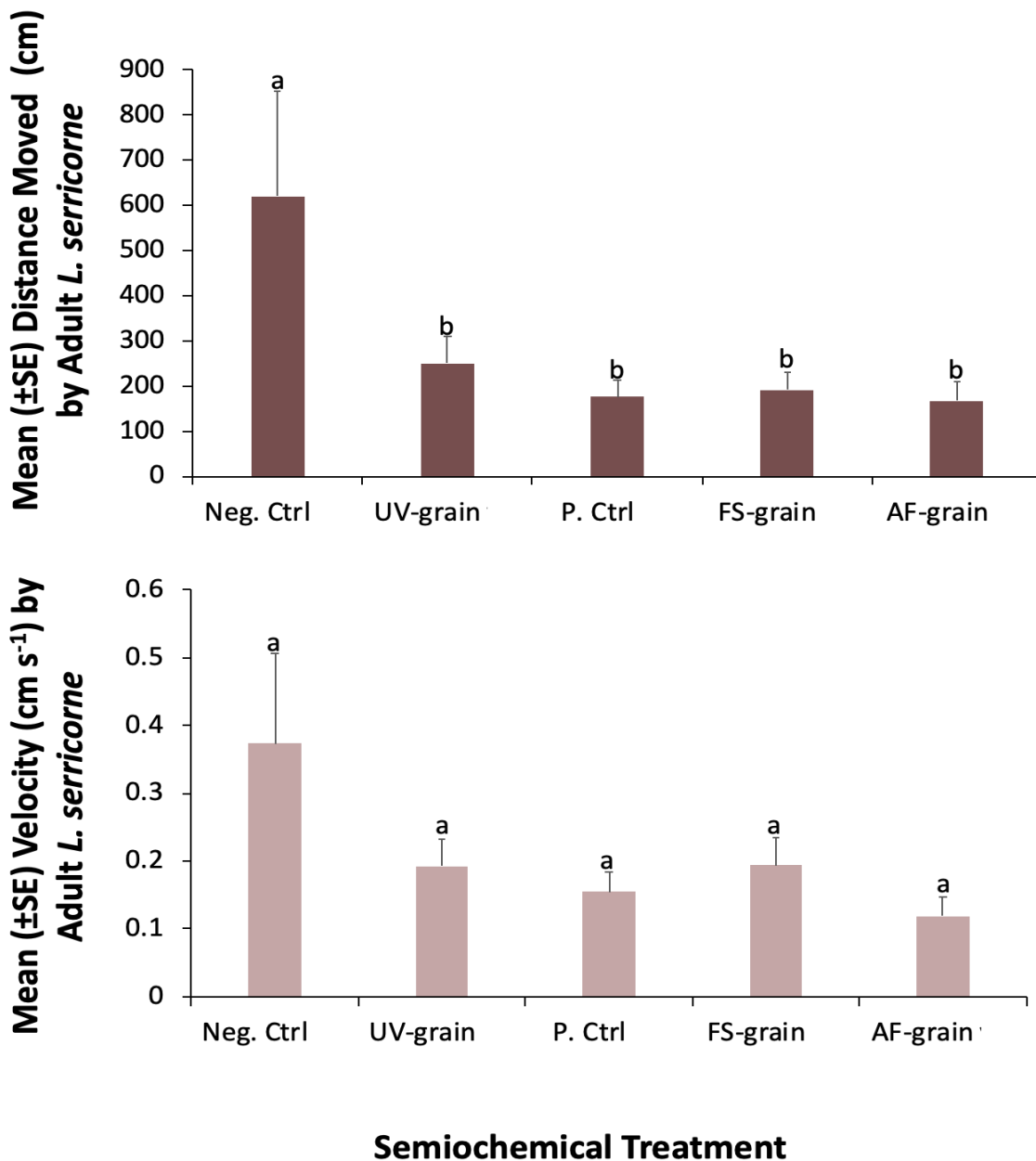


Figure 3-5. MVOC effects on adult *L. serricorne* behavior in kernel zones. Treatment (dark orange bars) and control (dark yellow bars) metrics from movement assay. Frequency of entering each kernel zone (top), duration spent in each zone (middle), and latency to finding each zone (bottom). Conducted at USDA-ARS Center for Grain and Animal Health Research. n = 20 adults per treatment, totaling 50 hours of data. Statistical comparisons: Tukey HSD ($\alpha = 0.05$) and paired t-tests (Bonferroni correction). Abbreviations: Neg. Ctrl – negative control; UV-grain – UV-sanitized grain; P. Ctrl – uninoculated grain; FS-grain – *Fusarium verticilloides*-inoculated grain; AF-grain – *A. flavus*-inoculated grain

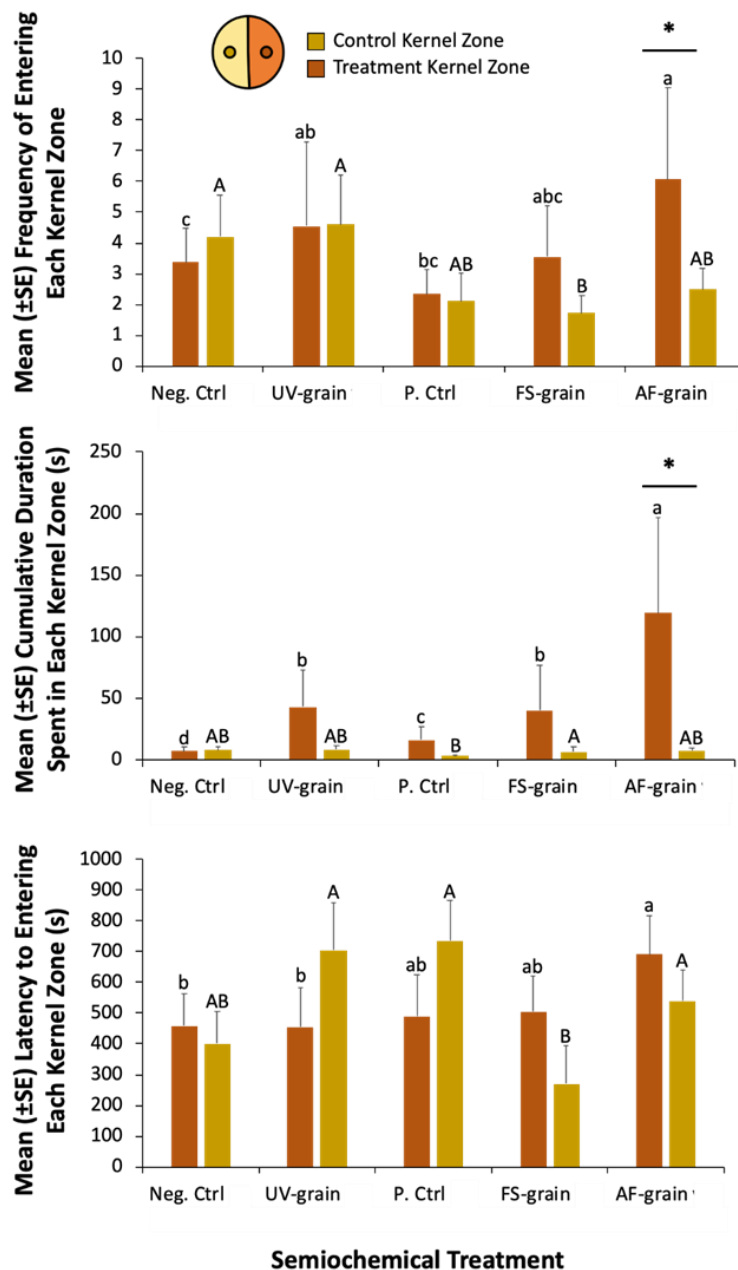


Figure 3-6. Treatment and control zone metrics for adult *L. serricorne* in a movement assay using video-tracking. Effects of MVOCs on zone frequency, cumulative duration, and latency. USDA-ARS Center for Grain and Animal Health Research, Manhattan, KS. n=20 adults per treatment, 30 min duration. Significant differences indicated by letters. Abbreviations: Neg. Ctrl – negative control; UV-grain – UV-sanitized grain; P. Ctrl – uninoculated grain; FS-grain – *Fusarium verticilloides*-inoculated grain; AF-grain – *A. flavus*-inoculated grain.

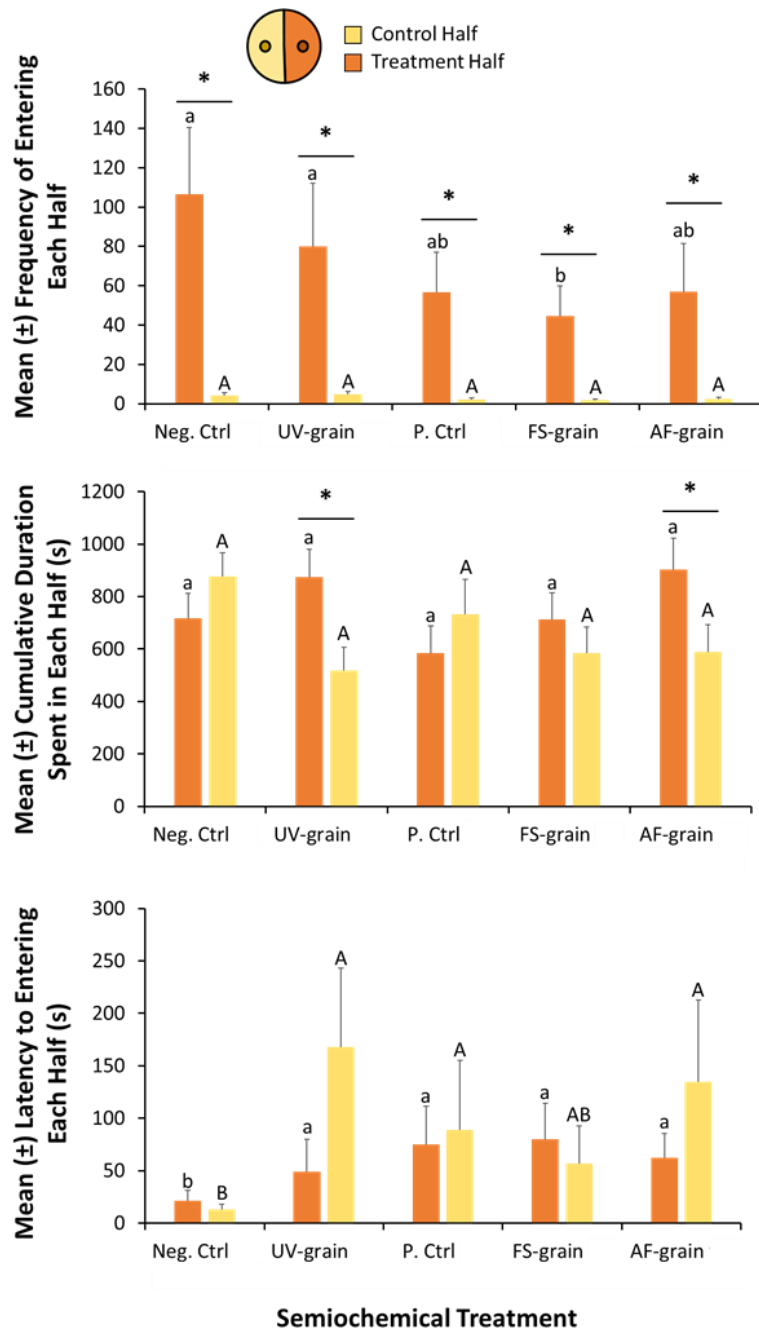


Figure 3-7. The mean percentage of adult *L. serricorne* recaptured in the release-recapture assay in response to MVOCs and grain used as lures in commercially-available monitoring pitfall traps, with a total of 20 adult, mixed-sex *L. serricorne* released in large plastic bins and placed in a large, walk-in environmental chamber under constant abiotic conditions and given 24 h to respond at the USDA-ARS Center for Grain and Animal Health Research in Manhattan, KS for a total of $n = 8$ replicate releases per treatment. Bars with shared letters are not significantly different from each other (Tukey HSD, $\alpha = 0.05$).

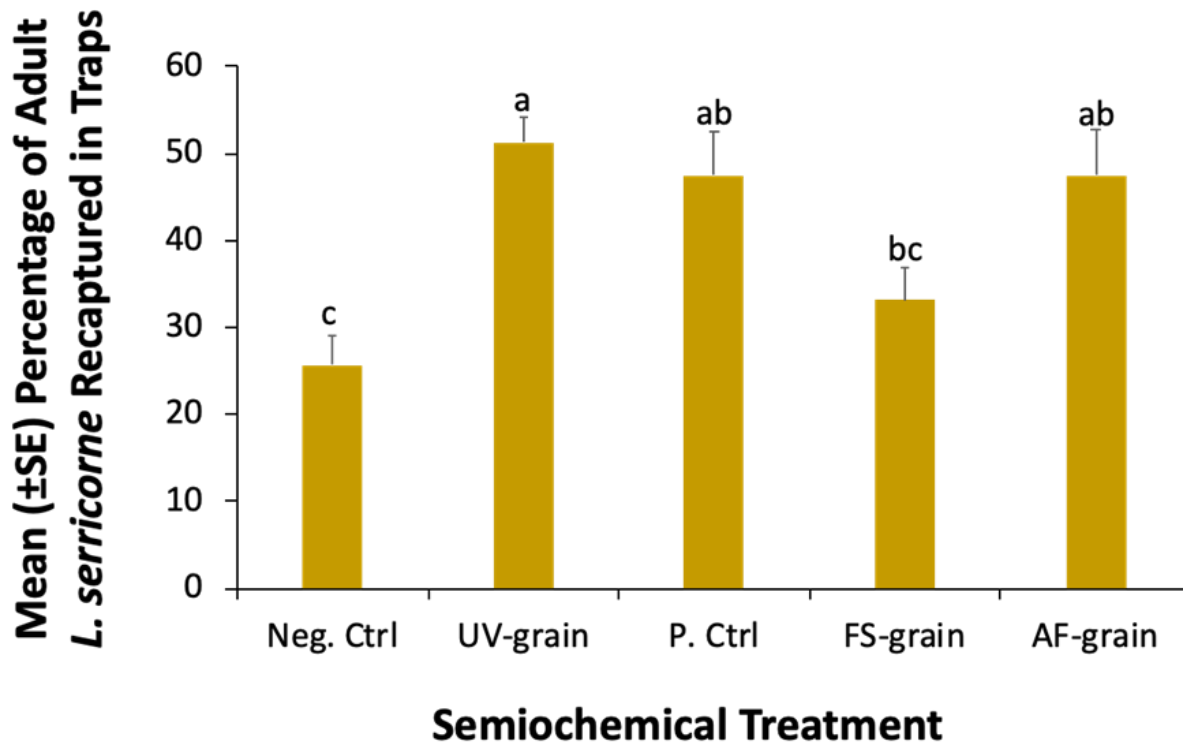


Figure 3-8. Non-metric multi-dimensional scaling ordination plot based on pairwise Bray-Curtis similarity index calculated between each combination of volatile emissions (ng h⁻¹ g⁻¹ grain) by samples after headspace collection for 3 h from 20 g of grain that was UV sanitized for 10 min (blue circles), inoculated with *A. flavus* (yellow circles), inoculated with *Fusarium* spp (red circles), or uninoculated (brown circles). There were n = 7 replicates per semiochemical treatment. Polygons represent convex hulls drawn between samples and are color coded similarly to treatments. There were a total of n = 1000 permutations, and stress was < 0.10, enabling adequate interpretation. Abbreviations: UV-grain – UV-sanitized grain; P. Ctrl – uninoculated grain; FS-grain – *Fusarium verticilloides*-inoculated grain; AF-grain – *A. flavus*-inoculated grain.

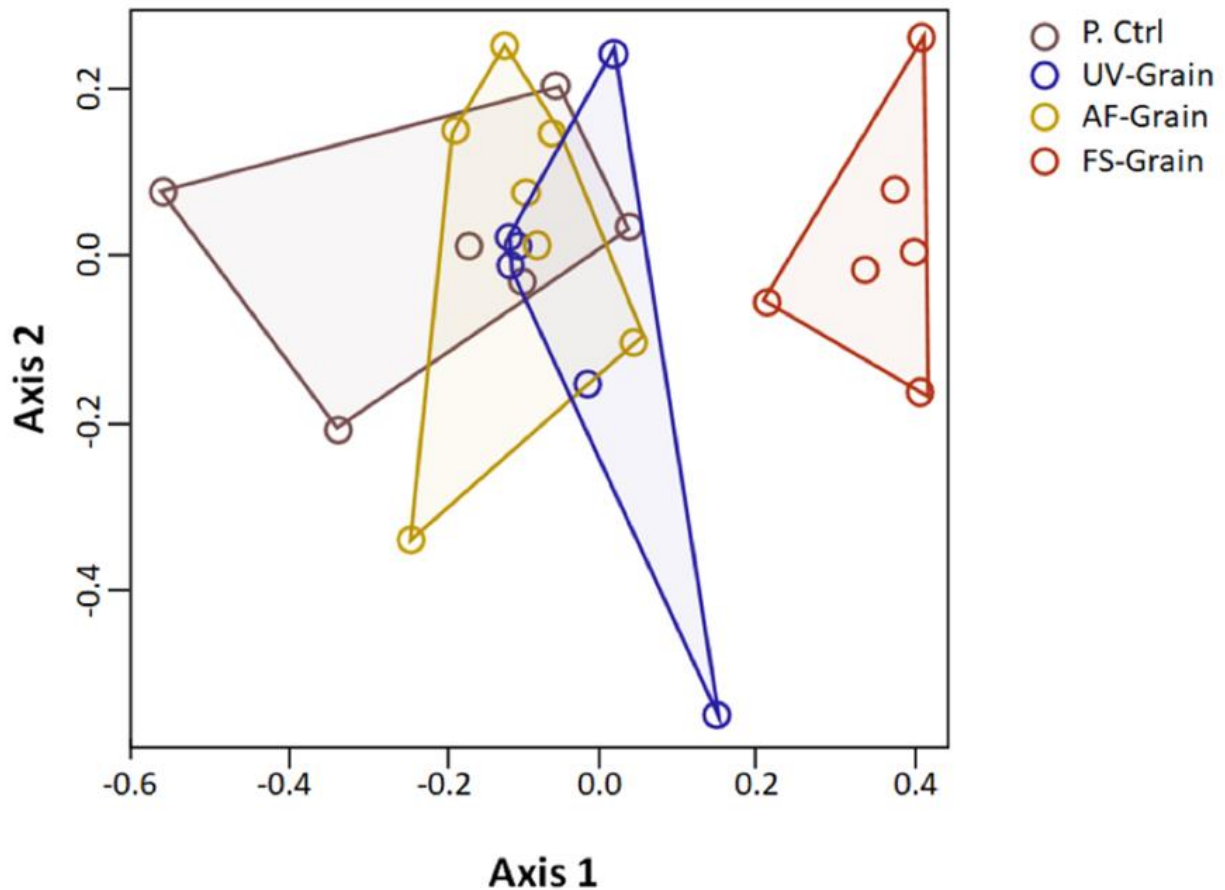
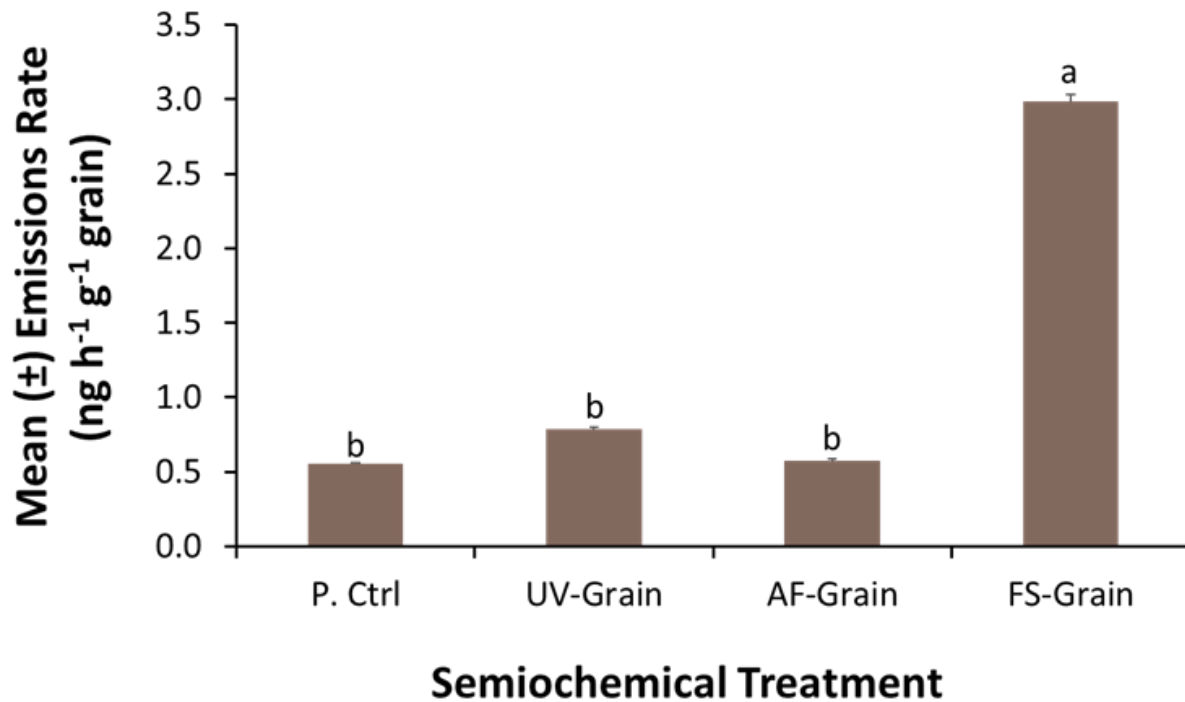


Figure 3-9. Mean emissions rate from the semiochemical treatments (in ng h⁻¹ g⁻¹ grain) during headspace collection in 500-mL capacity containers after adsorption to Porapaq-Q volatile collection trap, then elution with 150 µl dichloromethane. Bars with shared letters are not significantly different from each other (Tukey HSD, $\alpha = 0.05$). Abbreviations: UV-grain – UV-sanitized grain; P. Ctrl – uninoculated grain; FS-grain – *Fusarium verticilloides*-inoculated grain; AF-grain – *A. flavus*-inoculated grain.



