ABSTRACT

Title of Dissertation:	APPLYING INSECT ECOLOGY AND BEHAVIOR TO IMPROVE SUSTAINABLE PEST MANAGEMENT FOR *DROSPHILA SUZUKII*
	Margaret Theresa Lewis, Doctor of Philosophy, 2021
Dissertation directed by:	Dr. Kelly Hamby, Associate Professor, Department of Entomology

The introduction of the invasive vinegar fly *Drosophila suzukii* (spotted-wing drosophila) to the continental United States substantially disrupted integrated pest management (IPM) in fall-bearing caneberries (raspberries and blackberries). Prior to *D. suzukii*'s introduction, the caneberry pest complex was primarily composed of plant pathogenic fungi and fruit rot pathogens, with few interventions needed to control insect damage. However, tolerance for *D. suzukii* larvae in fruit is low, and a lack of management options has necessitated calendar-based insecticide applications, significantly increasing pesticide usage. As part of a larger effort to restore IPM in caneberries, my dissertation aims to advance our knowledge of *D. suzukii*'s ecology towards more sustainable pest management. Part of this work includes evaluating insecticide spray coverage on diversified fruit farms, with the overarching objective of improving spray coverage in the regions of the caneberry canopy that have the highest

D. suzukii activity levels. Optimizing spray coverage may increase the impact and efficacy of each insecticide application, suppressing *D. suzukii* populations with fewer insecticide applications. Improved spray coverage can also benefit disease management in caneberries. I additionally investigated interactions between *D. suzukii* and fungal microbes; both yeasts and hyphal fungi interact with *D. suzukii* throughout its life history, representing weak points that may be exploited for pest management. I tested how different species of yeast impact fitness and feeding behavior in larval *D. suzukii* and evaluated potential vectoring associations between *D. suzukii* and fruit rot fungi. Although further work is needed to fully understand *D. suzukii*'s patterns of microbial resource use, these studies demonstrate that interactions between *D. suzukii* and fungal microbes have the potential to alter both insect and pathogen pressure. Advancing our understanding of these interactions may facilitate the development of new pest management tactics. For example, yeasts could be used to develop species-specific insecticidal baits or lures for monitoring. Likewise, an epidemiological link between *D. suzukii* and fruit rot fungi would indicate that improved control of *D. suzukii* also provides benefits for pathogen management.

APPLYING INSECT ECOLOGY AND BEHAVIOR TO IMPROVE SUSTAINABLE PEST MANAGEMENT FOR *DROSOPHILA SUZUKII*

by

Margaret Theresa Lewis

Dissertation submitted to the Faculty of the Graduate School of the
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Preface

This dissertation is composed in part of previously published work, included here as Chapters 1 and 3 with the recommendation of the dissertation director, dissertation committee members and department graduate director. The citation for these publications are as follows:

Lewis, M. T. and K. A. Hamby. 2020. Optimizing caneberry spray coverage for *Drosophila suzukii* (Diptera: Drosophilidae) management on diversified fruit farms. *Journal of Economic Entomology.* 113: 2820-2831

Lewis, M. T. and K. A. Hamby. 2019. Differential impacts of yeast on feeding behavior and development in larval *Drosophila suzukii* (Diptera: Drosophilidae). *Scientific Reports.* 9: 13370

As directed in the graduate catalog, I state that I was responsible for the inception of the manuscript and the majority of manuscript preparation. Both publications were reformatted to meet university guidelines. Minor typographical errors were corrected and supplementary material was placed in appendices, but otherwise, these publications have been exactly reproduced. They are cited as appropriate throughout the dissertation. Please refer to Appendix D for a signed letter stating that these chapters were included with the approval of the dissertation director, dissertation committee members, and the graduate director.

Dedication

To my mother, Robine Lewis.

Acknowledgements

This work would not have been completed without the help of many individuals. First, I would like to thank my advisor, Kelly Hamby for her continuous support, guidance, and encouragement throughout this entire process. I would not be the scientist that I am today without her mentorship. I also would like to thank my committee members, Galen Dively, Kathryne Everts, Megan Fritz, and Raymond St. Leger, for the helpful guidance, advice, and feedback they provided as I developed these projects. I also thank my collaborator, Mengjun Hu, for his help designing and implementing the studies in the second chapter of this dissertation.

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Finally, to my family and my husband, thank you for the all the love, all the laughs, and all the unconditional support you have given me throughout the years. I wouldn't have made it without you.