Chapter 4 - A Microbial vectoring capacity by an internal- and external-infesting stored product insect after varying dispersal periods between novel food patches: An underestimated risk

Submitted as: Marco A. Ponce^{1*}, Jacqueline M. Maille,³ Alexander Bruce,⁴ Tania N. Kim¹, Erin D. Scully,⁵ and William R. Morrison III⁵. Microbial vectoring capacity by an internal- and external-infesting stored product insect after varying dispersal periods between novel food patches: An underestimated risk. Food Microbiology. Submitted.

Abstract

Knowing the vectoring capacity of internal- and external-infesting stored product insects is important for knowing the relative risk that microbes are to be introduced to postharvest commodities after immigration from the landscape. Thus, the aim of the current study was to evaluate and compare the microbial growth in novel food patches at different dispersal periods by different populations of *Sitophilus oryzae* (an internal-infesting pest) and *Lasioderma serricorne* (an external-infesting pest). To accomplish this, both species were individually isolated from lab colonies or field-captured populations and either placed immediately in a novel food patch, or given a dispersal period of 24 or 72 h in a sterilized environment before entering a new food patch. Vectored microbes in new food patches were imaged after 3 or 5 d of foraging, and microbial growth was processed using ImageJ, while the identity of microbes were understood through sequencing the ITS4/5 ribosomal subunit. Increasing dispersal time resulted in multiple-fold reductions in microbial growth in novel food patches by *L. serricorne*, but not *S. oryzae*. This was likely attributable to differences in baseline mobility by each species. In addition, we identified 14 taxa and nine genera from 34 samples. A total of 56% of the samples were from *Aspergillus* spp, which was the most speciose genus. *Aspergillus flavus* was the most frequently identified species. Our data suggests that there is a more persistent risk of microbial contamination by *S. oryzae* than by *L. serricorne*, which has important food safety implications at food facilities.

Key words: *Lasioderma serricorne*, *Sitophilus oryzae*, postharvest, wheat, microbial ecology, cigarette beetle, rice weevil

Introduction

Insects and microbes represent two of the largest threats to the postharvest supply chain, and create US \$100 billion losses annually worldwide (Wacker, 2018). The presence of plant pathogens (Shafiekhani and Atungulu 2017) and stored product insects (Tigar et al. 1994) prior to harvest has been partially linked with their continued infestation after harvest at food facilities. For instance, fungicides used prior to harvest can affect the microbial community of grain after harvest during storage (Shafiekhani and Atungulu 2017), while stored product insects may start to infest crops prior to harvest and continue damaging the crop during storage (Tigar et al. 1994).

While there is this important connection between plant pathogens before and after harvest, there has also been increasing concern globally about food safety. Contamination of stored grain with insect fragments as well as microorganisms remains a major concern for the grain industry as it affects the grains in both quantitative and qualitative losses (Yadav et al., 2014). Insects and microbes affect the safety, quality and properties of the grains. A survey of flour found *Alternaria*, *Penicillium*, *Aspergillus*, and *Fusarium* to be major parts of the microbiome after harvest (Minutillo et al., 2022). Some of these molds can produce harmful mycotoxins and pose serious health risk for consumers (Magan, 2013; Ponce et al., 2021). In complex ways, these same molds and other microorganisms have developed interspecific interactions with stored-product insects (reviewed in Ponce et al. 2021). Within each agricultural environment (e.g., preharvest and postharvest), there is an abundant opportunity for plant product-microbe, insect-microbe, and insect-plant product-microbe interactions.

The importance of stored-product arthropods in mediating problems with off-odor grain and mycotoxins has been increasing for the grain industry, in part due to the complex relationships between insects and microbes, and perhaps compounded by climate change (Gerken and Morrison

2022). Previous research has found that the presence of insects such as *Sitotroga cerealella* (Olivier) (Lepidoptera: Gelechiidae) on grain kernels provided a favorable environment for the growth of *Fusarium verticillioides*, as well as promoted the production of fumonisin B₁, an important mycotoxin (Rosa et al., 2019). Another study found that *Fusarium verticillioides* did not enter the cuticle of *Sitophilus zeamais* (Motschulsky) (Coleoptera: Curculionidae) and fumonisin B₁ extract had no negative effects on the fitness of conspecifics (Usseglio et al., 2020). However, in that study, the progeny of *S. zeamais* increased. Furthermore, hotspots of insect infestation may significantly elevate the moisture and temperature in the grain mass, leading to microbial growth and deterioration of durable commodities (Khan et al., 2016). Positive associations were found between the survival rate of *R. dominica* and *S. oryzae* in grain and aflatoxin levels. However, benefits may also accrue to stored-product arthropods by the presence of mycoflora on grain. For example, Ponce et al. (2023) found that the presence of both *S. oryzae* and *Aspergillus* spp. in a grain mass altered the abiotic conditions in ways that were favorable to both, and it also resulted in elevated progeny production by *S. oryzae* compared to when microbes were absent. As a result, there seems to be a close relationship between plant pathogenic fungi and stored product insects.

As mobile organisms, insects are key mediators of all three of these types of interactions with microbes (plant product-microbe, insect-microbe, insect-microbe-plant product), because they have the ability to disperse and possibly to vector microbes in these environments. For example, if we see the post-harvest environment as a successional ecosystem (Fandohan et al., 2003; Gerken and Morrison 2023), there will be primary or internal-infesting insect pest or microbial colonizers using undamaged kernels, followed by the colonization of secondary or external-infesting pests that can take advantage of damaged kernels and grain dust to propagate their existence. The plant pathogens *Aspergillus* and *Penicillium* spp. were found on 71% of 988

L. serricornis adults captured in traps placed in people's homes (Nakagawa et al., 2008). That study used scanning electron microscopy, and found *Aspergillus* spores on the pronotum, forewing, labrum, mandible, scutellum, abdomen, femur and tibia. Hubert et al. (2021) found the genera *Bacillus*, *Staphylococcus*, and *Kocuria* in the diet where mites had fed. That study also found the microbial profile differed among mite eggs, mite bodies, and spent growth medium from mites. Stored product pest species may bring their own suite of microbes, with specific microbial spores associated within the cuticle or within the gut of each individual (Fandohan et al., 2003; Lamboni 2009) causing deterioration in food commodities when conspecifics find a food source and feeds on it. For example, the fungal community in bagged coffee was found to shift from being dominated by *Sacharomycetales* during the first six months to dominated by *Wallemia* sp. thereafter (Vargas et al., 2018). Thus, plant pathogenic fungi and bacteria seem to be intimately associated with stored product insects.

Insect movement is a key mediator between microbes and insects. Insect movement can be divided between inter-patch movement for short distances and long-distance dispersal (Skendžić et al., 2019). A recent study evaluated the behavioral response of *Sitophilus oryzae* (L.) (Coleoptera: Curculionidae), an internal-infesting stored product pest to grain volatiles and grain inoculated with *Aspergillus flavus*, whereby it was demonstrated that while fungal cues were important for foraging at close-range attraction, grain volatiles were more important for attraction at longer distances (Ponce et al., 2022). By contrast, for the external-infesting pest, *Lasioderma serricornis* (L.) (Coleoptera: Ptinidae), fungal cues were important for attraction both at close-range and at a distance. More generally, a systematic review by Ponce et al. (2021) found that the behavioral response by the stored product insect community to microbial volatiles may be context and species dependent. Thus, whether passively or actively, during dispersal within and among

food patches, stored product insects may possibly vector microbes, but to what extent they do this, how this varies by species, and which species are vectored are mostly unknown. In addition, it is also unclear how dispersal period may affect these patterns. One hypothesis is that as stored product insects disperse, they may dislodge spores from their body, and may therefore vector fewer microbes with increasing dispersal time. A competing hypothesis may be that with increasing dispersal, adults pick up a variety of new spores, making their communities more diverse and abundant when they reach their new food patch. So far as we are aware, neither of these hypotheses have been formally tested with stored product insects.

In addition, when dispersing, some of the microorganisms vectored by stored product insects may be of public health importance to the food system. For example, many of the most common microbes already found in grain, including *Aspergillus* and *Fusarium*, have mycotoxin-producing strains that are carcinogenic to humans in very low doses and could potentially be spread by multiple insects (Magan et al., 2003). Certainly, there is already a problem in many parts of the developing world with aflatoxin contamination of grains found at local markets (Nleya et al., 2018). As insects disperse, they may also inadvertently or purposefully spread mycotoxin-producing strains of fungi that are important for human health, making this study all the more vital. In a recent study by Picciotti et al. (2023), it was highlighted that vectors may be specific for a particular microbe, if the vector of interest is monophagous, then the epidemiological process or pest control intervention is straightforward to study and, therefore hypothetically easier to manage in the field. On the other hand, if the vectors are polyphagous, the epidemiological process and pest control intervention is more complex to study and manage efficiently (Chuche and Thiéry, 2014; Bragard et al., 2019). Since many stored product insects are polyphagous and attack grain under different conditions (e.g., whole and intact vs broken or damaged), understanding this aspect

is imperative for improving integrated pest management (IPM) after harvest. As a consequence, it is important to assess the relative risk posed by *S. oryzae* and *L. serricornis* for introducing microbes to postharvest commodities after the immigration to novel food patches from the landscape to food facilities.

Apart from representing two extremes in life history, both *S. oryzae* and *L. serricornis* were chosen as study species for multiple important reasons. They are both economically significant pests of stored products (Edde 2019; Hagstrum and Subramanyam 2006), causing severe economic damage at food facilities. In addition, *L. serricornis* has been proposed as a model insect for beetle-fungus interactions, because of its important symbiosis with the symbiont fungus *Symbiotaphrina*, and the wealth of research performed on this species in the 1920s and 1970s (Martinson 2020). Likewise, *S. oryzae* has also been found to harbor obligatory endobacterial symbionts, without which it dies (Kosewska et al. 2023). Given the presence of fungal spores on the cuticle of *L. serricornis* (Nakagawa et al. 2008), it appears microbe-insect interactions are important to both species, but there are still gaps in knowledge about how these insects may be moving them in their environment. Because stored product insect movement is an important consideration after harvest affecting many management tactics, and few studies have evaluated whether and how stored product insects may vector microbes among food facilities or patches, our aims were to: 1) evaluate whether stored product insects reliably vector microbes, 2) determine the identity of those microbes through sequencing, 3) understand how species may affect vectoring capacity using *L. serricornis* and *S. oryzae* as models, and finally 4) elucidate how dispersal period (0, 24, 72 h) and population origin (colony, field, or those from *A. flavus*-enriched environments) affect inoculation of new food patches with microbes.

Materials and Methods

Experimental Insects and Source of Grain

Both *L. serricorne* and *S. oryzae* were obtained from the USDA Agricultural Research Service's (ARS) Center for Grain and Animal Health Research (CGAHR) in Manhattan, KS, USA. Colonies of *Ss. oryzae* (field-derived strain from Hudson, Kansas in 2012) were reared on organic whole kernel wheat after tempering it to 15% grain moisture from an initial 10.6% moisture. To assess the baseline moisture level of the source grain, a grain moisture meter was used (DICKEY-john, GAC2100, Auburn, IL, USA). Colonies with 4- to 8-week-old adults were kept in 950-mL mason jars (8.5 D × 17 cm H) and stored in an environmental chamber (136VL, Percival Instruments, Perry, IA, USA) set at constant conditions ($27.5 \pm 0.01^{\circ}\text{C}$, $60 \pm 3\%$ RH, and 14:10 L:D). Hard whole winter wheat used for colonies and inoculations below were obtained from a local Kansas farmer and held in cold storage ($\sim 4^{\circ}\text{C}$) at the USDA-ARS Center for Grain and Animal Health Research (CGAHR) for no more than ~ 3 years until needed for experiments, then frozen at -4°C for at least 72 h prior to use to prevent infestation by other arthropods. Adult *L. serricorne* were used from colonies collected in 2012 from a rice will in AR and continuously maintained on a diet of 95% bleached wheat flour and 5% brewer's yeast with oats sprinkled on top and a moistened, crumpled towel added. Insects were subcultured by sieving (No. 30 sieve, 594×594 μm mesh, W.S. Tyler Co., Cleveland, OH, USA) 150 mixed-sex *L. serricorne* adults and adding them into mason jars (950-ml capacity) filled two-thirds of the way to the top with diet, then placing inside environmental chambers under constant conditions at 27.5°C , 65% RH, and 14:10 L:D photoperiod. Individuals from each species were never tested more than once for the experiments below. These were termed the colony population of *L. serricorne* and *S. oryzae* hereafter.

Vectoring Assay for Sitophilus oryzae and Lasioderma serricorne

To determine whether *L. serricorne* and *S. oryzae* vectored microbes and how this was affected by dispersal time, a vectoring assay was performed for each species. A total of 32 g of potato dextrose agar (Merck, Darmstadt, Germany) was mixed with 900 mL of deionized water in a 1000-mL glass media bottle with a magnetic stirring rod placed inside the bottle. The agar solution was autoclaved (533LS, Getinge, Rochester, NY, USA) for 30 min and then stirred on a hot plate (Fisher Scientific, Waltham, M, USA) to slowly cool down for 20 min. Before pouring the agar solution into sterilized Petri dishes (100 × 15 W:H), the biosafety cabinet (75 × 73 × 95 cm L:H:W, #302381101, Labconco, Kansas City, MO, USA) was sanitized with 70% ethanol and exposed to UV light for 10 min. A total of ~30 Petri dishes with potato dextrose agar were allowed to solidify overnight for ~12 h. Fresh Petri dishes were used for each experimental replicate.

The vectoring assay consisted of *L. serricorne* or *S. oryzae* being provided with one of three different dispersal periods (0 h, 24 h, 72 h) prior to entering a novel food patch (e.g., clean agar dish) (Supplementary Fig. 1), followed by photographing microbial growth after allowing insects to forage within the patch for 3 and 5 d at 30°C, 60% RH, and 14:10 L:D photoperiod. Each novel food patch consisted of an agar petri dish sealed with parafilm in their own environmental chamber. Adult *L. serricorne* or *S. oryzae* were singly removed from colony containers with sterilized implements, then either placed immediately in the center of an agar Petri dish for the 0 h dispersal period, or given a 24 or 72 h dispersal period in an autoclaved 4 L-capacity glass container and stored at constant conditions of 25°C, 60% RH, and 14:10 L:D photoperiod prior to being added to the agar. In a second round of testing, adult *L. serricorne* or *S. oryzae* dispersed in autoclaved 950-mL glass mason jars for 24 h and 72 h prior to transferring

them to agar dishes. To further avoid contaminants in the second round, all glass mason jars were first cleaned with 70% ethanol and then sanitized with UV light for 10 min in a walk-in environmental chamber prior to placing single adults in each. Transfer of *L. serricorne* or *S. oryzae* adults from containers to agar at the conclusion of the dispersal period was performed inside the biosafety cabinet to prevent contamination of dishes.

Pictures of the agar dishes and corresponding microbial growth were taken using a DSLR camera (EOS 7D Mark II, Canon, Tokyo, Japan) mounted to 3D imaging StackShot (CogniSys, Inc., Traverse City, MI, USA) equipped with a dual flash (MT-26EX-RT, Canon, Tokyo, Japan). Light was diffused using a partially cut frosted plastic jar (15.2 × 7.6 cm D:H) making a total of $n = 60$ replicates per treatment combination (of dispersal time, species, and time in patch). The pictures taken were processed using ImageJ 1.53a (Wayne Rasband, National Institutes of Health, USA) to quantify the microbial growth in the agar dishes. The images had their backgrounds subtracted, then were processed with the “find edges” tool. Finally, they were converted to binary and either dilated or eroded to conform to the original image parameters. A circle encompassing the Petri dish was created and the mean grayscale, standard deviation of the grayscale value, and count of pixels was measured as a surrogate for microbial growth on the dishes. This allowed a quantitative measure of microbial growth by creating an average in a given image. The mean grayscale value could range from 0 (full white), indicating no microbial growth, to 255 (full black), indicating full microbial growth on the entire dish. Finally, visually, microbial morphospecies richness was assigned to each image given the number of unique morphospecies on the plate as a surrogate for species diversity.

Individuals from Microbially-Enriched Environments for Vectoring Assay

In order to determine the effect of how high microbial load from an initial food patch can affect subsequent vectoring patterns, we examined the vectoring capacity of *S. oryzae* exposed to grain inoculated with *A. flavus*. Grain inoculated by *Aspergillus flavus* is referred to as AF, hereafter. To prepare the *A. flavus*-inoculated grain, 600 g of grain was put in a stainless-steel pot filled with water and placed on a hot plate at 500°C. Once boiling for 15 min, the water was drained and the grain was evenly spread out on sterile wipes (38.1 × 42.5 cm, 3 ply, Tech wipes, Skilcraft, NIB, Alexandria, VA) that laid inset an autoclaved metal tray inside a laminar fume hood until dry (ca. 3 h). Afterwards, grain was evenly divided (~300 g) and placed in 2 separate autoclaved mason jars (950-mL capacity). A single hole was pierced through each lid and lined with a cotton ball. The jars were then sealed with aluminum foil and were autoclaved (533LS, Getinge, Rochester, NY, USA) for 30 min. To inoculate with *A. flavus*, a 3-inch strip of an isolation of *A. flavus* (Ponce et al. 2023) fungi that was growing in pure culture on a potato dextrose agarose petri dish was placed into each jar containing the grain. The *A. flavus* isolation had been colonized and growing without contamination in a petri dish for 7 d in an environmental chamber set to 30°C, 60% RH, and 14:10 L:D photoperiod. Jars with freshly prepared AF-inoculated grain sat at room temperature for roughly 10 d or until the *A. flavus* evenly covered as much the grain as possible. Batches of inoculated grain were used within 10–15 d of preparation. Grain was never used more than once for each replicate of every trial in each assay experiment to prevent cross contamination. A total of 75 insects were added to 300 g of AF-inoculated grain in a 950-ml mason jar and allowed to forage for 2 weeks prior to use in the vectoring experiment. The same dispersal periods and time in patch was included in this

experiment. The mean grayscale value and microbial morphospecies richness was recorded for each image. There were a total of $n = 30$ replicates per treatment combination.