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Introduction

The introduction of the invasive pestiferous vinegar fly, *Drosophila suzukii* Matsumura (spotted-wing drosophila), to the continental United States in 2008 significantly disrupted integrated pest management (IPM) in soft-skinned fruit production. Unique among *Drosophila* species, female *D. suzukii* have a serrated ovipositor that enables them to cut through the intact skin of ripening fruit to lay eggs. Subsequent larval feeding creates soft sunken areas within fruit, and both adult and larval feeding may introduce fungal pathogens, causing highly visible crop damage (Walsh et al., 2011). Primocane (fall-fruiting) caneberries (raspberry and blackberries) often bear the brunt of this damage. In addition to exhibiting a strong preference for caneberry crops (Bellamy et al., 2013), *D. suzukii* populations progressively build up over the course of a growing season so that their peak populations overlap with fall fruit (Hamby et al., 2014). Tolerance for *D. suzukii* damage is low, and detection of a single larva in a raspberry punnet can cause an entire shipment of fruit to be rejected (Farnsworth et al., 2017). Therefore, *D. suzukii* has dramatically changed caneberry pest management, and growers are often forced to rely on calendar applications of broad-spectrum insecticides for consistent control of *D. suzukii* (Digiacomo et al., 2019; Farnsworth et al., 2017; Goodhue et al., 2011; Van Timmeren & Isaacs, 2013).

Management options for *D. suzukii* remain limited, necessitating insecticide-based programs. However, recent research efforts have focused on advancing various cultural control tactics that include exclusion netting, habitat manipulation, and harvest management (Leach et al., 2016; Leach & Isaacs, 2018; Schöneberg et al., 2020). Many of these tactics show promise, but additional work is needed for widespread implementation

(Schöneberg et al., 2021). Likewise, promising biological control agents for *D. suzukii* have been identified (Biondi et al., 2021; Wang et al., 2018; Wang et al., 2021), and are currently being studied in controlled quarantine facilities for approval for release. Complementing these recent advances in *D. suzukii* management, interactions with fungal microbes provide another opportunity for developing new management tactics. Indeed, *D. suzukii* interacts with a diverse fungal community that includes single celled fungi that reproduce by budding (yeasts) as well as fungi with hyphal growth, with each interaction presenting a weak point in their life history that may potentially be exploited.

Like many species of *Drosophila*, *D. suzukii* has developed a close association with naturally occurring yeasts. Yeast microbes are widespread within fruit and do not typically cause pre-harvest fruit rot symptoms. Instead, some species of yeast restrict hyphal fungal growth and have been explored as biocontrol agents for fruit rot (Benbow & Sugar, 1999; Janisiewicz et al., 2010). Many frugivorous insects, including *Drosophila* spp. feed directly on yeasts. Yeast microbes provide insects with important dietary nutrients, including protein, lipids, and sterols that are otherwise absent in fruit, a carbohydrate rich resource (Fanson & Taylor, 2012; Hardin et al., 2015; Madden et al., 2018; Nasir & Noda, 2003). Indeed, previous surveys of *D. suzukii*'s yeast associations have found that adults and larvae feed on a distinct yeast community that is predominated by one species, *Hanseniaspora uvarum* (Hamby et al., 2012; Lewis et al., 2019). These yeast associations positively impact many aspects of *D. suzukii*'s fitness, including adult longevity, adult survivorship, and fecundity (Mori et al., 2016; Tochen et al., 2016). Yeast fermentation volatiles are also highly attractive to *D. suzukii* and can be used to capture adults (Burrack et al. 2015; Hamby et al. 2014; Iglesias et al. 2014). However, these traps lack specificity

and also attract a number of non-pestiferous *Drosophila* in addition to *D. suzukii,* limiting their commercial utility. Expanding our understanding of *D. suzukii*'s yeast associations may facilitate the development of more selective attractants as well as other novel management tools (Hamby & Becher, 2016).

In addition to yeast, *D. suzukii* may also interact with plant pathogenic fungi. Both Botrytis and Cladosporium fruit rots are important pests in mid-Atlantic raspberry production. *Botrytis cinerea*, or grey mold, is a widespread necrotrophic fungal pathogen that infects over 200 host plants, including raspberries (Elad et al., 2007; Williamson et al., 2007). In raspberries, *B. cinerea* causes fruit tissue collapse, degradation, and foliar necrosis (Ellis et al. 1991); post-harvest losses due to *B. cinerea* can be as high as 85% when fruit is stored at room temperature for 8 days (Ellis et al., 2008). In contrast, *C. cladosporioides* is a biotrophic fungus that primarily grows at the raspberry fruit surface (Martin et al., 2017), leaving the interior tissues largely undisturbed. Cladosporium fruit rot is currently considered a minor, post-harvest pathogen of raspberries. However, recent sampling in the mid-Atlantic red raspberries have reported pre-harvest *Cladosporium* infection rates as high as 32% (Swett et al. 2019), suggesting its prevalence has been underestimated.