Field-Collected Individuals for Vectoring Assay

To assess how the species richness and microbial growth of the vectored community may differ from laboratory adults, *L. serricorne* and *S. oryzae* individuals were also collected from the field. However, there were a few exceptions to the above protocol here, because field-caught individuals were only assessed after a dispersal period of 0 h dispersal time period. This was because of the relatively short lifespan in captivity, and difficulty in procuring live adults. Unlike colony populations and those from *A. flavus*-enriched environments, field-collected adults were acquired from four different field sites, which consisted of locations around the area of greater Manhattan, KS: 1) including a site with a pre-harvest wheat field bordered by woodlands (39°14'26.2"N, 96°34'59.1"W), 2) local apartment complex consisting of end consumers (39°11'43.6"N, 96°36'07.4"W), and 3) Kansas State University Agronomy Farm with storage silos (39°12'23.7"N, 96°35'43.2"W), and 4) a private residence adjacent to a working cattle farm (39°12'23.7"N, 96°35'43.2"W). In each location, a total of three 4-funnel Lindgren traps (Bioquip, Rancho Dominguez, CA, USA) were deployed at least 10 m apart at about 1 m height on rebar or hung from a tree along the perimeter of the location site, and were baited with a multi-species lure containing both *L. serricorne* sex pheromone and *Sitophilus* spp. pheromone (PTL bullet lure, #IL-108, and *Sitophilus* spp. bullet lure, #IL-703, Insects Limited, Westfield, IN, USA). In addition, three ground traps were deployed that consisted of commercially-available pitfall traps (Dome®, Storgard, Trécé, Adair, OK, USA) with two connectible pieces (Doud and Phillips 2020; Doud et al. 2021), containing a central well where a *Sitophilus* spp. lure was added along with a 5 g maize kairomone bait. Pheromone lures were changed every 60

d. No kill mechanism was added, because adults needed to be alive. Traps were checked on a daily basis for capture of new adults, and brought immediately back into the laboratory in separate unused, sterilized containers for addition to agar dishes. The procedure as outline above was then followed. The mean grayscale value and microbial morphospecies richness was recorded for each image. There were a total of $n = 30$ replicates per treatment combination.

Microbial Morphotype Sequencing

To identify microbes associated with the cuticle of *L. serricorne* and *S. oryzae*, fungal morphotypes were isolated from cuticles and cultured for the purpose of sequencing. Pure isolations from the selected morphotypes were made by excising a 1×1 cm agar plug of fungi and subculturing it onto a new potato dextrose agar dish, sealed with parafilm to obtain a pure culture. DNA was extracted from pure cultures after 7 d of growth in an environmental chamber set to 30°C, 60% RH, and 14:10 L:D photoperiod using the Quick-DNA Fecal/Soil Microbe Miniprep Kit (D6010, Zymo Research Corp, Irvine, CA, USA). The concentration of DNA and quality were assessed using the Take 3 Assay on a microplate reader (Gen5™, BioTek Instruments, Winooski, VT, USA) before performing the PCR.

Polymerase chain reaction (PCR) was used to amplify the internal transcribed spacer (ITS) region for each morphologically identified fungi using primer sets: ITS4 5'-TCCTCCGCTTATTGATATGC-3' and ITS5 5'-GGAAGTAAAAGTCGTAACAAGG-3' (White et al. 1990). For example, for the morphologically identified *Fusarium* sample, the translation elongation factor 1 alpha (TEF1 α) region and the RNA polymerase II subunit (RBP2) region were also amplified to facilitate species levels identification. The following primer pairs were used for TEF1 α and RBP1, respectively: EF1 (5'-ATGGGTAAGGARGACAAGAC-3')/EF2 (5'-GGARGTACCAGTSATCATGTT-3') and RBP2-5F2 (5'-GGGGWGAYCAGAAGAAGGC-3') /

RPB2-7cR (5'-CCCATRGCTTGYTTRCCCAT-3') (O'Donnell et al. 1998; O'Donnell et al. 2007; LeBlanc et al. 2015). Each reaction contained 1.0 µL extracted DNA, 1.0 µL of each primer (10 µM), 9.5 µL of nuclease free water, and 12.5 µL of master mix containing 50 units/mL of Taq DNA polymerase master mix (Hot Start Taq 2X Master Mix, Promega, Madison, WI, USA). Briefly, the PCR program consisted of 2 min of initial denaturation at 95°C, followed by 30 cycles of 95°C for 30 s, 55°C for 1 min, and 72°C for 1.5 min, and a final extension at 72°C for 5 min (S1000 Thermal Cycler, Bio-Rad, Hercules, CA, USA). PCR products were treated with ExoSAP-it prior to sequencing following the manufacturer's protocol (ThermoFisher Scientific, Waltham, MA, USA). Amplicons were sequenced bidirectionally on an ABI 3730XL instrument (Eurofins Scientific, Brussels, Belgium), and the resulting sequences were quality-filtered and aligned using Geneious Prime 2021.0.3 (Biomatters Ltd, Auckland, New Zealand).

The consensus sequences were searched against NCBI's nucleotide database (nt) using the BLASTn algorithm (Altschul et al. 1997). In order to circumvent taxonomic misassignments, the ITS consensus sequences were also checked against the UNITE Database using the Ribosomal Database Project Classifier algorithm (Wang et al. 2007). The consensus sequence for the ITS sequence of *A. flavus* was submitted to GenBank under OM490684 while the ITS, TEF1 α , and RBP2 sequences from *F. verticillioides* were deposited under OM460744, OM542207, and OM542208. The community composition sequences were deposited under MMXXXXXX–ZZXXXXXX.

Statistical Analysis

A linear mixed model was used to examine the microbial growth (mean grayscale value) in agar dishes (e.g., novel food patches), with separate models by population. Dispersal period

(0, 24, or 72 h), time in patch (3 or 5 d), and species (*L. serricorne* or *S. oryzae*) were used as fixed, explanatory variables in the model. Date was a random blocking variable. An initial model was created without interactions to inspect residuals and diagnostic plots, and if there were deviations from normality or homoscedasticity a log-transformation was used where appropriate, which corrected the problem. Upon a significant result from the overall model, Tukey HSD was used for multiple comparisons. For this and all other tests, R Software was used (R Core Team, 2022), with $\alpha = 0.05$.

In addition, a linear mixed model was used to analyze microbial species richness in agar dishes, with separate models by population, and using the same model form as above. Dispersal period (0, 24, or 72 h), time in patch (3 or 5 d), and species (*L. serricorne* or *S. oryzae*) were used as fixed, explanatory variables in the model. Date was a random blocking variable. Residuals were inspected, and datasets with deviations from assumptions were log-transformed. Tukey HSD was used for multiple comparison upon a significant result from the model.

Results

Effects of Dispersal on Lasioderma serricorne and Sitophilus oryzae

For *L. serricorne*, initial microbial growth at 3 d decreased significantly by 92% as dispersal period increased from 0 h to 72 h (Table 1; Figure 1). Similar results were shown for total microbial growth after 5 d in the food patch, with a 43% decrease as dispersal period increased from 0 h to 72 h (Figure 2). By contrast, for *S. oryzae*, initial microbial growth at 3 d did not change as dispersal period increased (Table 1), while the total microbial growth after 5 d decreased from 0 h to 24 h but then stabilized after 72 h (Tukey HSD, Figure 1). This was also corroborated visually (Figure 1). Clear trails of microbes from movement of both species were found in the novel food patches with tarsal imprints apparent (Figure 1). Microbial inoculation of

new environments quickly proceeded with time spent in patch for *L. serricorne* and *S. oryzae* (Table 1), with 2.2- and 1.7-fold, respectively, more microbial growth at 5 d than at 3 d (Figure 2). Bacteria appeared to dominate at 3 d, while fungi dominated by 5 d. While the interaction between dispersal period and time in patch significantly affected microbial growth for *S. oryzae*, it did not for *L. serricorne* (Table 1).

In evaluating the microbial species richness vectored by *L. serricorne*, there was no significant reduction with dispersal period (Table 1). Unlike *L. serricorne*, *S. oryzae* showed a significant reduction in microbial species richness with increasing dispersal period (Table 1), with a 43% reduction in species richness from 0 h to 72 h (Figure 3). Time in patch significantly affected species richness of microbes vectored by *L. serricorne* and *S. oryzae*, with 2.1- and 1.1-fold, respectively, more species vectored at 5 d compared to 3 d (Figure 3). There were significant two-way interactions between dispersal period and time in patch that affected microbial species richness for *L. serricorne* and *S. oryzae*. For example, the species richness of microbes vectored by *L. serricorne* was reduced by almost 70% at 5 d in patch when comparing 0 h to a 72 h dispersal period, but the species richness was only reduced by 48% at 3 d in patch between the two periods (Figure 3).

Effects of Dispersal from a Microbially-Enriched Environment by Sitophilus oryzae

For individuals dispersing from microbially rich environments, dispersal period significantly affected microbial growth of vectored microbes by *S. oryzae* (Table 2). In particular, microbial growth was reduced by 31% on average after *S. oryzae* dispersal increased from a 0 to 72 h (Figure 4). However, time in patch did not significantly change the amount of microbes vectored by *S. oryzae* (Table 2), because it remained relatively stable between 71–76 (mean grayscale) at 3 to 5 d (Tukey HSD, Figure 4). Nonetheless, there was a significant

interaction between dispersal period and time in patch on the microbial growth induced by *S. oryzae* (Table 2), but this was a quantitative interaction, with no change in the effect direction.

In terms of species richness, dispersal period significantly affected microbial richness of vectored microbes by *S. oryzae* when they were sourced from microbially-rich environments (Table 2). There were 1.2-1.3-fold more diverse fauna vectored by *S. oryzae* after a 24 h dispersal period compared to 0 or 72 h, respectively (Figure 4). In addition, the time in patch significantly affected the species richness of microbes vectored by *S. oryzae* (Table 2). On average, there were 1.2-fold more species vectored by *S. oryzae* at 5 d compared to 3 d. Finally, there was also a significant interaction between dispersal period and time in patch on species richness of microbes vectored by *S. oryzae*.

Effects of Population Origin on Vectoring Capacity

In comparing population origin, neither dispersal period nor time in patch by *S. oryzae* had a significant effect on microbial growth (Table 3). Importantly, population origin significantly affected microbial growth inculcated by dispersing *S. oryzae* (Figure 5). There was 1.7–1.8-fold more microbial growth from field populations of stored product insects than *S. oryzae* from laboratory colonies or *A. flavus*-inoculated grain, respectively.

Species richness of microbes vectored by *S. oryzae* was significantly affected by dispersal period (Table 3). The diversity of microbes was decreased by 31% from *S. oryzae* when dispersal period was increased from 0 to 72 h (Figure 5). Moreover, time in patch significantly affected species richness, with 10% greater diversity at 5 d compared to 3 d. Population origin significantly affected microbial species richness by dispersing *S. oryzae* (Table 3). There was 1.2–1.4-fold more microbial growth from field populations of stored product insects than *S. oryzae* from colonies or *A. flavus* inoculated grain, respectively (Figure 5).

Effects of Species on Vectoring Capacity

Species significantly affected microbial growth in novel food patches (Suppl. Table 1), with 2.3-fold more microbial growth in patches with *S. oryzae* than *L. serricornis* (Suppl. Figure 1). There was also a significant two-way interaction between time in patch and species (Suppl. Table 1). However, this was only a quantitative interaction, with no change in the direction of the effect size. At 5 d, *S. oryzae* and *L. serricornis* had created 1.7–2.2-fold more microbial growth than at 3 d in the food patches. Finally, there was also a significant three-way interaction among all the variables in the model (Suppl. Table 1). Apparent microbial load was greater on *S. oryzae* than *L. serricornis* at all time points.

Diversity of vectored microbes was significantly affected by species in food patch (Suppl. Table 1), with 1.4-fold greater diversity when *S. oryzae* was present than *L. serricornis* (Suppl. Figure 2). There was a significant interaction between species and dispersal period but not species and time in patch on microbial species richness (Suppl. Table 1; Suppl. Figure 2). In particular, there was no significant difference in the diversity of microbes between the two species at 0 h dispersal, but by 24 and 72 h dispersal period, *L. serricornis* appeared to more quickly suffer a loss in the diversity of microbes being vectored compared to *S. oryzae* (Suppl. Figure 2). Finally, there was a significant three-way interaction among all the variables (Suppl. Table 1).

Community Composition of Microbes through Sequencing

In total, there were 34 samples collected from 14 taxa and nine genera (Table 4, Figure 6). A total of 56% of the samples belonged to *Aspergillus* spp., while 41% were *A. flavus*. The next two most abundant species were *Fusarium* spp. (8.8%) and *Penicillium cinnamopurpureum* (8.8%). The remaining taxa comprised 3–5.8% of the total samples (Figure 6).

Discussion

For the first time, we have documented that *L. serricorne* and *S. oryzae* will readily vector bacteria and fungi when introduced into new environments after even extended dispersal periods, even in sterilized transit environments. We generally found increasing dispersal period resulted in fewer and less diverse microbes being vectored by both species, though a significant amount was still vectored by each and this pattern was more pronounced for *L. serricorne* than *S. oryzae*. Because this was done with stored product beetles dispersing in sterilized environments, our results are likely conservative estimates of the risk. In addition, as time in patch increased, so too did microbial growth by multiple-fold, likely as the combined result of movement by insects and spores around a dish and incubation at favorable temperatures. In prior work, *Aspergillus* spores have been detected on the cuticle of *L. serricorne* using scanning electron microscopy (Nakagawa et al. 2008), and from the current study, we know now *L. serricorne* as well as *S. oryzae* are capable of depositing these spores and having them germinate in new food patches.

In the vectoring assay, we found individual trails of microbes created by the tarsal prints of *S. oryzae* and *L. serricorne*. This is likely from the deposition of spores while walking on the agar during the time in the factitious patches. Although we did not make direct observations, it is possible that other behaviors such as self-grooming, antennation, resting, or frass deposition could be additional sources of microbes during the time that insects are on the agar (Meurant 2012), as spores may become dislodged from the insect or embedded in the agar during these behaviors. While deposition of spores through walking may be a passive way that microbes are being vectored, some insects have specialized structures for storing microbial symbionts (Martinson, 2020, Dowp, 1992), and it could be that the spore density on the cuticle varies by the structural exoskeleton features for *S. oryzae* and *L. serricorne*. In other systems, the microbial

abundance of wild-caught butterflies depended on body part, age, sex and species, with microbial abundance greater on the tarsi and proboscis than on the thorax (Olson et al., 2023). Therefore, in the future, we should evaluate where spore density is highest anatomically on these species.

Prior recent research has found that *L. serricorne* is associated in a long-standing symbiosis with the yeast-like fungus, *Symbiotaphrina kochii* (Martinson, 2020), but our study is one of first to illuminate how it may vector other environmental microbes or plant pathogens in the postharvest environment. Indeed, our study shows that *L. serricorne* is more affected by dispersal period after leaving a food patch than *S. oryzae* in its ability to vector microbes. The initial microbial growth of *L. serricorne* decreased significantly as the dispersal period increased, while the initial microbial growth of *S. oryzae* remained relatively constant. Additionally, the total microbial growth of *L. serricorne* decreased as the dispersal period increased, while that of *S. oryzae* decreased initially, but then stabilized up to 72 h after dispersal. These results suggest that *L. serricorne* is more sensitive to the stochastic effects experienced during a dispersal period and is less effective at vectoring microbes to new food patches compared to *S. oryzae*. This could be due to differences in their dispersal behavior, anatomy, or their mobility. Evidence has shown that *L. serricorne* is a strong flyer and highly mobile, dispersing from food facilities to other habitats in the field (Shinoda and Fujisaki, 2001), and is thus a landscape level threat (Edde 2019, Arthur et al. 2014). Campbell et al. (2002) studied an index of dispersal for pheromone trap captures in a warehouse for *L. serricorne* and found that they were mostly captured in areas storing the least amount of packaged food. The results in our study suggest that as *L. serricorne* disperses among habitats, it is likely bringing a community of microbes with it, which may inoculate new foraging patches. This strongly suggests microbes and stored product insects should be managed in concert to ameliorate the risks from each.

In addition, we found that microbial species richness vectored by *L. serricorne* and *S. oryzae* varies with dispersal time as well. For example, the species richness of microbes vectored by both *L. serricorne* and *S. oryzae* decreased with dispersal time, though in this case the pattern was more pronounced for the latter. Additionally, time in patch significantly affected species richness of microbes vectored by both species, with greater diversity at 5 d compared to 3 d more so for *L. serricorne* than *S. oryzae*. This suggests that there may be complex interactions between dispersal period, time in patch, microbial species richness, and mobility. In particular, because *S. oryzae* is highly mobile through walking (Morrison et al. 2021), it is possible there are fewer gains in species richness from 3 to 5 d, while during this time *L. serricorne* is able to increase its species richness because of its lower baseline walking mobility (Ponce et al. 2023). It is likely that further research is needed to understand these interactions more fully. It is also possible the conditions in the chamber and on the agar are selecting for certain microbial species, and the limited space hinders or abrogates growth of microbes on the dish. For example, at 3 d, the microbial population was less diverse due to the limited time for growth and interaction, and plates were never filled. By contrast, at 5 d, the microbial population exhibited greater diversity, but plates were often (though not always) fully covered, suggesting possible limitation. In some cases, there were dominant microbial species such as *Aspergillus* spp., which appeared to be suppressing the growth of other microbial species, leading to reduced microbial diversity.

A variety of microbes can be found in grain, including bacteria, fungi, and yeasts. Some of the most common bacteria include *Bacillus*, *Enterobacter*, and *Pseudomonas*, while some of the most common fungi include *Aspergillus*, *Fusarium*, and *Penicillium* spp. (Magan et al. 2003). The presence of these microbes generally has a negative effects on grain quality and safety. For instance, some bacteria and fungi can produce toxins that are harmful to human and animal

health, and can also reduce the quality and shelf life of the grain. In addition, some microbes can produce areas in the grain with elevated temperature and moisture that are ideal for insect development, termed hotspots (Ponce et al. 2021). But, there are also beneficial fungi that can be used antagonistically to occupy the same niche as mycotoxin-producing fungi, but without its deleterious effects (Wambacq et al. 2016). In fact, in this study we found that *L. serricorne* and *S. oryzae* vectors *Aspergillus*, *Fusarium*, and *Penicillium* spp. One of the most frequent microbial species found in this study was *Aspergillus flavus* (Eurotiales: Trichocomaceae), which is responsible for the production of a mycotoxin called aflatoxin B1 and secondary metabolites that helps the fungus colonized and infect maize kernels (Antiga et al., 2020). Our results demonstrate that stored product insects such as *L. serricorne* and *S. oryzae* maybe contributing to the spread of *Aspergillus flavus* as they disperse. This could be particularly relevant to the pathogenesis of *Aspergillus flavus*, as the fungus may benefit from a vector to spread and infect new hosts. This is perhaps also a key potential indicator finding for other microbial species, who may share a similar disease cycle. Other studies have also shown how fungal cues from *Aspergillus flavus* affect the close- and longer-range foraging ecology of both primarily and secondary stored product insects (Ponce et al., 2023; Ponce et al., 2022). In prior work, progeny production of *S. zeamais* increased in the presence of *Fusarium verticillioides* (Usseglio et al. 2020). Therefore, it is important to manage microbial populations in grain to ensure that they are safe, nutritious, and of high quality.