Contrasting yeasts, *D. suzukii*'s interactions with hyphal fungi may be antagonistic. Volatile odorants commonly associated with *B. cinerea* deter oviposition in multiple frugivorous insects, including *D. suzukii*, the light brown apple moth (*Epiphyas postvittana*), and the grape berry moth (*Lobesia botrana*) (Cha et al., 2019; Chin et al., 2018; Rizvi & Raman, 2017; Rizvi & Raman, 2016; Tasin et al., 2018; Wallingford et al., 2017), and recent studies have leveraged this behavior to develop repellants as part of a

push-pull system (Wallingford et al., 2017, 2018). Despite this antagonism, larval *D. suzukii* still associate with both *Botrytis* and *Cladosporium* at a low rate. Both fungal genera were isolated from the frass of field-collected larvae, indicating feeding. In concurrent surveys of raspberry fruit, we also observed low rates of co-occurrence between larvae and fungi (Lewis et al. 2019).

Dissertation Overview

Together, this body of literature provides evidence for tripartite interactions between *Drosophila suzukii,* caneberry fruit, and fungi (a category that includes both yeasts and fungi with hyphal growth). These interactions may simultaneously influence insect damage and disease incidence within caneberry production, and advancing our understanding of them will be critical for developing economic and ecologically sustainable integrated pest management programs. In this dissertation, my primary objectives are to advance our understanding of these tripartite interactions and leverage these interactions for more sustainable pest management. Each chapter approaches this question from a unique perspective, considering different aspects of *D. suzukii*'s interactions with fungi and raspberries and addressing both applied and basic ecological research questions.

First, I evaluate how different species of naturally occurring yeast impact fitness and feeding behavior in larval *D. suzukii*. This work builds off a rich body of literature on *D. suzukii*'s yeast associations and provides further evidence for a close association between *D. suzukii* and *Hanseniaspora uvarum*. Understanding and characterizing these yeast interactions has important implications for sustainable pest management; for

example, facilitating targeted management tactics such as *D. suzukii* – specific lures or insecticidal baits.

Second, I study *D. suzukii*'s interactions with hyphal fungi, specifically assessing the potential for adult flies to alter disease incidence by acting as a vector. To determine if the fungal associations that we observed in larvae extend to adult flies, I surveyed adult fungal associations over a three-year period, isolating and identifying fungi from field-collected flies. Additional laboratory vectoring assays were conducted to test whether it is feasible for adult flies to transmit fungi to new substrates. Together, the results from this study provide evidence for a potential vectoring association that, if persisting into the adult life stage, adds to existing evidence that integrating insect and pathogen management tactics may improve overall pest control within raspberry production.

Finally, I evaluate how spray coverage affects *D. suzukii* management on diversified fruit farms, to guide the development of IPM programs that minimize pesticide use. I conducted laboratory bioassays to correlate spray coverage with management of *D. suzukii*, demonstrating that it is an important factor for insecticide efficacy. Through field spray coverage trials, I found that the dense foliage exhibited by caneberry plants can block pesticide dispersion to the interior and lower regions of the plant canopy, a trend that is problematic given that adult flies tend to have the highest activity levels and egg laying rates in those regions. Given the results of these field spray coverage trials, future research should focus on developing strategies to improve interior canopy coverage, which will benefit both *D. suzukii* and pathogen management.

Chapter One: Differential Impacts of Yeast on Feeding Behavior and Fitness in Larval *Drosophila suzukii* (Diptera: Drosophilidae)

Abstract

Larval *Drosophila* encounter and feed on a diverse microbial community within fruit. In particular, free-living yeast microbes provide a source of dietary protein critical for development. However, successional changes to the fruit microbial community may alter host quality through impacts on relative protein content or yeast community composition. For many species of *Drosophila*, fitness benefits from yeast feeding vary between individual yeast species, indicating differences in yeast nutritional quality. To better understand these associations, we evaluated how five species of yeast impacted feeding preference and development in larval *Drosophila suzukii.* Larvae exhibited a strong attraction to the yeast *Hanseniaspora uvarum* in pairwise yeast feeding assays. However, larvae also performed most poorly on diets containing *H. uvarum*, a mismatch in preference and performance that suggests differences in yeast nutritional quality are not the primary factor driving larval feeding behavior. Together, these results demonstrate that yeast plays a critical role in *D. suzukii*'s ecology and that larvae may have developed specific yeast associations. Further inquiry, including systematic comparisons of *Drosophila* larval yeast associations more broadly, will be necessary to understand patterns of microbial resource use in larvae of *D. suzukii* and other frugivorous species.

Introduction

Microorganisms can play a critical role in the nutritional ecology of insects and other animals (Douglas, 2009). Obligate symbionts, including bacteria and fungi that colonize the insect digestive tract, aid in the detoxification and digestion of phloem, wood,

and other low-nutrient plant materials (Douglas, 2009; Hammer & Bowers, 2015; Hansen & Moran, 2014; A. C. Wong et al., 2014). Some gut microbial symbionts also help their host synthesize essential amino acids (Shigenobu et al., 2000), vitamins (Douglas, 2017; Salem et al., 2014), or sterols (Bos et al., 1976; Nasir & Noda, 2003; Noda & Koizumi, 2003) otherwise lacking in the insect's diet. In addition to obligate gut symbionts, insects can compensate for nutritional deficiencies within their food by supplementing their diets with free-living microbes, including bacteria, fungi, or yeast.