Even though this study was done under controlled environmental conditions, temperature and humidity are important factors that can affect insect-microbe interactions in the postharvest supply chain. Beti et al. (1990) found that the combination of *S. zeamais* with maize positively increased grain moisture, and increase grain moisture was correlated with higher aflatoxin

content from *A. flavus*. In addition, some studies have shown that high temperatures can be used to manage risk by insects by reducing the survival and reproduction of insect pests, and thus reduce the risk of microbial contamination (Skendžić et al., 2021; Silva and Elliot, 2016). Similarly, low humidity can also reduce the survival and reproduction of some insect pests (Norhisham et al., 2013). Furthermore, pH levels, modified atmospheres (e.g., oxygen levels), and nutrients available are other important factors that could be affecting microbial growth as well as diversity. However, it is noteworthy that the optimal temperature and humidity conditions for insect pests and microbes may vary depending on the species involved, and other factors, such as the type of commodity and the duration of storage. For example, grain commodities such as maize, sorghum or wheat can have different physiological properties that may benefit the reproduction and diversification of different microbial species (Dabija et al., 2021; Singh et al., 2023).

In this study, we also evaluated the emigration of *S. oryzae* from microbe-rich environments to understand how this may change the dynamics of microbial growth and species richness over different dispersal times and time foraging in food patch. We found that there was a much less marked pattern of decreasing microbial growth with increasing dispersal time. In addition, we also observed quick saturation of the plate with microbes already by 3 d after introduction and little change after 5 d. This suggests that even over the extended dispersal periods, spores will readily dislodge in the new environment and fungi will colonize quickly if conditions are favorable. Prior work found maize exposure to *S. zeamais* from 7 to 21 d with weevils previously inoculated with *A. flavus* ended up increasing the aflatoxin B1 content of the commodity (Beti et al. 1995). Those authors concluded that *S. zeamais* facilitated the growth of *A. flavus* in maize by increasing the surface area susceptible to fungal infection and increasing

the moisture content as a result of insect activity. Stored product mites appear to come from microbe-rich environments, and this is essential for their success (Hubert et al. 2021).

In addition, future work should evaluate the effect of habitat foraging preferences during dispersal and how that affects choice and subsequent progeny production by *L. serricorne*.

Overall, our study sheds some light on the relative risk of microbial contamination by two stored product insect species (e.g., an internal-infesting and external-infesting pest) in postharvest commodities. Our findings suggest that there is a greater and more persistent risk of microbial contamination by *S. oryzae* than by *L. serricorne*, and this could vary among other stored product insects species as well. This information could be useful for food safety management in the postharvest supply chain, as it could help identify which insect species pose the greatest risk of microbial contamination and which ones should be targeted for management measures.

Ultimately, this research helps highlight an underestimated risk to the food safety of the postharvest supply chain by the joint action of insects and microbes.

References

- Antiga L, La Starza SR, Miccoli C, D'Angeli S, Scala V, Zaccaria M, Shu X, Obrian G, Beccaccioli M, Payne GA, Reverberi M. (2020). *Aspergillus flavus* Exploits Maize Kernels Using an “Orphan” Secondary Metabolite Cluster. *International Journal of Molecular Sciences.*; 21(21):8213. <https://doi.org/10.3390/ijms21218213>
- Arthur, F. H., Campbell, J. F., & Toews, M. D. (2014). Distribution, abundance, and seasonal patterns of stored product beetles in a commercial food storage facility. *Journal of Stored Products Research*, 56, 21-32..
- Beti, J. A., Phillips, T. W., & Smalley, E. B. (1995). Effects of maize weevils (Coleoptera: Curculionidae) on production of aflatoxin B1 by *Aspergillus flavus* in stored corn. *Journal of Economic Entomology*, 88(6), 1776-1782..
- Bragard C, Dehnen-Schmutz K, Gonthier P, Jacques M. A., Jaques Miret J.A., Justesen A.F., MacLeod A., Magnusson C.S., Milonas P., Navas-Cortes J.A., Parnell S., Potting R., Reignault P.L., Thulke H.H., van der Werf W., Vicent Civera A., Yuen J., Zappala L, Candresse T., Lacomme C., Bottex B., Oplaat C., Roenhorst A., Schenk M., Di Serio F. (2019) *Scientific opinion on the pest categorisation of non-EU Cicadomorpha vectors of Xylella spp.* EFSA J 17:5736
- Broissin-Vargas, L. M., Snell-Castro, R., Godon, J. J., González-Ríos, O., & Suárez-Quiroz, M. L. (2018). Impact of storage conditions on fungal community composition of green coffee beans *Coffea arabica* L. stored in jute sacks during 1 year. *Journal of Applied Microbiology*, 124(2), 547-558.
- Campbell, J. F., Mullen, M. A., & Dowdy, A. K. (2002). Monitoring stored-product pests in food processing plants with pheromone trapping, contour mapping, and mark-recapture. *Journal of Economic. Entomology.*, 95(5), 1089-1101.

- Chuche, J., & Thiéry, D. (2014). Biology and ecology of the Flavescence dorée vector *Scaphoideus titanus*: a review. *Agronomy for Sustainable Development*, 34, 381-403.
- Chung, S. H., Rosa, C., Scully, E. D., Peiffer, M., Tooker, J. F., Hoover, K., Luthe, K& Felton, G. W. (2013). Herbivore exploits orally secreted bacteria to suppress plant defenses. *Proceedings of the National Academy of Sciences*, 110(39), 15728-15733.
- Dabija, A., Ciocan, M. E., Chetrariu, A., & Codină, G. G. (2021). Maize and sorghum as raw materials for brewing, a review. *Applied Sciences*, 11(7), 3139.
- Edde, P. A. (2019). Biology, ecology, and control of *Lasioderma serricorne* (F.)(Coleoptera: Anobiidae): A review. *Journal of Economic Entomology*, 112(3), 1011-1031.
- Fandohan, P., Hell, K., Marasas, W. F. O., & Wingfield, M. J. (2003). Infection of maize by *Fusarium* species and contamination with fumonisin in Africa. *African Journal of Biotechnology*, 2(12), 570-579.
- Gerken, A. R., & Morrison, W. R. (2022). Pest management in the postharvest agricultural supply chain under climate change. *Frontiers Agronomy*, 4, 918845. doi: 10.3389/fagro.
- Gerken, A.R., & Morrison, III W.R. (2023). Viewing Farm2Fork agriculture through the lens of Community Ecology: The who, why, and what of IPM in the postharvest agricultural supply chain. *Frontiers Agronomy*, in press.
- Gurung, K., Wertheim, B., & Falcao Salles, J. (2019). The microbiome of pest insects: it is not just bacteria. *Entomologia Experimentalis et Applicata*, 167(3), 156-170.
- Hammer, T. J., Janzen, D. H., Hallwachs, W., Jaffe, S. P., & Fierer, N. (2017). Caterpillars lack a resident gut microbiome. *Proceedings of the National Academy of Sciences*, 114(36), 9641-9646.

- Hubert, J., Nesvorna, M., Green, S. J., & Klimov, P. B. (2021). Microbial communities of stored product mites: variation by species and population. *Microbial ecology*, 81(2), 506-522.
- Khan, T., Shahid, A. A., & Khan, H. A. A. (2016). Could biorational insecticides be used in the management of aflatoxigenic *Aspergillus parasiticus* and its insect vectors in stored wheat? *PeerJ*, 4, e1665.
- Kosewska, O., Przemieniecki, S.W., & Nietupski, M. (2023). The effect of antibiotics on bacteriome of *Sitophilus oryzae* and *Rhyzopertha dominica* as a factor determining the success of foraging: A chance for antibiotic therapy in grain stores. *Applied Sciences* 13(3), 1576.
- Lamboni, Y., & Hell, K. (2009). Propagation of mycotoxigenic fungi in maize stores by post-harvest insects. *International Journal of Tropical Insect Science*, 29, 31-39.
- Magan, N., Hope, R., Cairns, V., & Aldred, D. (2003). Post-harvest fungal ecology: impact of fungal growth and mycotoxin accumulation in stored grain. *Epidemiology of Mycotoxin Producing Fungi: Under the aegis of COST Action 835 'Agriculturally Important Toxigenic Fungi 1998–2003'*, EU project (QLK 1-CT-1998–01380), 723-730.
- Martinson V. G. (2020). Rediscovering a forgotten system of symbiosis: Historical perspective and future potential. *Genes*, 11(9), 1063. <https://doi.org/10.3390/genes11091063>
- Mason, C. J., Jones, A. G., & Felton, G. W. (2019). Co-option of microbial associates by insects and their impact on plant–folivore interactions. *Plant, Cell & Environment*, 42(3), 1078-1086.
- Meurant, G. (2012). Insect-Fungus Interactions. United Kingdom: *Elsevier*, pp 325.
- Minutillo, S. A., Ruano-Rosa, D., Abdelfattah, A., Schena, L., & Malacrino, A. (2022). The Fungal Microbiome of Wheat Flour Includes Potential Mycotoxin Producers. *Foods*,

- 11(5), 676. Morrison III, W. R., Arthur, F. H., & Bruce, A. (2021). Characterizing and predicting sublethal shifts in mobility by multiple stored product insects over time to an old and novel contact insecticide in three key stored commodities. *Pest Management Science*, 77(4), 1990-2006.
- Nakagawa, A., Kawakami, Y., Hashimoto, K., Hatakeyama, Y., Uchida, A., Takahashi, H., Iwano, H., (2008). Adhesion rate and patterns of fungi spores on the body surface of the *Lasioderma serricorne*. *Journal of Chemical Ecology* 46, pp. 1059-1068.
- Norhisham, A. R., Abood, F., Rita, M., & Hakeem, K. R. (2013). Effect of humidity on egg hatchability and reproductive biology of the bamboo borer (*Dinoderus minutus* Fabricius). *SpringerPlus*, 2(1), 9. <https://doi.org/10.1186/2193-1801-2-9>
- Picciotti, U., Araujo Dalbon, V., Ciancio, A., Colagiero, M., Cozzi, G., De Bellis, L., & Porcelli, F. (2023). “Ectomosphere”: Insects and Microorganism Interactions. *Microorganisms*, 11(2), 440.
- Ponce M., Kim T.N., Morrison W.R. (2023). The interaction between *Sitophilus oryzae* (Coleoptera: Curculionidae) and *Aspergillus flavus* jointly affects microclimate, grain quality, and each other's fitness. *Environmental Entomology*. in press.
- Ponce, M. A., Kim, T. N., & Morrison III, W. R. (2021). A systematic review of the behavioral responses by stored-product arthropods to individual or blends of microbially produced volatile cues. *Insects*, 12(5), 391.
- Ponce, M. A., Lizarraga, S., Bruce, A., Kim, T. N., & Morrison III, W. R. (2022). Grain inoculated with different growth stages of the fungus, *Aspergillus flavus*, affect the close-range foraging behavior by a primary stored product pest, *Sitophilus oryzae* (Coleoptera: Curculionidae). *Environmental Entomology*, 51(5), 927-939.

- Ponce, M. A., Sierra, P., Maille, J. M., Kim, T. N., Scully, E. D., & Morrison III, W. R. (2023). Attraction, mobility, and preference by *Lasioderma serricorne* (Coleoptera: Ptinidae) to microbially-mediated volatile emissions by two species of fungi in stored grain. *Scientific Reports*, 13(1), 6176.
- Rosa Junior, O. F., Dalcin, M. S., Nascimento, V. L., Haesbaert, F. M., Ferreira, T. P. D. S., Fidelis, R. R., ... & Santos, G. R. D. (2019). Fumonisin production by *Fusarium verticillioides* in maize genotypes cultivated in different environments. *Toxins*, 11(4), 215.
- Shafiekhani, S., & Atungulu, G.G. (2017). Kinetics of mold growth and rice quality metrics during storage as affected by pre-harvest fungicide application. *Proceedings of the American Society of Agricultural and Biological Engineers*, Paper#1700818, doi:10.13031/aim.201700818.
- Shinoda, K., & Fujisaki, K. (2001). Effect of adult feeding on longevity and fecundity of the cigarette beetle, *Lasioderma serricorne* F.(Coleoptera: Anobiidae). *Applied Entomology and Zoology*, 36(2), 219-223.
- Silva, F. W. S., & Elliot, S. L. (2016). Temperature and population density: interactional effects of environmental factors on phenotypic plasticity, immune defenses, and disease resistance in an insect pest. *Ecology and Evolution*, 6(11), 3672–3683.
<https://doi.org/10.1002/ece3.2158>
- Singh, A., Singh, S., Kansal, S. K., Garg, M., & Krishania, M. (2023). Production and characterization of anthocyanin-rich beer from black wheat by an efficient isolate *Saccharomyces cerevisiae* CMS12. *Scientific Reports*, 13(1), 5863.

- Skendžić, S., Zovko, M., Živković, I. P., Lešić, V., & Lemić, D. (2021). The impact of climate change on agricultural insect pests. *Insects*, 12(5), 440.
- Tigar, B. J., Key, G. E., Flores-s, M. E., & Vazquez-a, M. (1994). Field and post-maturation infestation of maize by stored product pests in Mexico. *Journal of Stored Products Research*, 30(1), 1-8.
- Usseglio, V. L., Dambolena, J. S., Martinez, M. J., & Zunino, M. P. (2020). The Role of Fumonisin in the Biological Interaction between *Fusarium verticillioides* and *Sitophilus zeamais*. *Journal of chemical ecology*, 46(11-12), 1059-1068.
- Wambacq, E., Vanhoutte, I., Audenaert, K., De Gelder, L., & Haesaert, G. (2016). Occurrence, prevention and remediation of toxigenic fungi and mycotoxins in silage: A review. *Journal of the Science of Food and Agriculture*, 96(7), 2284-2302.
- Yadav, D. N., Anand, T., Sharma, M., & Gupta, R. K. (2014). Microwave technology for disinfestation of cereals and pulses: An overview. *Journal of food science and technology*, 51, 3568-3576.
- Zhao, T., Kandasamy, D., Krokene, P., Chen, J., Gershenzon, J., & Hammerbacher, A. (2019). Fungal associates of the tree-killing bark beetle, *Ips typographus*, vary in virulence, ability to degrade conifer phenolics and influence bark beetle tunneling behavior. *Fungal Ecology*, 38, 71-79.

Table 4-1. Summary of ANOVA models for mean grayscale value (microbial growth) and microbial diversity (e.g., species richness) after the introduction of *L. serricorne* or *S. oryzae* on PDA agar dishes after 0, 24, or 72 h dispersal from laboratory colonies.

Variable	Mean Grayscale Value			Species Richness	
	df	<i>F</i>	<i>P</i>	<i>F</i>	<i>P</i>
<i>L. serricorne</i>					
Dispersal Period	2	6.05	0.003	2.26	0.11
Time in Patch	1	10.0	0.002	22.0	0.0001
Interaction	2	0.20	0.82	11.4	0.0001
Residuals	174				
<i>S. oryzae</i>					
Dispersal Period	2	1.39	0.25	39.4	0.0001
Time in Patch	1	76.6	0.0001	140	0.0001
Interaction	2	17.1	0.0001	33.0	0.0001
Residuals	174				

Table 4-2. Summary of ANOVA models for mean grayscale value (microbial growth) and microbial diversity (e.g., species richness) after the introduction of *S. oryzae* on PDA agar dishes after 0, 24, or 72 h dispersal from grain inoculated with *A. flavus*.

Variable	Mean Grayscale Value			Species Richness	
	df	<i>F</i>	<i>P</i>	<i>F</i>	<i>P</i>
<i>S. oryzae</i>					
Dispersal Period	2	26.7	0.0001	28.1	0.0001
Time in Patch	1	1.87	0.17	38.2	0.0001
Interaction	2	4.20	0.02	13.1	0.0001
Residuals	174				

Table 4-3. Summary of ANOVA models for mean grayscale value (microbial growth) and microbial diversity (e.g., species richness) after the introduction of *S. oryzae* from different populations (colony, *A. flavus*-inoculated grain, and field) on PDA agar dishes after a 0, 24, or 72 h dispersal period.

Variable	Mean		Grayscale		Species Richness	
	Value					
	df	F	P	F	P	
<i>S. oryzae</i>						
Time in Patch	1	0.93	0.33	18.8	0.0001	
Population Origin	2	22.6	0.0001	8.44	0.001	
Time in Patch: Population Origin	2	1.25	0.26	1.84	0.16	
Residuals	196					

Table 4-4.. Summary of microbial identifications from each species via sequencing the ribosomal ITS4/5 subunit.

Name	Source		Identification	Percent		
	Population	Species		Identity	Query Cover	Accession
ITS4_Rep1_Tr1_9 ITS5	Lab	CB	<i>Alternaria brassicae</i>	99%	99.6	JF439435.1
ITS4_Rep1_Tr1_3 ITS5	Lab	CB	<i>Aspergillus flavus</i>	98%	99.5	MW789017.1
ITS4_Rep1_Tr1_5 ITS5	Lab	CB	<i>Aspergillus flavus</i>	56%	95.7	MK087749.1
ITS4_Rep1_Tr1_7 ITS5	Lab	CB	<i>Aspergillus flavus</i>	100%	98.0	MT645322.1
ITS4_Rep1_Tr1_8 ITS5	Lab	CB	<i>Aspergillus flavus</i>	71%	98.6	MG640049.1
DM2R1 ITS5	Field	D	<i>Aspergillus flavus</i>	97%	99.8	MN856376.1
DM7R1 ITS5	Field	D	<i>Aspergillus flavus</i>	99%	99.3	MT629885.1
DM8R1 ITS5	Field	D	<i>Aspergillus flavus</i>	98%	99.5	MW789017.1
DMR1 ITS5	Field	D	<i>Aspergillus flavus</i>	98%	99.7	MW789017.1
F11R1 ITS5	Field	W	<i>Aspergillus flavus</i>	98%	100	MN856403.1
F12R1 ITS5	Field	W	<i>Aspergillus flavus</i>	96%	100	MN856426.1
F21R1 ITS5	Field	W	<i>Aspergillus flavus</i>	98%	99.7	MW789017.1
F22R1 ITS5	Field	W	<i>Aspergillus flavus</i>	98%	98.3	MW789017.1
F5R1 ITS5	Field	W	<i>Aspergillus flavus</i>	99%	99.0	MT629.885.1
M4R1 ITS5	Lab	RW	<i>Aspergillus flavus</i>	97%	99.8	MN856376.1
F10R1 ITS5	Field	W	<i>Aspergillus neotritici</i>	99%	99.5	KC923428.1
DM5R1 ITS5	Field	D	<i>Aspergillus niger</i>	98%	99.3	MN907662.1
F3R1 ITS5	Lab	RW	<i>Aspergillus niger</i>	99%	99.3	MH237633.1
ITS4_Rep1_Tr1_6 ITS5	Lab	CB	<i>Aspergillus oryzae</i>	98%	100	MK503967.1

F1R1_ITS5	Field	W	<i>Aspergillus terreus</i>	98%	100	JQ697519.1
M3R1_ITS5	Lab	RW	<i>Fomitopsis meliae</i>	96%	100	KC585351.1
F20R1_ITS5	Field	W	<i>Fusarium spp.</i>	96%	99.8	MH569610.1
ITS4_Rep1_Tr1_10_ITS4	Lab	CB	<i>Fusarium verticillioides</i>	99%	94.3	KX281171.1
F2R1_ITS5	Field	W	<i>Fusarium verticillioides</i>	97%	100	OM956083.1
DM4R1_ITS5	Field	D	<i>Hyphopichia butonii</i>	97%	99.5	KY103598.1
M5R1_ITS5	Lab	RW	<i>Hyphopichia butonii</i>	97%	99.3	MN310410.1
ITS4_Rep1_Tr1_1_ITS4	Lab	CB	<i>Irpex lacteus</i>	99%	99.5	MF161243.1
F13R1_ITS5	Field	W	<i>Penicillium cinnamopurpureum</i>	98%	99.8	KU847973.1
F14R1_ITS5	Field	W	<i>Penicillium cinnamopurpureum</i>	98%	99.8	KU847973.1
M7R1_ITS5	Lab	RW	<i>Penicillium cinnamopurpureum</i>	93%	84.6	MH858869.1
M9R1_ITS5	Lab	RW	<i>Rhizopus arrhizus</i>	98%	98.9	LC557494.1
ITS4_Rep1_Tr1_4_ITS5	Lab	CB	<i>Syncephalastrum contaminatum</i>	92%	92.2	MK799842.1
ITS4_Rep1_Tr1_11_ITS5	Lab	CB	Unknown	-	-	-
ITS4_Rep1_Tr1_2_ITS4	Lab	CB	Unknown	-	-	-

¹CB—cigarette beetle, *L. serricorne*, D—dermestids, most likely cigarette beetle, W—weevil, either *S. oryzae* or *S. zeamais*, RW—rice weevil, *S. oryzae*.

Figure 4-1. Habitus images of factitious foraging patches from the vectoring assay showing that microbes are transmitted physically by *S. oryzae* (left orange and green boxes) and *L. serricornis*, (right red box) but at decreasing frequency and diversity with increasing dispersal periods (0 h, 24 h, 72 h) prior to entering a foraging patch. *Sitophilus oryzae* originally from laboratory colonies (orange box) or *Aspergillus flavus*-inoculated grain (e.g., microbially-enriched environments; green box). The longer that *S. oryzae* and *L. serricornis* were in foraging patches (3 vs. 5 d), the greater the microbial growth under constant conditions (30°C, 60% RH, and 14:10 L:D photoperiod). Pictures are representative images taken from a total of n = 30 replicates for each dispersal period and foraging time in a food patch.

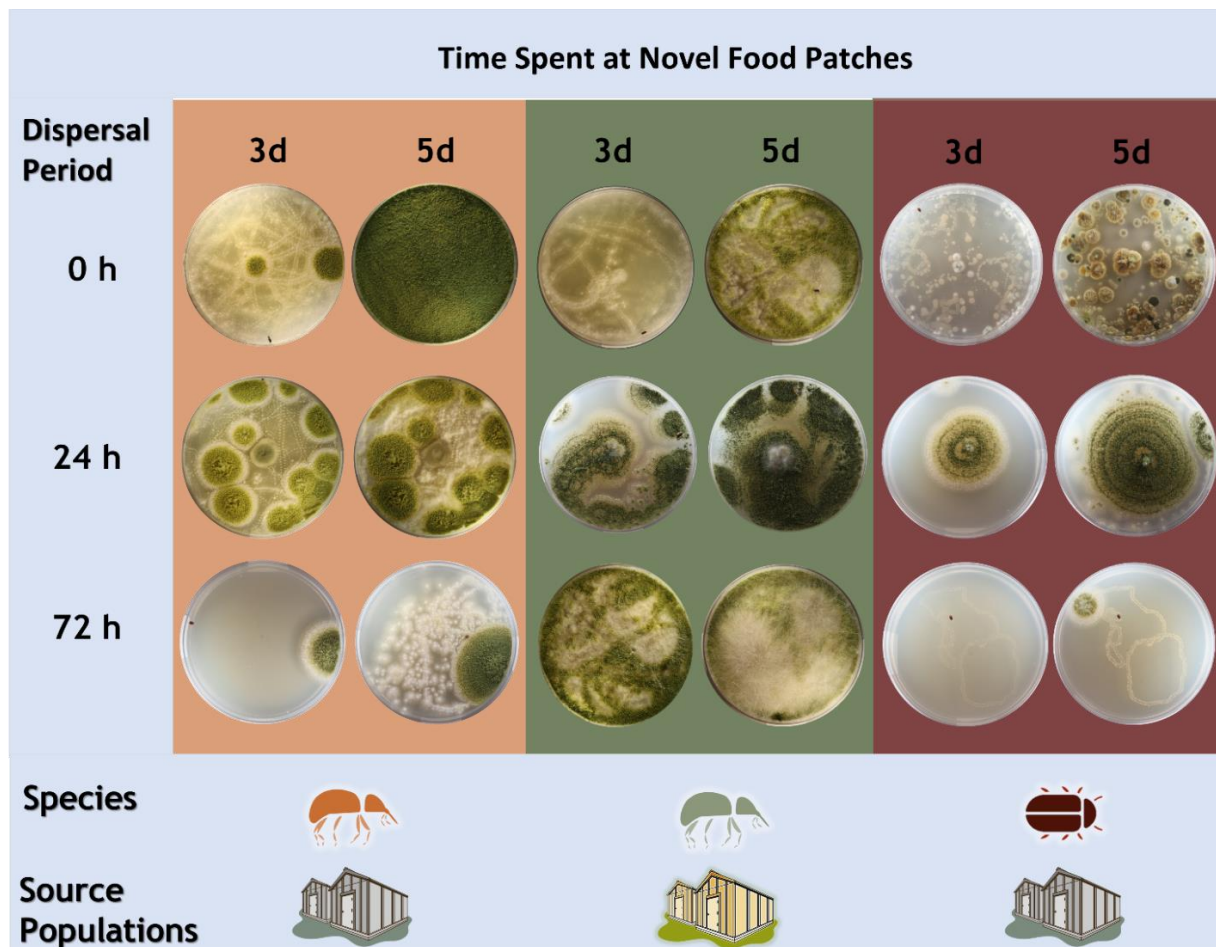


Figure 4-2. Mean grayscale value (e.g., surrogate measure of microbial growth) for microbes vectored by *L. serricorne* (top panel) and *S. oryzae* (bottom panel) adults from the colony population after they were given a dispersal period of 0, 24, or 72 h and then placed in factitious foraging patches for 3 d (green bars) or 5 d (yellow bars), which consisted of potato dextrose agar dishes that were photographed using a 3D imaging system and processed with ImageJ. There were a total of $n = 30$ replicate adults tested per bar. Bars with shared letters are not significantly different from each other (Tukey HSD, $\alpha = 0.05$). The grayscale value can range from 0, indicating no microbial growth, to 255, indicating the full agar dish was covered with microbes.

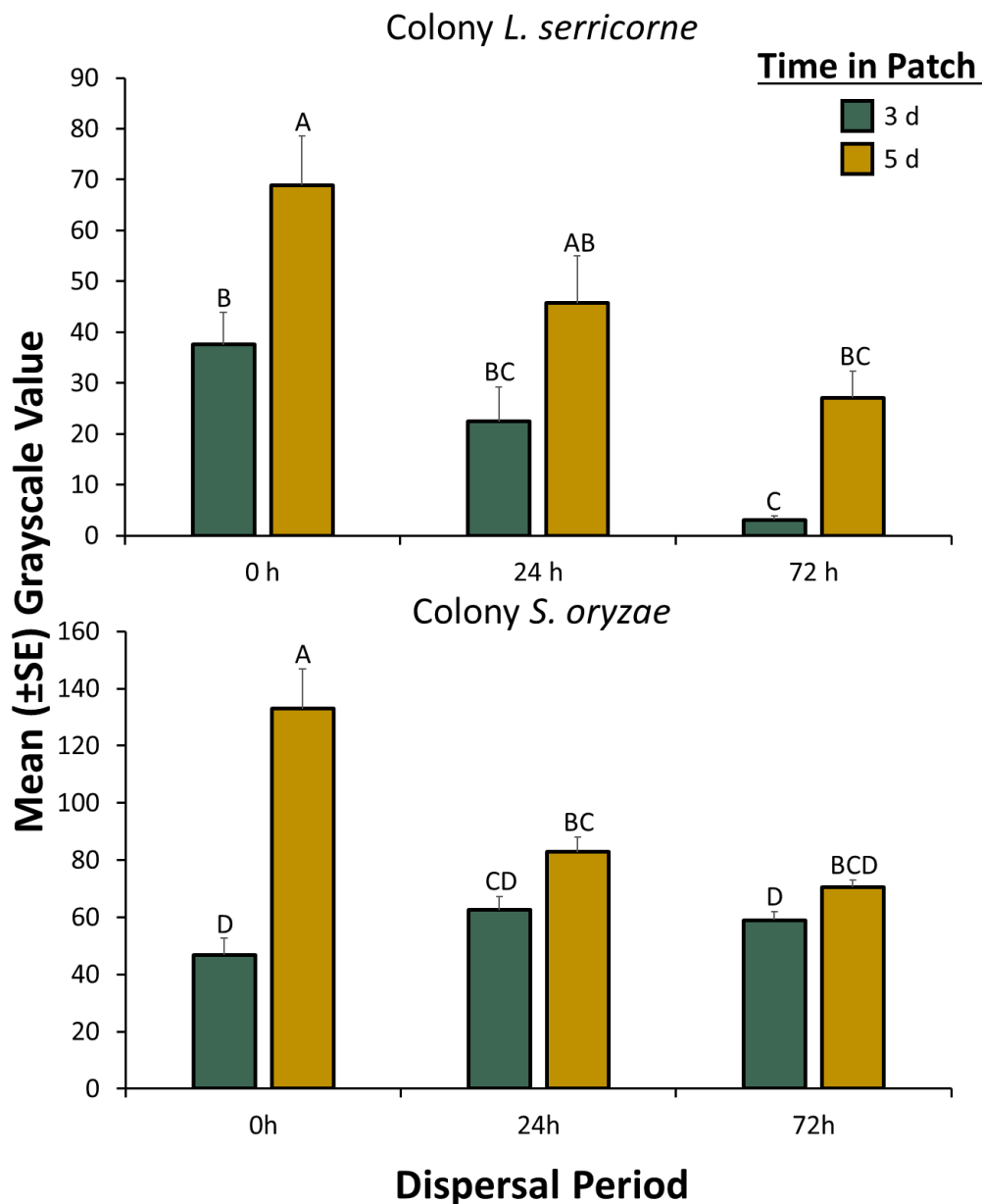


Figure 4-3. Mean species richness (e.g., surrogate measure of microbial diversity) for microbes vectored by *L. serricorne* (top panel) and *S. oryzae* (bottom panel) adults from the colony population after they were given a dispersal period of 0, 24, or 72 h and then placed in factitious foraging patches for 3 d (green bars) or 5 d (yellow bars), which consisted of potato dextrose agar dishes that were photographed using a 3D imaging system and processed with ImageJ. There were a total of $n = 30$ replicate adults tested per bar. Bars with shared letters are not significantly different from each other (Tukey HSD, $\alpha = 0.05$). Species richness represents the number of microbial morphotypes in each food patch.

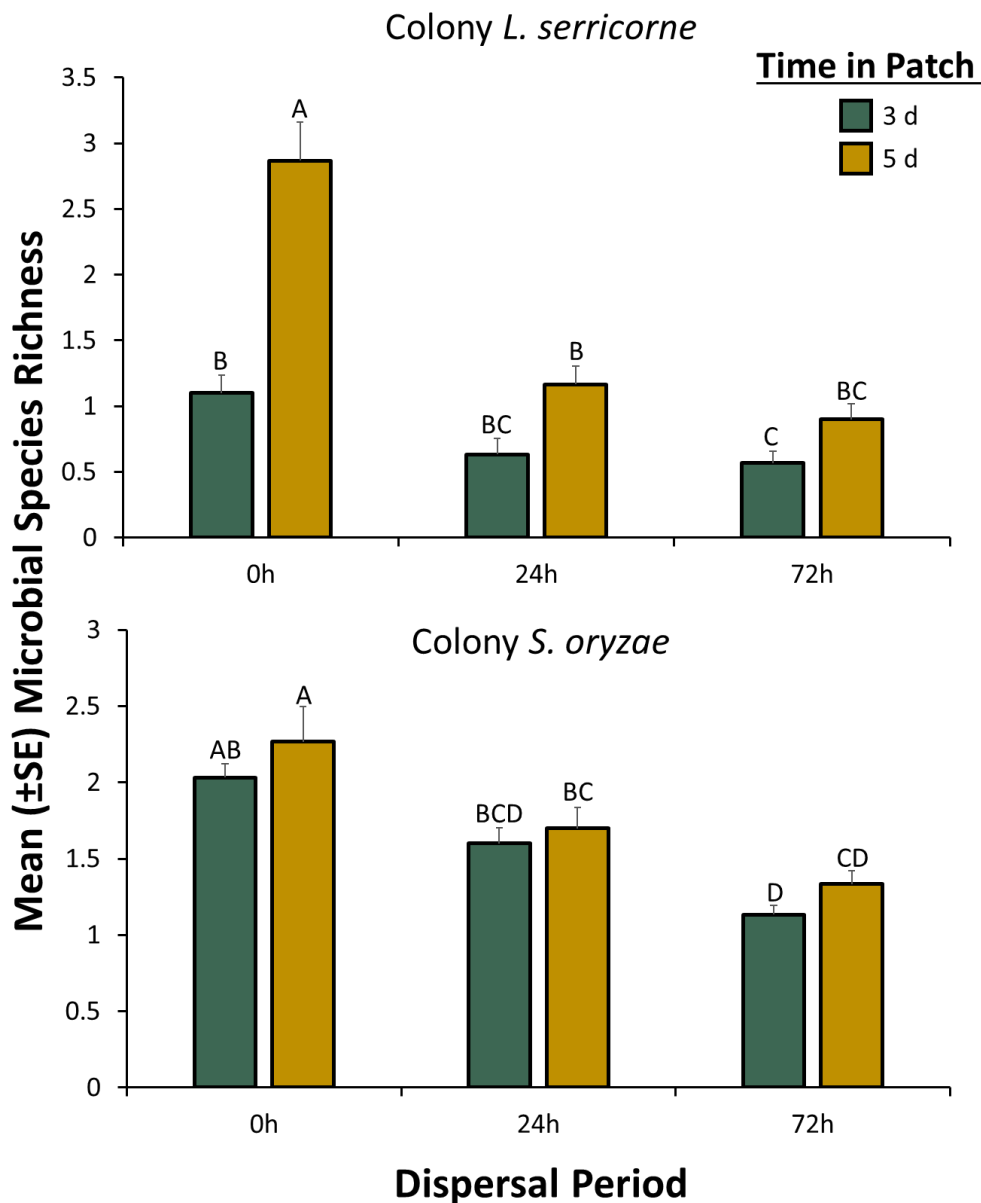


Figure 4-4. Mean grayscale value (e.g., surrogate measure of microbial growth; top panel) or mean species richness (e.g., surrogate measure of microbial diversity; bottom panel) for microbes vectored by *S. oryzae* from *A. flavus*-inoculated grain after they were given a dispersal period of 0, 24, or 72 h and then placed in factitious foraging patches for 3 d (green bars) or 5 d (yellow bars), which consisted of potato dextrose agar dishes that were photographed using a 3D imaging system and processed with ImageJ. There were a total of $n = 30$ replicate adults tested per bar. Bars with shared letters are not significantly different from each other (Tukey HSD, $\alpha = 0.05$). The grayscale value can range from 0, indicating no microbial growth, to 255, indicating the full agar dish was covered with microbes. Species richness represents the number of microbial morphotypes in each food patch.

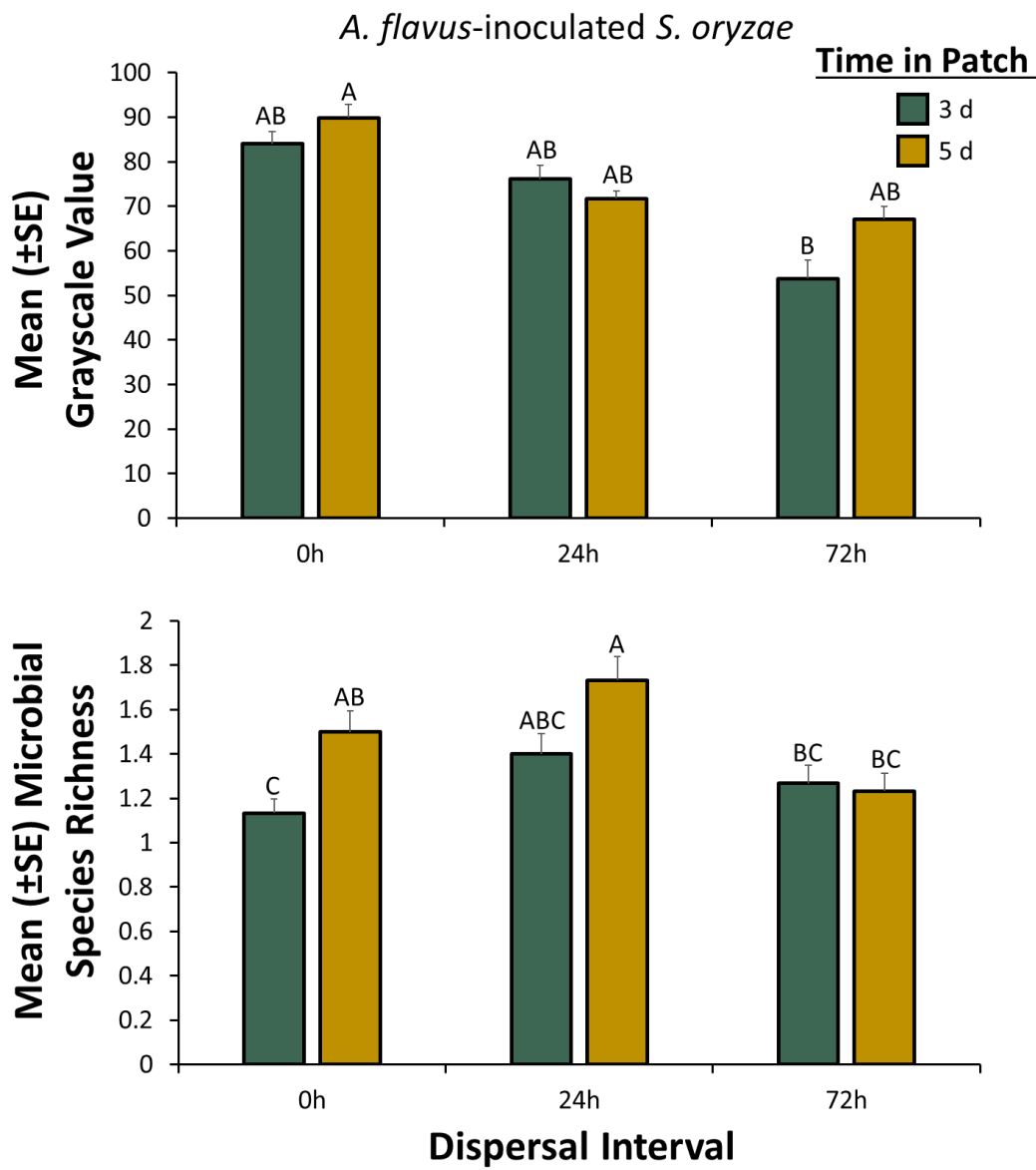


Figure 4-5. Mean grayscale value (e.g., surrogate measure of microbial growth; top panel) or mean species richness (e.g., surrogate measure of microbial diversity; bottom panel) for microbes vectored by *S. oryzae*, depending on population source (from *A. flavus*-inoculated grain, AF; laboratory colonies, Colony; or field-captured adults, Field) after they were given a dispersal period of 0, 24, or 72 h and then placed in factitious foraging patches for 3 d (green bars) or 5 d (yellow bars), which consisted of potato dextrose agar dishes that were photographed using a 3D imaging system and processed with ImageJ. There were a total of $n = 30$ replicate adults tested per bar. Bars with shared letters are not significantly different from each other (Tukey HSD, $\alpha = 0.05$). The grayscale value can range from 0, indicating no microbial growth, to 255, indicating the full agar dish was covered with microbes. Species richness represents the number of microbial morphotypes in each food patch.