This latter category of nutritional interactions is particularly well documented within the genus *Drosophila* (Diptera: Drosophilidae). For many frugivorous species of *Drosophila*, yeasts provide a source of dietary protein otherwise absent from ripening fruit, a carbohydrate-rich resource (Hardin et al., 2015). While these carbohydrates are important for many aspects of adult *Drosophila* fitness, including their life span, fecundity, and survivorship (K. P. Lee, 2015; Rendon et al., 2019; Tochen et al., 2016), yeast-associated protein also plays a critical role in fitness, particularly during the larval life stage. In general, *Drosophila* larvae exhibit lower survivorship in yeast-free or low yeast substrates (Becher et al., 2012; Bellutti et al., 2017; Bing et al., 2017), and increasing the ratio of dietary protein to carbohydrates within the larval diet improves survivorship, reduces larval development time, and increases adult body mass (Keebaugh et al., 2018; Silva-Soares et al., 2017; Young et al., 2018). Choice and no-choice behavioral studies suggest that *Drosophila* larvae preferentially feed on protein-rich food sources and will carefully regulate their food intake to consume protein quantities optimal for larval fitness (Silva-Soares et al., 2017).

However, protein abundance within fruit and other fermenting larval substrates change over time, impacting nutritional quality for *Drosophila* larvae. During fermentation, the yeast microbial community undergoes a series of successional changes in both its species composition and density (Morais et al., 1995). In particular, the protein to carbohydrate (P:C) ratio increases as fermentation progresses (Matavelli et al., 2015; Silva-Soares et al., 2017). This microbial succession is frequently mirrored by sucessional colonization of different *Drosophila* species (Morais et al., 1995), because individual *Drosophila* vary in their nutritional requirements (Matavelli et al., 2015). Expanding our understand of the nutritional ecology of different *Drosophila* species may provide insight into larval resource partitioning and will also contribute to our knowledge of *Drosophila suzukii* Matsumura, a close relative of the model organism *Drosophila melanogaster* Meigen and a major agricultural pest in small fruit crops.

Drosophila suzukii is an invasive fruit fly that occupies a unique ecological niche among frugivorous *Drosophila*. Unlike other species, female *D. suzukii* possess a serrated ovipositor that enables them to lay eggs in ripening fruit (Walsh et al., 2011) during the early stages of fermentation. In contrast, most other frugivorous *Drosophila* species wait until fruit is decaying to deposit eggs. Consequentially, *D. suzukii* larvae develop under relatively protein-poor and carbohydrate-rich conditions, a nutritional niche that corresponds with larval performance in laboratory development assays. When reared on intermediate protein diets (e.g. 1:2 or 1:4 P:C ratio), larval *D. suzukii* exhibit faster development times, larger adult body sizes, and higher female ovariole numbers relative to *Drosophila biarmipes* Malloch (Silva-Soares et al., 2017), a close relative of *D. suzukii* that colonizes decaying fruit. Furthermore, diets too rich in microbiota may have

deleterious effects on larval *D. suzukii* fitness. The median lifespan of amicrobial *D. suzukii* reared on nutrient-rich sucrose-yeast diets (71 days) decreased when their natural microbiota was present (47 days). The presence of microbiota also decreased adult body size by 0.32 mg (female) and 0.11 mg (male), while slightly increasing the development period from 11.94 to 12.19 days (Bing et al., 2017). In contrast, the microbiota/nutrient-rich diet combination does not appear to harm *D. melanogaster*; comparisons between amicrobial larvae and larvae containing their natural microbiota found no differences in larval development time (Storelli et al., 2011). These differences likely reflect adaptations by *D. suzukii* larvae to relatively nutrient-poor ripening fruit.

In addition to differences in yeast density, the composition and relative abundance of individual yeast species within a fruit changes over time. This can further impact fruit habitat suitability, as individual yeast species differentially impact larval fitness and development (Bellutti et al., 2017; Bing et al., 2017). For example, *D. melanogaster* exhibit lower survivorship and smaller adult body mass when reared on diets containing the yeast *Metschnikowia pulcherrima*, compared with diets containing either *Saccharomyces cerevisiae, Pichia toletana,* or *Kluyveromyces lactis* (Anagnostou et al., 2010). Different quantities of heat-killed yeasts are needed, depending on species, to support development in larval *D. melanogaster,* suggesting that yeasts vary in their nutritional quality (Keebaugh et al., 2018). Indeed, the concentration and composition of key nutrients such as lipids, amino acids, mannoproteins, and fatty acids differ between yeast species (Freeland & Gale, 1947; Lavrynenko et al., 2015; Sitepu et al., 2013). In addition to variably impacting larval development, it is possible that these nutritional differences influence larval feeding behavior.