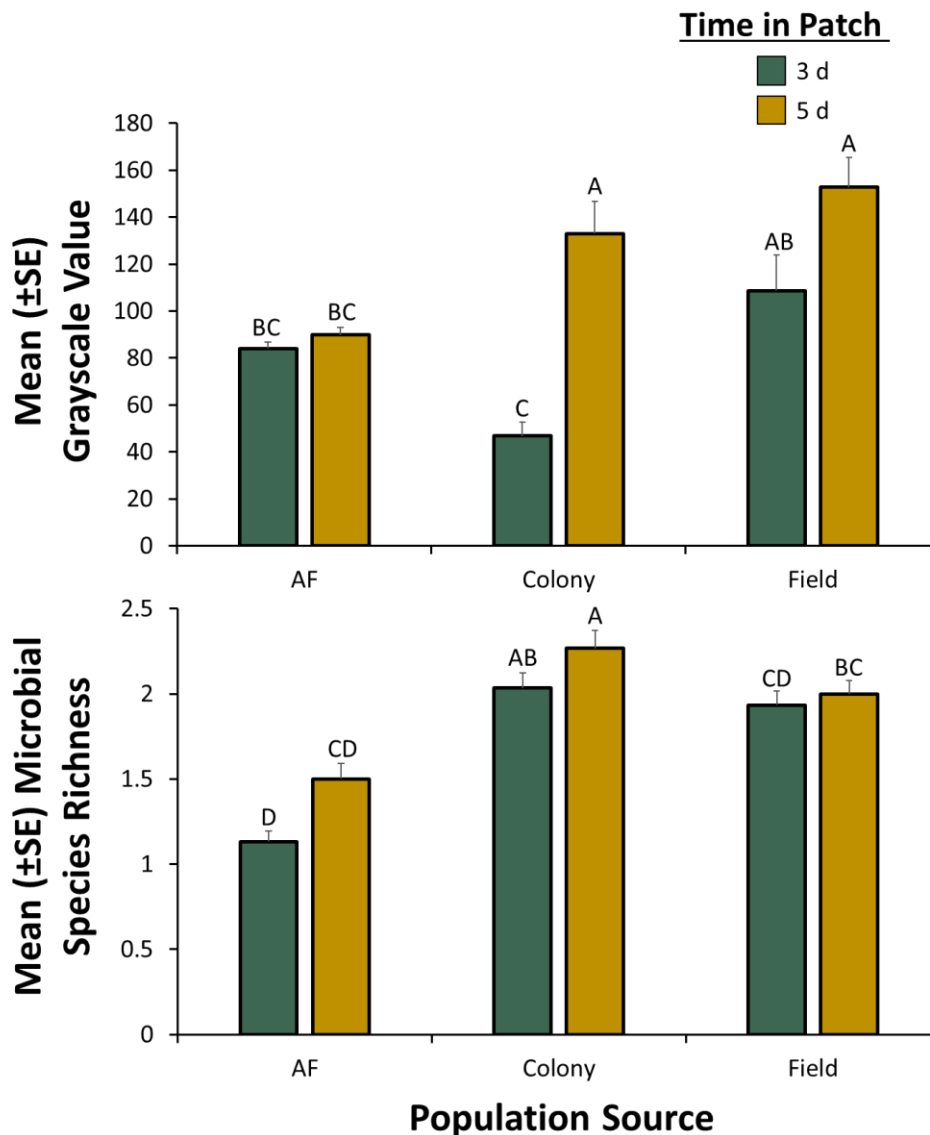
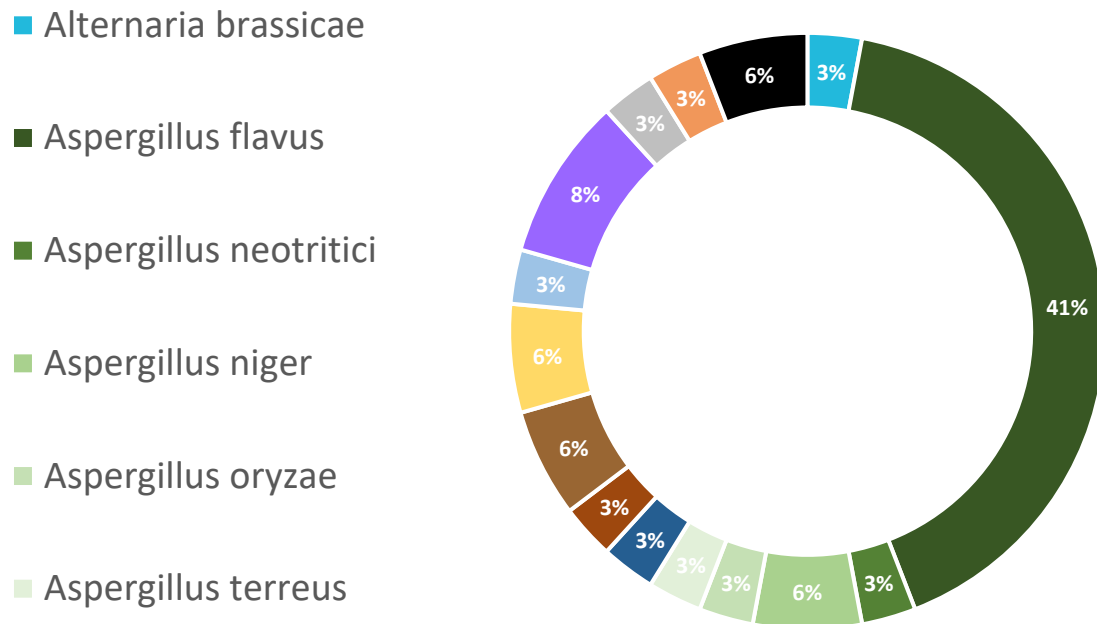


Figure 4-6. Community composition of microbes derived via sequencing the ITS4/5 ribosomal subunit from environmental fungi vectored and dispersed on PDA dishes by *S. oryzae* and *L. serricorne*. Percentages displayed for each taxa portrayed in circle. *Aspergillus* spp.



**Chapter 5 - Beetles choosing habitats poorly: Foraging preferences
of adult cigarette beetle and rice weevil do not match progeny
production in novel food patches with and without microbes**

Submitted as: Marco A. Ponce,^{1*} Tania N. Kim,¹ Erin D. Scully,² William R. Morrison

III². Beetles choosing habitats poorly: Foraging preferences of adult cigarette beetle and rice weevil do not match progeny production in novel food patches with and without microbes.

Journal of Stored Products Research. Submitted.

Abstract

Sitophilus oryzae and *Lasioderma serricorne* are two extremely damaging stored-product insects, and are intimately involved in insect-microbe interactions. Prior research has identified a key symbiosis for *L. serricorne*, but it is uncertain what modulates these insects' preference for and fitness in host habitats with high or low microbial loads. Thus, our aim was to evaluate if the dispersal preference and fitness by *L. serricorne* and *S. oryzae* is modulated by microbial infestation, uncontaminated grain, or sterilized grain in factitious food patches after a 72 h dispersal opportunity in pairwise combinations. Preference assays were performed by using a modified dispersal apparatus containing a release zone in the center where 30 insects were released in each replicate with two alternate habitat types on opposite ends, separated by 75 cm of PVC. Our study found that inoculation with *A. flavus* on wheat modulated the preference and progeny production of *S. oryzae* and *L. serricorne*, although in different ways. *Lasioderma serricorne* dispersed to *A. flavus*-inoculated wheat over sterilized wheat, and also produced more progeny. By contrast, *S. oryzae* dispersed to habitats with sterilized wheat, and also produced more progeny in those same patches. However, *S. oryzae* also produced more progeny in *A. flavus*-inoculated wheat compared to uncontaminated grain, showing the species may have behavioral flexibility in responding to its environment, despite poor decisions by the parental generation. Overall, our work suggests that microbes modulate the dispersal and fitness for both species, which may have implications for food safety and management at food facilities.

Key words: dispersal, behavioral, chemical ecology, wheat, *Sitophilus oryzae*, *Lasioderma serricorne*

Introduction

Understanding the foraging preferences of stored product insects is a crucial area of research in pest management after harvest. Stored product insects and microbes pose a significant threat to commodities, leading to substantial economic losses and food safety concerns (Wacker 2018). To effectively manipulate the behavior of these pests, it is essential to unravel the intricate factors that influence their foraging behavior and host patch use. Some of these factors include abiotic factors, such as microclimatic conditions (e.g., temperature, relative humidity [RH], and grain moisture). Numerous studies have delved into the influence of abiotic factors on foraging behavior of stored product insects (Mantzoukas et al., 2020; Rumbos and Athanasiou, 2017; Kavallieratos et al., 2011). For example, stored product insects generally are not active below 15°C (Arthur et al. 2020), while temperatures above 45–50°C are usually lethal for extended periods. Relative humidity can sometimes have species-specific, and conflicting effects on foraging, while most stored product insects generally prefer commodities with low moisture content.

Moreover, biotic factors may affect foraging as well. For example, these include important considerations like competitive (Quellhorst et al. 2023; Baliota et al. 2022) or mutualistic interactions (Martinson 2020), food availability and quality (Govindan and Swihart 2012), and the presence of microbes (Ponce et al. 2021) or chemical signals (Duehl et al. 2011). Better elucidating how especially biotic factors, which have historically been neglected, is vital to developing effective pest management strategies and strategically manipulating grain environments. With improved knowledge, it may become increasingly possible to alter the foraging behavior of stored product insects and effectively mitigate pest infestations.

Abiotic and biotic factors may interact with each other to affect foraging by altering the microclimate in commodities. The microclimate, by definition, differs from the climate of the surrounding area. One aspect that affects the microclimate in a grain mass is the metabolic activity of insects or microbes, which can create so-called “hotspots” of infestation that are ideal for further colonization by more insects or microbes. These areas then act as centers that produce microbial volatiles once infested (Ponce et al., 2021), and result in elevated moisture content, temperature, or RH that are ideal for commodity degradation and attract more pests. For example, Ponce et al. (2023) found that the presence of microbes increased the fitness of the rice weevil, *Sitophilus oryzae* (L.) (Coleoptera: Curculionidae) at high temperatures, while the presence of both types of pests modified abiotic conditions in ways that were mutually favorable. In addition, it also resulted in elevated progeny production by *S. oryzae* compared to when microbes were absent. Likewise, mite-microbe interactions were found to be essential for the growth of all mite life stages (Hubert et al. 2020).

Characterizing the behavioral ecology of stored-product insect foraging choices is critical for improving a fundamental understanding of species interactions at food facilities, but also for applications to long-term monitoring programs. Examining foraging ecology includes addressing questions related to dispersal to new habitats, preference for specific resources, time spent in a patch, oviposition choices, and progeny production in an area. Initial dispersal behavior may determine the overall patterns of movement and distribution of insects within storage facilities (Gerken and Campbell 2022; Jian 2019), often affecting progeny production in commodities (e.g., Arthur and Morrison 2020). The time spent in a food patch is indicative of the insect's perception of resource quality (Scheirs and De Bruyn, 2002) and can provide insights into optimal foraging strategies. Another important aspect is understanding the oviposition choices

that are crucial for population growth and, which can be influenced by multiple factors, including the nutritional quality of the resource and the presence of competitors or microbes (Yee et al., 2020). Nonetheless, a key component of understanding behavioral ecology is examining the preference of stored product insects between alternate habitat cues. However, this often not done in a way where there are natural odor gradients, where insects may freely choose their path over an extended distance, but are committed once they enter the commodities, and under realistic abiotic conditions for food facilities.

Microbial cues may significantly affect the behavioral ecology of foraging by stored product insects. Microbes, including fungi and other microorganisms, produce a panoply of volatile compounds during infestation, which can serve as important cues for stored product insects. These cues can attract or repel insects, influencing their behavioral ecology and decision-making as they forage (Ponce et al., 2021). For example, microbes serve as a significant source of behaviorally active semiochemicals for insects, playing a crucial role in multiple aspects of their mate-finding and oviposition activities (reviewed in Davis et al., 2013). In addition, microbial volatiles facilitate long-distance habitat finding (Ponce et al. 2023; Steiner et al. 2007), short-range host finding (Ponce et al. 2022), and mating behavior (Wang et al., 2023). For example, the microbially-produced volatile organic compound, 1-octen-3-ol, synergized attraction to traps with the conspecific aggregation pheromone for *Cryptolestes ferrugineus* Stephens (Coleoptera: Laemophloeidae) (Stevens et al., 2019). Other studies have shown that the red flour beetle, *Tribolium castaneum* (Herbst) (Coleoptera: Tenebrionidae), uses fungi associated with cotton seeds to locate nearby resources (Ahmad et al., 2013). More recent work by Ponce et al. (2022) and Ponce et al. (2023) have further contributed to this field by investigating the behavioral responses of *S. oryzae* and *L. serricorne* to microbial cues, showing

that *S. oryzae* only used them at close-range, while the latter uses microbial cues at both close- and long-range. Together, this body of research shows that microbial cues may significantly modify specific foraging, host patch use, and oviposition.

Understanding preference for specific resources or habitats can lead to improvement in behaviorally-based, semiochemical-mediated tactics to manipulate stored product insect behavior. This includes a suite of tactics to manipulate pest behavior to protect the commodity, including attract-and-kill, mating disruption, and push-pull (Morrison et al., 2021). Some of the most common compounds isolated from experimental grain bins were 3-methyl-1-butanol, 1-octen-3-ol, and 3-octanone (Sinha et al., 1987), which overlaps with some of the most common fungal volatiles (Ponce et al., 2021), including those emitted from grain inoculated with *Aspergillus flavus* (Ponce et al., 2023). These volatile compounds either in isolates or mixtures with other semiochemicals (e.g., kairomones, pheromones) hold promise for potential practical application in augmenting attract-and-kill and other strategies. By linking microbial volatiles to insect foraging preferences, and host patch behaviors (i.e., ovipositing), researchers can uncover novel ways to manipulate insect foraging behaviors and develop more effective and environmentally friendly control methods.

Two important, and cosmopolitan stored product insects are the internal-infesting *Sitophilus oryzae* (L.) (Coleoptera: Curculionidae) and the external-infesting *Lasioderma serricorne* (F.) (Coleoptera: Ptiniidae) (Hagstrum and Subramanyam, 2006). These insects appear to interact with fungi in different ways. Nakagawa et al. (2008) found fungal spores on the cuticle of *L. serricorne*, while Ponce et al. (2023) found that both *S. oryzae* and *L. serricorne* readily vectored a variety of microbes to new food patches. *Fusarium* spp. considered weak pathogens emitted compounds from grain that were attractive or neutral, while relatively strong

Fusarium spp. pathogens had a repellent effect on *S. oryzae* (Selitskaya et al. 2014). Thus, interactions with microbes appear important for both of these species.

While microbial contamination is a common occurrence in post-harvest environments, it is unknown whether stored product insect prefer to disperse and reproduce in these areas. Thus, the aim of the current study was to evaluate if the habitat preference of *S. oryzae* and *L. serricorne* was modulated by microbial infestation in surrogate food patches after 3 d of foraging at a distance. We hypothesized that host habitats infested with microbes and emitting MVOCs may be more attracting to dispersing external-infesting, *L. serricorne* than internal-infesting stored product pest, *S. oryzae*, because they may signal a usable resource to the former but not the latter, and therefore we may also expect more progeny production in uninfested environments for *S. oryzae*.

Materials and Methods

Experimental Insects and Source of Grain

Adult beetles used in this study were obtained from colonies kept at the laboratory of the U.S. Department of Agriculture Agricultural Research Service's (ARS) Center for Grain and Animal Health Research (CGAHR) in Manhattan, KS, USA. In particular, colonies of *S. oryzae* (field-derived strain from North-Central Kansas in 2012) were reared on organic, whole kernel wheat (Heartland Mills, Marienthal, KS, USA) after tempering it to 15% grain moisture from an initial 10.6% moisture. To assess the baseline moisture level of the source grain, a grain moisture analyzer was used (DICKEY-john, GAC2100, Auburn, IL, USA). Colonies with 4- to 8-week-old adults were kept in 950-mL mason jars (8.5 D × 17 cm H) and stored in an environmental chamber (136VL, Percival Instruments, Perry, IA, USA) set at constant conditions (27.5 ± 0.01°C, 60 ± 3% RH, and 14:10 L:D). Wheat used for colonies and inoculations below were held

in cold storage (~4°C) until needed for experiments, then frozen at -4°C for at least 72 h prior to use to prevent mite or other insect infestation. Adult *L. serricorne* were used from insect colonies that were originally collected in 2012 from a processing facility in Arkansas and continuously maintained on a diet of 95% bleached wheat flour and 5% brewer's yeast with oats sprinkled on top and a moistened, crumpled towel added. Insects were subcultured by sieving (No. 30 sieve, 594 × 594 µm mesh, W.S. Tyler Co., Cleveland, OH, USA) 150 mixed-sex *L. serricorne* adults and adding them into mason jars (950-ml capacity) filled two-thirds of the way to the top with diet, then placing inside environmental chambers under constant conditions at 27.5°C, 65% RH, and 14:10 L:D photoperiod. Individuals from each species were never tested more than once in the experiments below. These were termed the colony populations of *L. serricorne* and *S. oryzae*.

Grain Treatment Preparation

Control Grain

To obtain a positive control, whole wheat grain was taken directly from storage, and used in the experiments below. Thus, it contained whatever natural microbiome was inherent to the grain during harvesting and storage, but there was no visible spoilage to the unaided human eye.

Autoclaved Grain

To obtain sterilized, autoclaved grain, whole wheat grain was evenly divided (~300 g) and placed in 2 separate previously autoclaved mason jars (950-mL capacity). A single hole was pierced through each lid and lined with a cotton ball to prevent the transfer of spores or other contaminants. The jars were then sealed with aluminum foil and were autoclaved on a dry cycle (533LS, Getinge, Rochester, NY, USA) for 30 min. Jars with autoclaved grain were allowed to acclimate to room temperature prior to experimental assays. This treatment was used for both species *S. oryzae* and *L. serricorne*. For *L. serricorne*, autoclaved grain was ground up in a

sterilized 1656 mL-capacity food processor (KFP0718BM, KitchenAid, St. Joseph, MI, USA), because it is an external-infesting insect and requires grain that is not intact.

Fungal-Inoculated Grain

In order to assess the effect of microbially rich environments on foraging decisions by stored product insects, *Aspergillus flavus* was inoculated onto grain through the following process: 600 g of organic, whole wheat was placed in a stainless-steel pot filled with water and placed on a hot plate at 500°C. Once boiling for 15 min, the water was drained and the grain was evenly spread out on sterile wipes (38.1 × 42.5 cm, 3 ply, Tech wipes, Skilcraft, NIB, Alexandria, VA) that lined an autoclaved metal tray inside a laminar fume hood until dry (ca. 3 h). Afterwards, grain was evenly divided (~300 g) and placed in 2 separate, autoclaved mason jars (950-mL capacity). A single hole was pierced through each lid and lined with a sterile cotton ball. The jars were then sealed with aluminum foil and were autoclaved (533LS, Getinge, Rochester, NY, USA) for 30 min. To inoculate with *A. flavus*, a 7.62-cm × 1-cm strip of a pure plug of *A. flavus* (Ponce et al. 2023) on a potato dextrose agarose petri dish was placed into each jar containing the wheat. The *A. flavus* isolation had been colonized and growing without contamination in a petri dish for 7 d in an environmental chamber set to 30°C, 60% RH, and 14:10 L:D photoperiod. Jars with freshly prepared *A. flavus*-inoculated grain sat at room temperature for roughly 10 d or until the *A. flavus* evenly covered as much of the wheat as possible. Batches of inoculated wheat were used within 10–15 d of preparation. Wheat was never used more than once for each replicate of every trial in each assay experiment to prevent cross contamination. This treatment was used for both species *S. oryzae* and *L. serricorne*, except in the latter's case, 150 g of the inoculated wheat was ground with 150 g of the control grain (positive control treatment) at a 1:1 ratio based on the life history as an external-infesting pest.

Dispersal and Progeny Production Habitat Preference Assay

To test the foraging preference of *L. serricorne* and *S. oryzae* when dispersing to novel food patches with and without microbes, a dispersal apparatus was employed using the treatments above. The dispersal apparatus consisted of two 2.5 cm diameter PVC of 75 cm length connecting an empty introduction glass jar (6.5 × 4.7 cm H:D) to one of two adjacent glass jars with alternate possible “food patches” (Figure 1). The introduction jar from the dispersal apparatus was drilled with two holes (each 2.22 cm D) through the hard plastic cap (Figure 1 B,E), with just one hole for each alternate food patch at the ends of the dispersal apparatus was drilled and centered over the glass jars to create a pitfall trap design (Figure 1 B,C). The three food patches were spaced 75 cm apart using a plywood plank (5 × 10 × 75 cm H:W:L) placed in serial connection (Figure 1 B) with round cored seating for the release jar and food patches. To increase traction and facilitate dispersal to novel food patches, a single piece of cotton twine of 83.5 cm length was threaded through the apparatus for the adults to walk along, with one end contacting the bottom of the introduction chamber and the other end terminating halfway into each food patch so that only one-way movement was possible once a decision to enter a new food patch was made (Figure 1 C, E). One of each of the three treatments as described above were compared against each other in a pairwise fashion for each species as follows, including the following treatments: 20 g of *Aspergillus flavus*-inoculated wheat or flour, 20 g of the positive control, and/or 20 g of the autoclaved wheat or flour.

For each replicate and combination treatment, 30 adult *L. serricorne* or *S. oryzae* were introduced into the release container of the dispersal apparatus and given a 72 h period to disperse to one of two habitats. At the end of the period, the number of adults dispersing were counted in each adjacent food patch. Further, any still located in the release chamber or in transit

in the PVC pipe were noted. In addition, the grain from each adjacent jar was transferred into clean glass jars and placed in an environmental chamber set to 30°C, 60% RH, and 14:10 L:D photoperiod (Figure 1 D) to then assess progeny production after 60 d by counting the number of adults minus the number of initial individuals captured. There were $n = 10$ replicates per treatment combination and species.

Progeny Morphometric

The size of progeny was recorded using a microscope (SMZ-18, Nikon Inc, Tokyo, Japan) at 10x magnification. Image settings were adjusted to capture clear and detailed images of the progeny. The microscope utilized a digital camera system and software (NIS-Elements, Nikon Inc, Tokyo, Japan) to acquire and analyzed the images. The magnification level of 10x was chosen to ensure an appropriate balance between capturing sufficient detail and maintaining a wide field of view for accurate measurements. To obtain accurate size measurements, a standardized calibration was used, allowing precise conversion of pixel measurements to actual physical dimensions.

Statistical Analysis

Results from the number of adults captured in each patch were analyzed with a generalized linear model based on a Poisson or Quasipoisson distribution (depending on whether overdispersion was a problem). A separate model was used for each species since initial models indicated a significant effect of species. Treatment (e.g., food patch habitat) was used as explanatory variables. Upon a significant result from the model, Tukey HSD was used for multiple comparisons. All statistical analyses were performed in R Software (R Core Team, 2023), using $\alpha = 0.05$

Results

Dispersal Preference Assay

A total of 63%, 66%, and 77% of the released *L. serricornis* dispersed to one of the habitats in the AF vs. UV, Ctrl vs. AF, and Ctrl vs. UV comparison, respectively, suggesting that reasonable interpretation is possible. The type of habitat significantly affected dispersal by *L. serricornis* (Table 1). For example, there were almost twice as many *L. serricornis* found in the *A. flavus*-inoculated wheat compared to the sterilized patches (UV), while there were 1.8-fold more conspecifics in the positive control wheat compared to the sterilized patches as well (Figure 2, Table 1).

A total of 98–100% of the released *S. oryzae* dispersed to one of the habitats in the AF vs. UV, Ctrl vs. AF, and Ctrl vs. UV comparisons, suggesting that reasonable interpretation is possible. Similar to *L. serricornis*, the type of food patch affected dispersal by *S. oryzae*, depending on comparison (Table 1). There was no significant preference in dispersal among *A. flavus*-inoculated wheat and unmanipulated wheat (Ctrl) or autoclaved wheat (UV) (Figure 3; Table 1). By contrast, *S. oryzae* preferred to disperse to sterilized wheat (UV) compared to unmanipulated grain (Figure 3), with 1.5-fold more adults present in the former.

Progeny Production Preference Assay

In total, *L. serricornis* produced 13,097 progeny, with 42%, 39%, and 18% of them in the AF vs. UV, Ctrl vs. UV, and Ctrl vs. AF comparison. The type of food patch significantly affected progeny production by *L. serricornis* in AF vs. UV patch comparison, with 2.2-fold more progeny in *A. flavus*-inoculated wheat compared to sterilized wheat (UV) (Figure 4, Table 2). However, there was no significant difference between food patches in progeny production by

L. serricorne for Ctrl vs. AF and Ctrl vs. UV (Table 2). In each pairwise comparison, progeny production only fluctuated between 117–122 and 237–277, respectively (Figure 4).

Overall, *S. oryzae* produced 19,528 progeny, with 35%, 35%, and 30% of them in the Ctrl vs. UV, Ctrl vs. AF, and AF vs. UV comparison. The food habitat type significantly altered progeny production in the AF vs. UV comparison, with 8-fold more progeny produced in the sterilized (UV) patch than in the *A. flavus*-inoculated patch (Figure 5; Table 2). Interestingly, progeny production was significantly affected in the opposite direction in the AF vs. Ctrl comparison (Table 2), with 10-fold more progeny by *S. oryzae* in *A. flavus*-inoculated habitats than in unmanipulated wheat (Ctrl) (Figure 5). There was no significant fitness advantage among either habitat in the UV vs. Ctrl comparison (Table 2).

Morphometric Analysis

The size of progeny produced by *S. oryzae* in the Ctrl vs. AF comparison was significantly affected by habitat (t-test: $df = 398$; $t = 30.1$; $P < 0.0001$). In particular, progeny from *A. flavus*-inoculated wheat were 21% smaller on average than those from the unmanipulated wheat (Ctrl) (Figure 6).

Discussion

The results of the current study provide valuable insights into the foraging behavior and habitat preferences of *L. serricorne* and *S. oryzae*, as well as the role that microbes play in modulating dispersal and fitness patterns. In particular, the findings suggest distinct species-specific responses, with *L. serricorne* preferring to disperse to and reproduce in *A. flavus*-inoculated environments compared to sterilized ones, while *S. oryzae* did not show a preference in dispersal between microbe rich or poor ones, and also various produce many and few progeny in microbe-rich environments. This shows that there may be more behavioral flexibility by *S.*

oryzae in whether to respond to microbes, and which may be the most important. However, choice by parental *S. oryzae* seems to have incredibly important fitness consequences for progeny. Prior work has explored differences among two competing hypothesis, including “mother knows best” whereby females prefer to oviposit on hosts that increase larval survival, and “optimal bad motherhood” whereby females prefer to oviposit on hosts that increase their own survival (Garcia-Robledo and Horvitz 2012, Ning et al. 2022). Some of that prior work has found that “mother knows best” prevails (e.g., Garcia-Robledo and Horvitz 2012). Below, we delve into specific findings from this study.

We found *L. serricorne* to more consistently seek out microbe-rich environments as well as have greater fitness in those environments. This preference may be driven by factors such as the resources in the environment made more abundant by microbes or other ecological conditions that enhance the survival and reproduction of *L. serricorne*. It is known that *L. serricorne* has a close association with endosymbionts (Martinson 2020), and conspecifics have been documented with fungal spores on their cuticle (Nakagawa et al. 2008). In fact, even after extended dispersal periods in sterilized environments, *L. serricorne* has been documented vectoring microbes, including *A. flavus*, to new environments (Ponce et al. 2023). The implications of *L. serricorne* seeking out areas in a commodity already infested with microbes is that conspecifics may exacerbate these hotspots through their metabolic activity (Hubert et al. 2018) and/or may passive vector the microbes from that location to new locations.

By contrast, *S. oryzae* partially preferentially dispersed to sterilized wheat habitats, and produced more progeny in sterilized wheat than in *A. flavus*-inoculated wheat. This preference for clean environments might be influenced by the life history of *S. oryzae*, which allows it to already take advantage of intact wheat kernels (Hagstrum and Subramanyam 2006), or factors

such as competition avoidance (MVOCs may be a sign of too much insect activity; Ponce et al. 2021) or resource utilization optimization (Liu et al., 2019). Prior work found that *S. oryzae* did not use microbial volatiles at a distance when foraging, but that they were more important for close-range interactions (Ponce et al. 2022). However, surprisingly, in the AF vs. Ctrl comparison, *S. oryzae* produced many more progeny in the *A. flavus*-inoculated wheat than in the unmanipulated wheat. This may show there is individual-level behavioral flexibility, depending on which genotypes are in an environment, and that there can be local adaptation to decisions made by the parental generation (Nicolaus and Edelaar 2018). In addition, it is possible that the unmanipulated control represents a variety of fungi, as has been shown in prior work from sequencing (Van Winkle et al. 2021), and some of these may have incurred a much greater fitness cost to *S. oryzae* than *A. flavus* inoculated on wheat. Overall, the results for *S. oryzae* suggest conspecifics forage for clean, microbial-free environments. The findings also indicate that the presence of microbes in the host habitat may strongly affect progeny production.

There are a variety of outstanding unanswered questions. Going forward, research should investigate landscape mosaics of microbial infestation by *S. oryzae* and *L. serricorne* to understand how dispersal and microbial infestation interact in determining distribution of conspecifics at different patches. For example, *T. castaneum* assesses present and future food patch quality when ovipositing (Halliday et al. 2019). It is possible that prior experience may modulate both foraging preference and fitness (Proffitt et al. 2015), so future work should evaluate insects reared only on sterilized grain compared those reared in microbe-rich environments. To fully understand the implications of the foraging preferences observed in this study, future work should systematically assess how these preferences affect grain quality in the chosen food patch. By exploring the direct consequences of foraging behavior on grain quality,

future research can provide valuable insights into the potential impact of these insect pests on agricultural productivity. Overall, these future avenues can guide the development of targeted pest management strategies by taking into account the specific preferences and requirements of *L. serricorne* and *S. oryzae*.

This study adds to the existing knowledge by highlighting that microbes can also modulate the foraging behavior of both *S. oryzae* and *L. serricorne*, albeit in different ways. Both these insects act not only as vectors of these microbes (Ponce et al. 2023), but these microbes also influence their behavior, fitness, and development. These findings emphasize the importance of considering microbial interactions in understanding the behavior and ecology of stored product insect pests, and highlight the need for further research to fully elucidate the implications of foraging preferences on grain quality, progeny production, and developmental parameters. Such insights will contribute to more effective pest management strategies tailored to the specific characteristics of each of these pests.

References

- Ahmad, F., Daglish, G. J., Ridley, A. W., Burrill, P. R., & Walter, G. H. (2013). Short-range resource location by *Tribolium castaneum* Herbst (Coleoptera: Tenebrionidae) demonstrates a strong preference for fungi associated with cotton seed. *Journal of Stored Products Research*, 52, 21-27.
- Arthur, F. H., Morrison III, W. R., & Trdan, S. (2020). Feasibility of using aeration to cool wheat stored in Slovenia: A predictive modeling approach using historical weather data. *Applied Sciences*, 10(17), 6066.
- Arthur, F., & Morrison III, W. R. (2020). Methodology for assessing progeny production and grain damage on commodities treated with insecticides. *Agronomy*, 10(6), 804.
- Baliota, G.V., D.S. Scheff, W.R. Morrison III, C.G. Athanassiou. (2022). Competition of *Prostephanus truncatus* with *Sitophilus oryzae* on maize: the species that gets there first matters. *Bulletin of Entomological Research*, 112, 520–527.
- Davis, T. S., & Landolt, P. J. (2013). A survey of insect assemblages responding to volatiles from a ubiquitous fungus in an agricultural landscape. *Journal of Chemical Ecology*, 39, 860-868.
- Duehl, A.J., Arbogast, R.T. & Teal, P.E.A. (2011). Density-related volatile emissions and responses in the red flour beetle, *Tribolium castaneum*. *Journal of Chemical Ecology*, 37, 525–532. <https://doi.org/10.1007/s10886-011-9942-3>
- García-Robledo, C., & Horvitz, C. C. (2012). Parent–offspring conflicts, “optimal bad motherhood” and the “mother knows best” principles in insect herbivores colonizing novel host plants. *Ecology and Evolution*, 2(7), 1446-1457.

- Gerken, A. R., & Campbell, J. F. (2022). Spatial and temporal variation in stored-product insect pest distributions and implications for pest management in processing and storage facilities. *Annals of the Entomological Society of America*, 115(3), 239–252.
- Germinara, G. S., De Cristofaro, A., & Rotundo, G. (2015). Repellents effectively disrupt the olfactory orientation of *Sitophilus granarius* to wheat kernels. *Journal of Pest Science*, 88, 675-684.
- Govindan, B.N. & Swihart, R.K. (2012). Experimental beetle metapopulations respond positively to dynamic landscapes and reduced connectivity. *PloS ONE*, 7(4), e34518.
- Hagstrum, D. W., & Subramanyam, B. (2006). Fundamentals of Stored-Product Entomology; AACC International: St. Paul, MN, USA, 323.
- Halliday, W. D., Slevan-Tremblay, I., & Blouin-Demers, G. (2019). Do female red flour beetles assess both current and future competition during oviposition?. *Journal of Insect Behavior*, 32, 181-187.
- Jian, F. (2019). Influences of stored product insect movements on integrated pest management decisions. *Insects*, 10(4), 100.
- Kavallieratos, N. G., Athanassiou, C. G., Hatzikonstantinou, A. N., & Kavallieratou, H. N. (2011). Abiotic and biotic factors affect efficacy of chlorfenapyr for control of stored-product insect pests. *Journal of Food Protection*, 74(8), 1288-1299.
- Liu, C., Wang, C., & Yao, H. (2019). Comprehensive resource utilization of waste using the black soldier fly (*Hermetia illucens* (L.))(Diptera: Stratiomyidae). *Animals*, 9(6), 349.
- Magan, N., Hope, R., Cairns, V., & Aldred, D. (2003). Post-harvest fungal ecology: impact of fungal growth and mycotoxin accumulation in stored grain. *Epidemiology of Mycotoxin*

- Producing Fungi: Under the aegis of COST Action 835 ‘Agriculturally Important Toxigenic Fungi 1998–2003’, EU project (QLK 1-CT-1998–01380), 723-730.
- Mantzoukas, S., Lagogiannis, I., Karmakolia, K., Rodi, A., Gazepi, M., & Eliopoulos, P. A. (2020). The Effect of grain type on virulence of entomopathogenic fungi against stored product pests. *Applied Sciences*, 10(8), 2970.
- Marin, S., Ramos, A. J., Cano-Sancho, G., & Sanchis, V. (2013). Mycotoxins: Occurrence, toxicology, and exposure assessment. *Food and Chemical Toxicology*, 60, 218-237.
- Martinson, V. G. (2020). Rediscovering a forgotten system of symbiosis: historical perspective and future potential. *Genes*, 11(9), 1063.
- Morrison III, W. R., Scully, E. D., & Campbell, J. F. (2021). Towards developing areawide semiochemical-mediated, behaviorally-based integrated pest management programs for stored product insects. *Pest Management Science*, 77(6), 2667-2682.
- Nakagawa, A., Kawakami, Y., Hashimoto, K., Hatakeyama, Y., Uchida, A., Takahashi, H., & Iwano, H. (2008). Adhesion rate and patterns of fungi spores on the body surface of the cigarette beetle, *Lasioderma serricorne*. *Medical Entomology and Zoology*, 59(2), 85-89.
- Nicolaus, M., & Edelaar, P. (2018). Comparing the consequences of natural selection, adaptive phenotypic plasticity, and matching habitat choice for phenotype–environment matching, population genetic structure, and reproductive isolation in meta-populations. *Ecology and Evolution*, 8(8), 3815-3827.
- Ning, Z., Chen, C., Cui, B., Zhang, Y., Xie, T., Wang, Q., ... & Bouma, T. J. (2022). ‘Mother knows best’: Maternal oviposition effects of a range-expanding insect herbivore degrade coastal wetlands by targeting juvenile foundation plant species. *Land Degradation & Development*, 33(16), 3023-3035.

- Phillips, T. W., & Throne, J. E. (2010). Biorational approaches to managing stored-product insects. *Annual Review of Entomology*, 55, 375-397.
- Ponce M., Kim T.N., Morrison W.R. (2023). The interaction between *Sitophilus oryzae* (Coleoptera: Curculionidae) and *Aspergillus flavus* jointly affects microclimate, grain quality, and each other's fitness. *Environmental Entomology*, in press.
- Ponce M., Maille M.J., Bruce A., Kim T.N., Scully D.E., Morrison W.R. (2023). Microbial vectoring capacity by an internal- and external-infesting stored product insect after varying dispersal periods between novel food patches: An underestimated risk, in press.
- Ponce, M. A., Kim, T. N., & Morrison III, W. R. (2021). A systematic review of the behavioral responses by stored-product arthropods to individual or blends of microbially produced volatile cues. *Insects*, 12(5), 391.
- Ponce, M. A., Lizarraga, S., Bruce, A., Kim, T. N., & Morrison III, W. R. (2022). Grain inoculated with different growth stages of the fungus, *Aspergillus flavus*, affects the close-range foraging behavior by a primary stored product pest, *Sitophilus oryzae* (Coleoptera: Curculionidae). *Environmental Entomology*, 51(5), 927-939.
- Ponce, M. A., Sierra, P., Maille, J. M., Kim, T. N., Scully, E. D., & Morrison III, W. R. (2023). Attraction, mobility, and preference by *Lasioderma serricorne* (Coleoptera: Ptinidae) to microbially-mediated volatile emissions by two species of fungi in stored grain. *Scientific Reports*, 13(1), 6176.
- Proffit, M., Khallaf, M. A., Carrasco, D., Larsson, M. C., & Anderson, P. (2015). 'Do you remember the first time?' Host plant preference in a moth is modulated by experiences during larval feeding and adult mating. *Ecology Letters*, 18(4), 365-374.

- Quellhorst, H.E., T.N. Kim, K.Y. Zhu, and W.R. Morrison III. (2023). Short-term spatial dispersion patterns between the larger grain borer and the maize weevil in grain columns with implications for management of stored maize. *Environmental Entomology*, in review.
- Rumbos, C. I., & Athanassiou, C. G. (2017). The use of entomopathogenic nematodes in the control of stored-product insects. *Journal of Pest Science*, 90, 39-49.
- Scheirs, J., & De Bruyn, L. (2002). Integrating optimal foraging and optimal oviposition theory in plant–insect research. *Oikos*, 96(1), 187-191.
- Selitskaya, O. G., Gavrilova, O. P., Schenikova, A. V., Shamshev, I. V., & Gagkaeva, T. Y. (2014). The effect of toxin-producing *Fusarium* fungi on behavior of the rice weevil *Sitophilus oryzae* (Coleoptera, Dryophthoridae). *Entomological Review*, 94, 820-825.
- Steiner, S., Erdmann, D., Steidle, J. L., & Ruther, J. (2007). Host habitat assessment by a parasitoid using fungal volatiles. *Frontiers in Zoology*, 4(1), 1-10.
- Stevens, M. M., Wood, R. M., & Mo, J. (2019). Monitoring flight activity of *Cryptolestes ferrugineus* (Coleoptera: Laemophloeidae) in outdoor environments using a commercial pheromone lure and the kairomone 1-octen-3-ol. *Journal of Stored Products Research*, 83, 227-235.
- Van Winkle, T., Ponce, M. A., Quellhorst, H. E., Bruce, A., Albin, C. E., Kim, T. N., Zhu, K.Y., & Morrison III, W. R. (2021). Microbial volatile organic compounds mediate attraction by a primary but not a secondary stored product insect pest in wheat. *Journal Chemical Ecology*, 48, 27-40.