Larval *Drosophila* often exhibit distinct yeast feeding preferences (Anagnostou, Dorsch, et al., 2010; Cooper, 1960; Fogleman et al., 1981; Hoang et al., 2015; Lindsay, 1958), though the level of selectivity can vary between species based on their host substrate. *Drosophila* that have a restricted host range tend to exhibit less selective feeding behavior. For example, larvae of the specialist cactophilic *Drosophila nigrospiracula, Drosophila mettleri,* and *Drosophila pachea* feed on yeast at the same frequency as yeast species occur within the larval substrate (Fogleman et al., 1982). This behavior may indicate that larvae with a restricted host range cannot afford to evolve specialized microbe feeding behaviors, as microbial communities are ephemeral and often vary between conspecific host substrates (Fogleman et al., 1982). In contrast, generalist *Drosophila* such as *Drosophila mojavensis* or *D. melanogaster* exhibit distinctive yeast feeding preferences in both field and laboratory settings (Fogleman et al., 1981; 1982).

This selective foraging behvior may reflect perceived differences in yeast resource quality. The larval chemosensory system contains an array of gustatory and olfactory neural receptors (Joseph & Carlson, 2015) that allow larvae to discriminate between food sources based on nutritional factors such as the identity and availability of sugars and amino acids (Apostolopoulou et al., 2015; Bjordal et al., 2014; Rohwedder et al., 2012). Therefore, larval *Drosophila* may selectively feed on yeasts that best support their fitness, with specific *Drosophila* – yeast associations dependent on the fruit microbial community and stage of fruit decay typically encountered.

Previous field surveys indicate that *D. suzukii* larvae feed upon a distinct yeast fauna, with one species of yeast, *Hanseniaspora uvarum*, predominating in the gut (Hamby et al., 2012; Lewis et al., 2019). *Hanseniaspora uvarum* is a widespread yeast species that

occurs at high frequency in the early stages of fruit fermentation (Cadez & Smith, 2011; Morais et al., 1995) and can be antagonistic to other species of fungi, including yeast (Liu et al., 2010). Therefore, larval feeding patterns may reflect *H. uvarum*'s abundant field density; alternatively, these patterns may indicate feeding preferences.

To better understand the nature of these interactions, we evaluated larval *D. suzukii* feeding preference and performance in response to diets prepared using five different species of yeast, including the model organism *S. cerevisiae* and natural yeast associates of larval *D. suzukii* (Lewis et al., 2019). We hypothesized that larvae would exhibit a significant preference for the yeast that best supported their fitness. While larvae did exhibit a strong preference for *H. uvarum* in laboratory preference assays, this preference negatively correlated with performance. Our results suggest larval *D. suzukii* yeast feeding preferences may be driven by factors beyond nutritional quality.

Materials and Methods

Flies and Yeast

A laboratory reared colony of *D. suzukii* was established using adults and larvae collected from raspberry fields (Germantown and Woodbine, MD, USA) as well as adults trapped in a residential riparian area (Beltsville, MD, USA) in 2014. Flies were reared for over 50 generations under a 16:8 hour light/dark cycle at 22°C on a modified Bloomington Drosophila Stock Center cornmeal, molassess, and yeast medium (consisting of 84.4% v/v water, 9.6% v/v cornmeal, 5.5% w/v yeast, 4.6% v/v molasses, 0.5% w/v agar, 0.5% w/v proprionic acid, and 0.01% w/v methyl 4-hydroxybenzoate). Our colony recipe contains a higher concentration of yeast compared to the Bloomington recipe and uses different

antifungals (proprionic acid and methyl 4-hydroxybenzoate instead of p-hydroxybenzoic acid methyl ester). However, all other ingredients and ratios were similar (Appendix A, Table S1). The colony was infected with an unknown insect pathogen, which presented symptoms similar to *Drosophila* C Virus (Ashburner et al., 2005); infected larvae typically exited the food at an early instar and developed a brownish-black coloration before dying. To minimize effects from this infection, fly bottles were carefully inspected prior to experiment, with flies only taken from bottles that did not exhibit active symptoms. Because development studies were completed using amicrobial larvae, we anticipate no confounding effects due to this infection.

Experiments were conducted using five different species of yeast. Four of those species, *Hanseniaspora uvarum*, *Pichia kluyveri*, *Issatchenkia terricola*, and *Wickerhamomyces pijperi*, were isolated from the fecal pools (frass) of field-collected *D. suzukii* larvae (Lewis et al., 2019) with individual yeast species selected based on the strength of their association with *D. suzukii*. In particular, *H. uvarum*, *P. kluyveri*, and *I. terricola*, were isolated from multiple populations of *D. suzukii* in both Maryland and California (Hamby et al., 2012; Lewis et al., 2019). A strain of *Saccharomyces cerevisiae* obtained from Red Star® Active Dry Yeast (LeSaffre Yeast Corporation, Milwaukee, WI, USA) was also included in laboratory assays as a positive control.

H. uvarum, *P. kluyveri*, *I. terricola*, and *S. cerevisiae* were used in all experiments described below, but *W. pijperi* was only included in the yeast preference assays. This species of yeast was only found in one field site in Maryland, but occured in 4 out of 12 larvae surveyed (Lewis et al., 2019). Given its strong prevelance at this single field site, we assayed larval yeast preference for *W. pijperi*. However, we excluded *W. pijperi* from

the larval development assays due to labor constraints; larvae did not show a strong preference for that yeast and *W. pijperi* is not commonly associated with *Drosophila* spp. (Chandler et al., 2012; Hamby et al., 2012).

Yeast Impacts on Larval Growth and Development

To evaluate yeast impacts on larval fitness and development, *D. suzukii* larvae were reared on the same colony diet previously describe using a standardized quantity of one of four yeast species: *H. uvarum, P. kluyveri, I. terricola*, and *S. cerevisiae* (Appendix A, Table S1). As a negative control, diets were also prepared with no yeast added. All diets were steam sterilized using an autoclave at 121°C for 20 minutes prior to use in experiments; this step killed all microbes and ensured that yeast quantity remained constant throughout the experiment. Approximately 18 grams of diet were poured into small (60 x 15 mm) petri dishes and cooled overnight in a sterile biosafety cabinet.

After the overnight cooling period, 20 amicrobial first-instar larvae were then added to each petri dish (Appendix A, Supplemental Methods). This created a density of 1.1 larvae g^{-1} diet, which is slightly below the threshold at which larval *D. suzukii* begin exhibiting significant competition effects (Hardin et al., 2015). The entire experiment was repeated on three separate dates (N=3 replicates). During each experimental replicate, we prepared six subsamples per treatment (six petri dishes of diet containing 20 *D. suzukii* larvae each). *D. suzukii* larvae were monitored daily, and we quantified suvivorship, development time, and adult body size (wing and thorax measurements) as measures of larval fitness.

Diet plates with larvae were held in a 22°C incubator on a 16:8 hour light/dark cycle and checked daily at approximately the same time that each experimental replicate was

initiated (generally between 10 AM and 12 PM). Any pupae that emerged were transferred from the diet into an individual 1.5 mL microcentrifuge tube and then monitored daily for adult emergence. Transferring the pupa into individual tubes ensured that the adult flies did not become stuck within the diet, thus allowing us to measure adult body size. Once emerged, adult flies were held in their tubes for 24 hours to harden before being frozen and stored for future body size measurements.

To quantify adult body size, we measured the wing and thorax length for every adult *D. suzukii* that successfully emerged in our trials, using a Leica M80 microscope with an optical reticle attached to the eyepiece. Measurements were adapted from previously described methods (Robertson & Reeve, 1952; Shearer et al., 2016). Briefly, to take thorax measurements, fine-tipped forceps were used to grasp each fly at the base of their legs, and the fly was oriented so its thorax was horizontal. Measurements were taken from the most anterior part of the mesothorax to the tip of the scutellum (Figure 1-1a). Once thorax measurements were complete, the right wing was removed from the specimen and slide mounted. Two measurements were taken to quantify wing length: from the origin of the 4th longitudinal vein to the posterior cross vein (L1; Figure 1-1b) and from the posterior cross vein to the intersection of the wing edge and the 4th longitudinal vein (L2; Figure 1-1b). To minimize measurement biases and errors, all flies within a given replicate were measured by the same individual.