- Wacker, F. 2018. Food waste and food losses – Importance of international partnerships and research. In: 12th International Working Conference on Stored Product Protection, 7–11 October 2018. Julius-Kuhn Institut, Berlin, Germany
- Wang, Z., Zhao, Y., Wang, Y., & Yujie, L. (2023). Population divergence in pheromone communication for mate choice of *Tribolium castaneum* and its association with gut bacteria. *Journal of Pest Science*, in press. <https://doi.org/10.1007/s10340-023-01632-6>
- Yee, D. A., Glasgow, W. C., & Ezeakacha, N. F. (2020). Quantifying species traits related to oviposition behavior and offspring survival in two important disease vectors. *Plos one*, 15(9), e0239636.

Table 5-1. Summary of t-tests with Bonferroni correction for number of *Lasioderma serricorne* and *Sitophilus oryzae* dispersing to new habitats.

Comparison	df	t	P
<i>Lasioderma serricorne</i>			
AF vs. UV	18	3.23	0.0047
Ctrl vs. AF	18	1.6	0.13
Ctrl vs. UV	18	3.99	0.0009
<i>Sitophilus oryzae</i>			
AF vs. UV	18	0.73	0.47
Ctrl vs. AF	18	1.78	0.09
Ctrl vs. UV	18	3.46	0.0028

¹ Abbreviations: AF - *A. flavus* inoculated wheat, UV - UV- autoclaved and surface sterilized wheat, Ctrl - unmanipulated wheat.

Table 5-2. Summary of t-tests with Bonferroni correction for progeny production by *Lasioderma serricorne* and *Sitophilus oryzae* dispersing in new habitats.

Comparison	df	t	P
<i>Lasioderma serricorne</i>			
AF vs. UV	18	6.15	0.0001
Ctrl vs. AF	18	0.14	0.89
Ctrl vs. UV	18	1.47	0.16
<i>Sitophilus oryzae</i>			
AF vs. UV	18	8.54	0.0001
Ctrl vs. AF	18	12.2	0.0001
Ctrl vs. UV	18	1.41	0.18

¹ Abbreviations: AF - *A. flavus* inoculated wheat, UV - UV- autoclaved and surface sterilized wheat, Ctrl - unmanipulated wheat.

Figure 5-1. Dispersal apparatus representation.

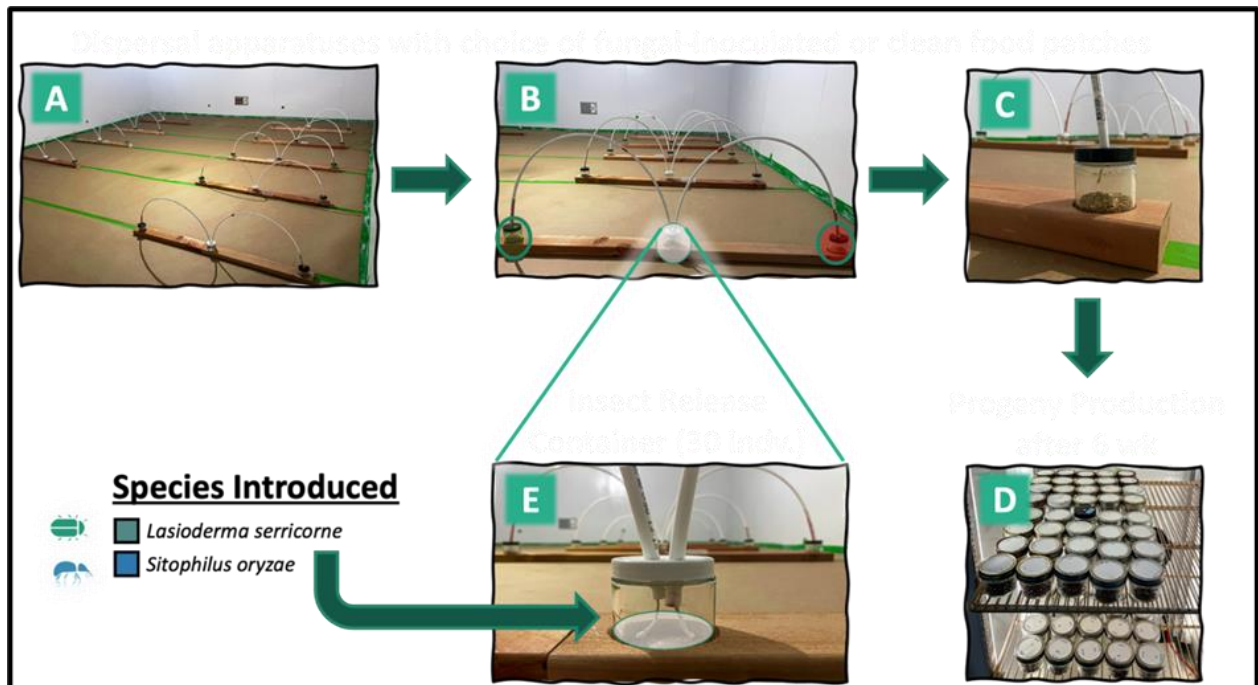


Figure 5-2. The mean number of *Lasioderma serricorne* adults dispersing to a novel food resource after 72 h in environmental chambers at 30°C and 65% RH in pairwise comparisons among food patches with 20 g of *Aspergillus flavus*-inoculated (AF), unmanipulated wheat (Ctrl, as positive control), or autoclaved and surface sterilized wheat (UV). Each bar represents the mean of 10 replicates, with 30 adults released in each replicate. for treatment combination. Abbreviations: ns – not significant (with a Bonferroni-corrected α -threshold using a t-test), ** - $0.01 < P < 0.001$, *** - $P < 0.0001$.

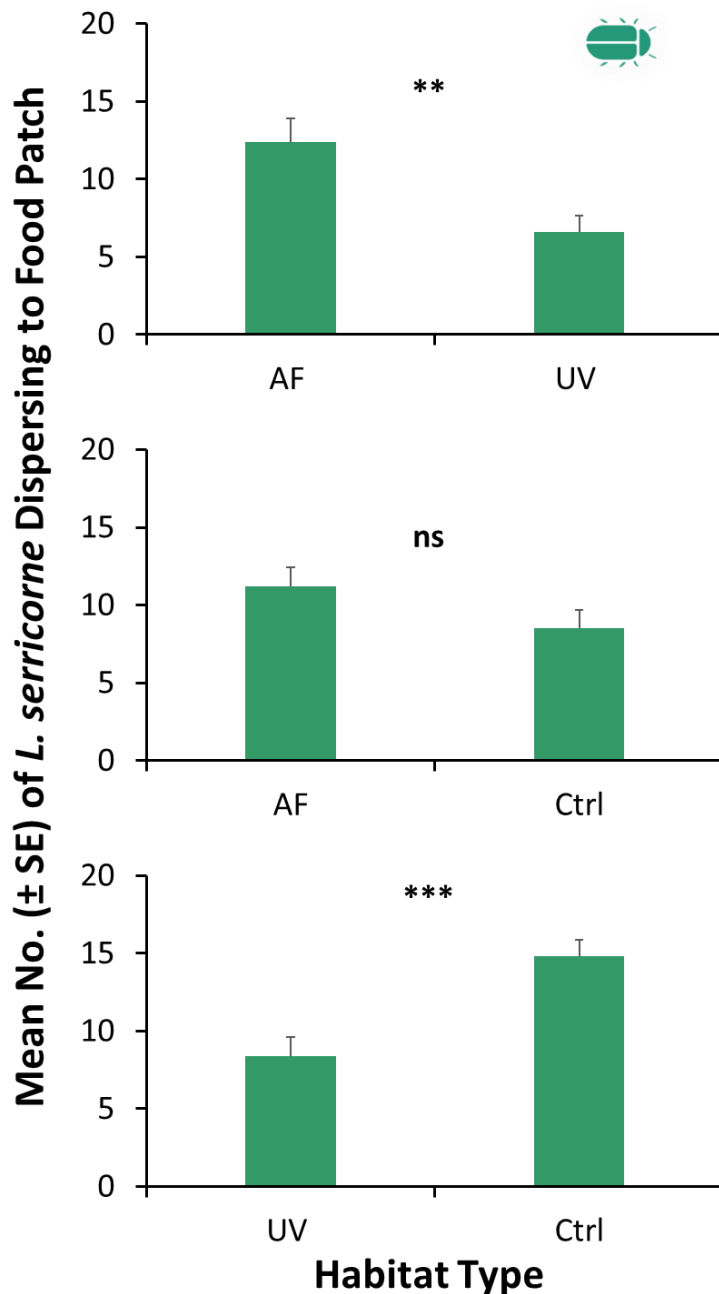


Figure 5-3. The mean number of *Sitophilus oryzae* adults dispersing to a novel food resource after 72 h in environmental chambers at 30°C and 65% RH in pairwise comparisons among food patches with 20 g of *Aspergillus flavus*-inoculated (AF), unmanipulated wheat (Ctrl, as positive control), or autoclaved and surface sterilized wheat (UV). Each bar represents the mean of 10 replicates, with 30 adults released in each replicate. for treatment combination. Abbreviations: ns – not significant (with a Bonferroni-corrected α -threshold using a t-test), ** - $0.01 < P < 0.001$, * - $P < 0.0001$.**

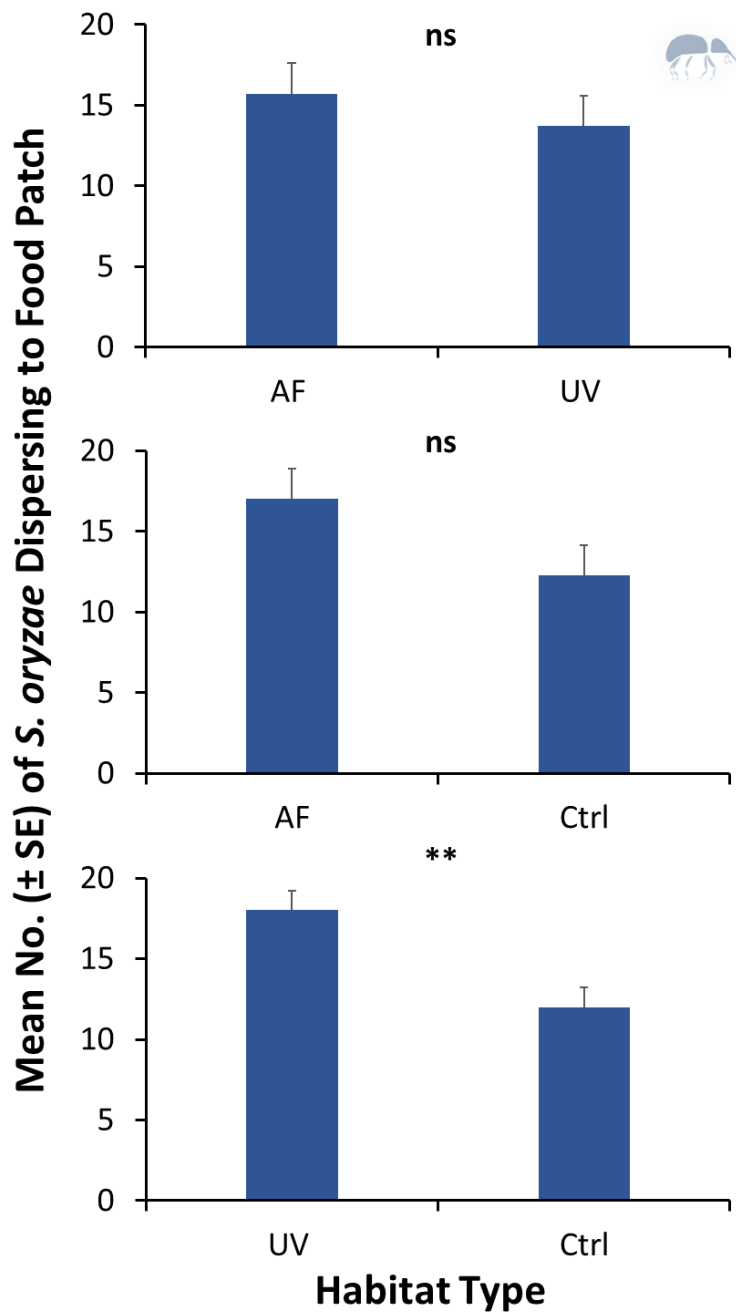


Figure 5-4. The mean progeny production by *Lasioderma serricorne* adults that dispersed to each novel food patch after 60 d of incubation at 30°C and 65% RH. The novel food patches consisted of 20 g of grain inoculated with *Aspergillus flavus*—(AF), unmanipulated wheat (Ctrl—as positive control), or autoclaved and surface sterilized wheat (UV). Each bar represents the mean of 10 replicates, with 30 adults released in each replicate. for treatment combination. Abbreviations: ns – not significant (with a Bonferroni-corrected α -threshold using a t-test), ** - $0.01 < P < 0.001$, *** - $P < 0.0001$.

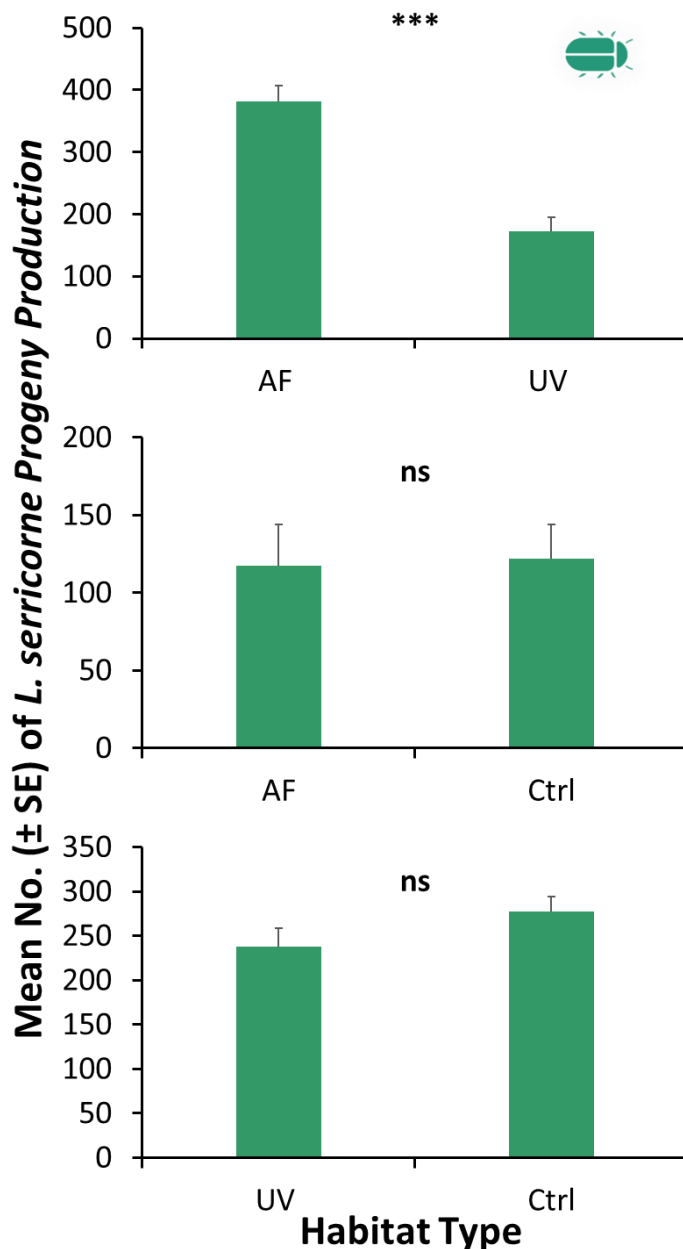


Figure 5-5. The mean progeny production by *Sitophilus oryzae* adults that dispersed to each novel food patch after 60 d of incubation at 30°C and 65% RH. The novel food patches consisted of 20 g of grain inoculated with *Aspergillus flavus*—(AF), unmanipulated wheat (Ctrl—as positive control), or autoclaved and surface sterilized wheat (UV). Each bar represents the mean of 10 replicates, with 30 adults released in each replicate. for treatment combination. Abbreviations: ns – not significant (with a Bonferroni-corrected α -threshold using a t-test), ** - $0.01 < P < 0.001$, *** - $P < 0.0001$.

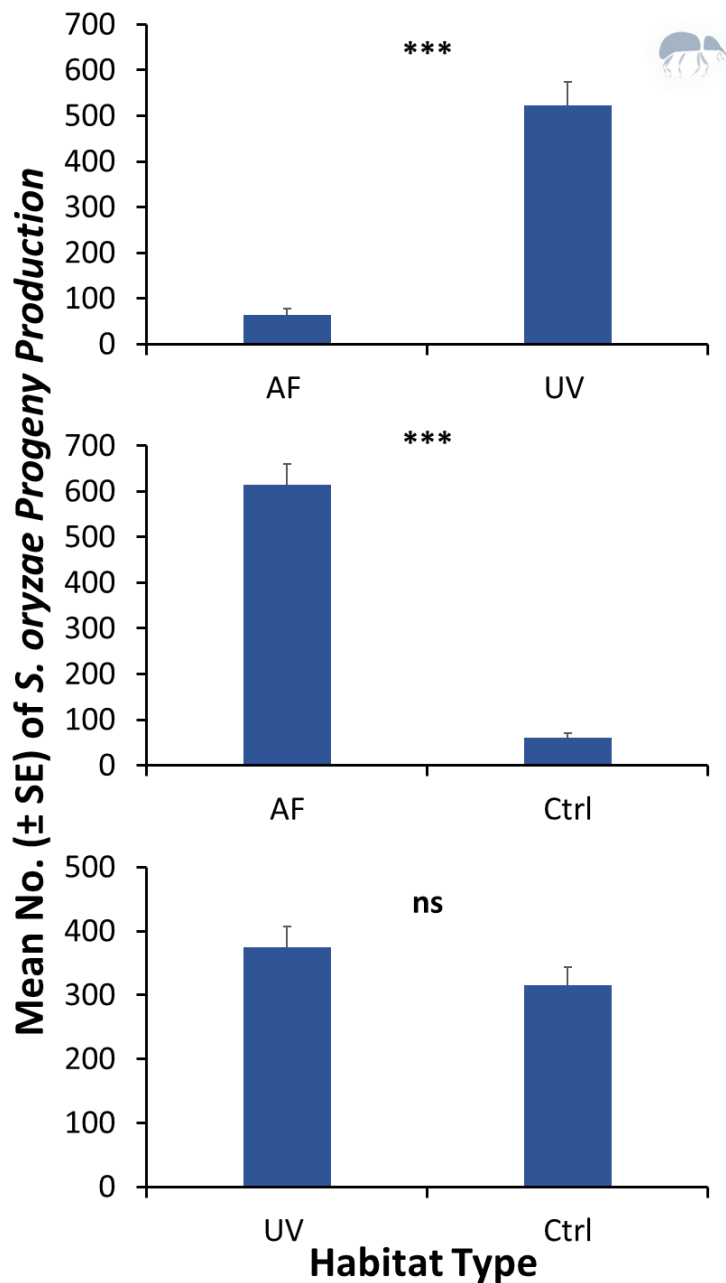


Figure 5-6. Mean body length ($\pm$ SE, mm) of progeny produced after 60 d by *Sitophilus oryzae* that dispersed to habitats inoculated with *Aspergillus flavus* (AF), and food patches with unmanipulated wheat (Ctrl) after an initial 72 h period at 30C and 65% RH in an environmental chamber. Bars represent the means of 200 randomly selected adults from each habitat, and bars with shared letters are not significantly different from each other (t-test, $\alpha = 0.05$). A habitus image above bar shows size difference between adult *S. oryzae* in a side-by-side comparison.