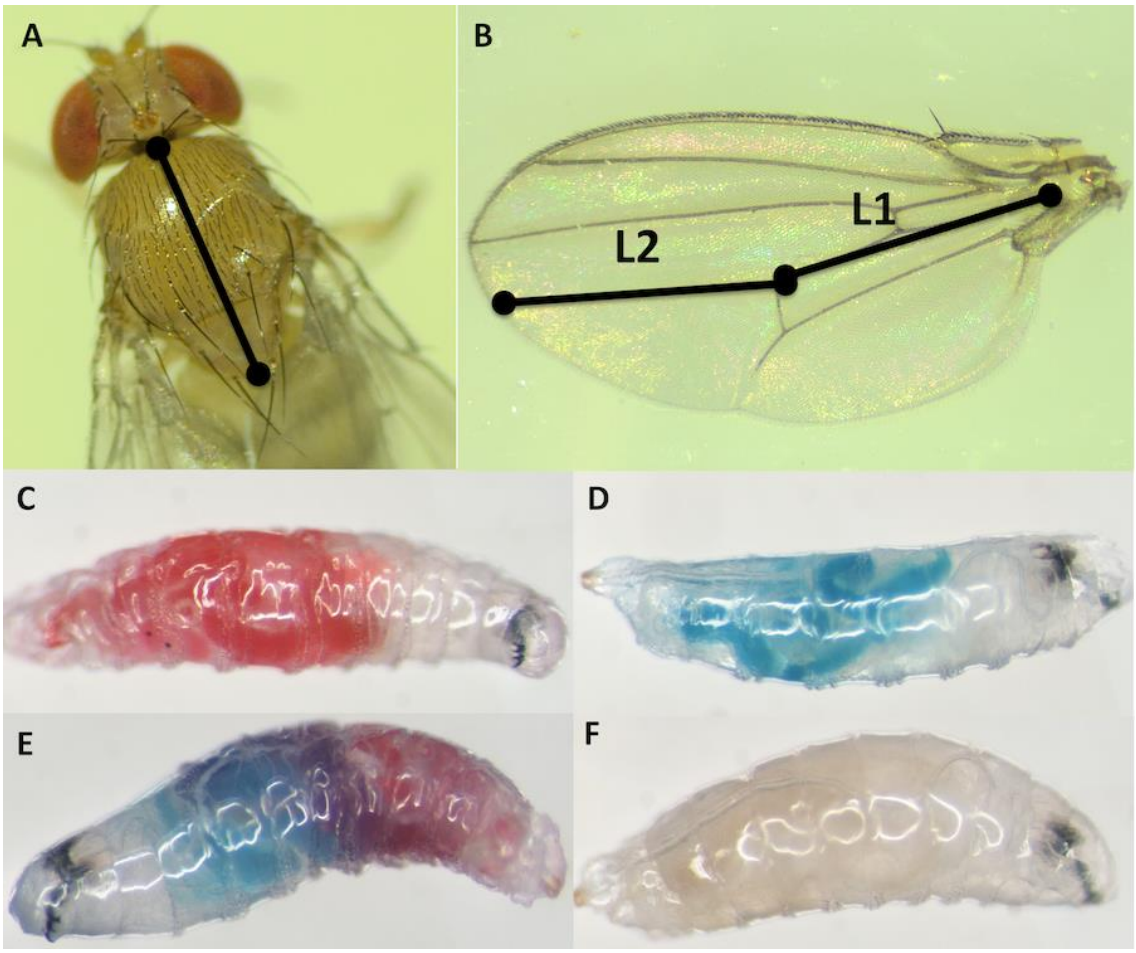

Figure 1-1. Methods for assessing larval development and yeast preferences. (A) Thorax length measurements were taken from the most anterior part of the mesothorax to the tip of the scutellum using a Leica M-80 microscope with an occular retical attached. (B) To quantify wing length, one wing was removed from each adult specimen and mounted on a glass slide. Two measurements were taken on each wing: L1 = the distance between the origin of the 4th longitudinal vein to the posterior cross vein; L2 = the distance between posterior cross vein to the intersection of the wing edge and the longitudinal fourth vein. In larval yeast preference assay, larvae were scored as either (C) red, (D) blue, (E) purple, or (F) white.

Statistical Analysis.

All statistical analysis were conducted using R.3.4.1 (R Core Team, 2017). Data were averaged across subsamples within an individual trial (N = 3 replicate trials). Survivorship rates were calculated as the percentage of larvae that sucessfully pupated (larval survivorship), the percentage of pupae that sucessfully emerged as adults (pupal survivorship), and the percentage of larvae that sucessfully emerged as adults (total

survivorship). For each category, data were analyzed using a linear mixed model, with the percent survivorship as the response variable, yeast treatment included as a categorical predictor, and replicate included as a random effect. Model residuals were checked for the assumptions of normality of variance and homogeneity of variance using Shapiro-Wilk and Levenes Tests. In all three analysis, assumptions were satisfied using untransformed data. Significant results were followed by pairwise mean comparisons using Tukey's adjustment in the lsmeans package (Lenth, 2016).

Development time to pupation (larval development), time from pupation to adult eclosion (pupal development), and total development (1^{st} instar larva to adult) were analyzed separately using a mixed-model ANOVA in the lme4 (Bates et al., 2015) and lmerTest (Kuznetsova et al., 2016) packages in R, with yeast treatment included as a main effect and trial replicate as a random effect. We confirmed data met the assumption of normality of variance with Shapiro-Wilk tests. However, weighted least squares methods [weighting factor: $(\text{treatment residual variance})^{-1}$] were used for larval and total development times due to difficulties satisfying the assumption of homogeneous variance. No weighting factor was required for analysis of pupal development time.

Body size measurements were also analyzed using a mixed-model ANOVA using the lme4 and lmerTest packages and models again included the yeast treatment as a fixed effect and trial replicate as a random effect. Data were transformed as necessary to meet assumptions of normality and heterogeneity of variance. Due to difficulties satisfying assumptions of normality and heterogeneity of variances, the analyses were conducted separately for male and female flies, with female wing and thorax measurements analyzed using weighted least squares [weighting factor: $(\text{treatment residual variance})^{-1}$]. Significant

results were again followed by pairwise mean comparisons using Tukey's adjustment in the lsmeans package (Lenth, 2016).

Quantifying Diet Quality

To compare the nutritional content of our experimental diets with the standard laboratory rearing diet, we conducted proximate nutrient analysis on each experimental diet as well as a diet prepared using freeze-dried *S. cerevisiae*. On two separate dates, diets were prepared using the same protocol described previously. After autoclaving, all diets were poured into sterile 50 mL falcon tubes, refrigerated, and shipped to an off-site facility for analysis within three days of preparation. All analyses were conducted by Medallion Labs (General Mills D.B.A. Medallion Labs, Minneapolis, MN). Analysis were conducted 28 November 2018 and 9 January 2019 using standard testing protocols (Appendix A, Supplemental Methods).

Evaluating Larval Yeast Preference

Using binary choice feeding assays, we evaluated larval *D. suzukii*'s preference for five species of yeast previously desribed. Bioassay arenas were constructed following methods adapted from previous larval *Drosophila* feeding assays (Hoang et al., 2015). Briefly, in each experimental replicate, larvae were presented with two yeast species stained red and blue with food coloring. After one hour, larvae were removed and visually scored for yeast feeding preferences.

Second-instar *D. suzukii* larvae were starved for one hour prior to starting the assay (see Supplemental Methods in Appendix A). Forty larvae were then transferred to the center of one assay arena using an ethanol sterilized paintbrush and left in dark conditions

for one hour, during which time they were free to crawl around and feed on either yeast option. At the end of the hour assay period, larvae were individually removed from the arena, and scored for feeding preference using a Leica M80 stereomicroscope based on the color of their abdomen (Figure 1-1). Each larva could be classified as either red, blue, purple (indicating that they fed on both yeasts), or white (indicating that no choice was made).

Statistical Analysis

Any larvae that died or went missing during the hour-long assay period were excluded from the analysis. Prior to analysis, the number of larvae that chose to feed on each yeast option within an assay arena were standardized using a preference index described in Hoang et al. (2015) :

$$(1)\ \text{Larvae with colored (red or blue) abdomen} + \frac{\text{Larvae with purple abdomen}}{2}$$

Adjusted larval counts were analyzed using a paired t-test (R Core Team, 2017), with each assay arena of 40 larvae treated as an experimental replicate. Data were graphically checked for outliers using both box plots and Q-Q normality plots, and the assumption that the sampling distribution of mean differences was normally distributed was assessed using Anderson Darling test for normality in R with the 'nortest' package (Gross & Ligges, 2015). Data is reported as the percentage of larvae that chose feed on each yeast.

Preference Assay Controls

To ensure that food coloring did not impact larval performance, we alternated which color each yeast option was stained between replicates. Additionally, a series of control preference assays was also conducted, in which larvae were presented with the

same species of yeast in both food colors. Food coloring did not impact larval preference for any of the yeasts assayed (Appendix A, Table S2).

To confirm that our visual assessments of larval feeding matched their actual feeding behavior, we also performed a set of separate confirmation assays and sequence identified the gut microbial community for a subset of experimental larvae (Appendix A, Supplemental Methods); results indicated that larval yeast feeding corresponded with the color of their abdomen, with few exceptions (Appendix A, Table S3).

Results

Larval Development Assays

We evaluated how three species of yeasts isolated from field collected *D. suzukii* larvae (*H. uvarum, Pichia kluyveri,* and *Issatchenkia terricola*) and diets without yeast (negative control) impacted fitness and development in larval *D. suzukii* (Appendix A, Table S1). As a positive control, we also prepared diets using commerical *Sacchromyces cerevisiae*, because this species is frequently used as a model organism to study *Drosophila* – yeast interactions. To ensure the diet microbial community remained static and to remove confounding effects due to yeast growth rate, development assays were conducted with standardized amounts of frozen yeast and diets were autoclave sterilized to heat-kill all microbes. Diet treatments were monitored daily for both pupation and adult eclosion, and emergence data were used to calculate larval (1^{st} instar larvae to pupa) survivorship and development time, pupal (pupa to adult) survivorship and development time, and total (1^{st} instar larvae to adult) survivorship and development time. Thorax and wing length measurements were also taken to quantify the body size of any emerged adults. Diets were

prepared on three separate occasions (N=3) with 6 dishes per treatment for which subsamples were averaged prior to analysis.

Survivorship.

Individual yeast species significantly affected larval survivorship (1st instar larvae to pupa; $F_{3,6} = 6.688$, $P = 0.024$). Larvae reared on a yeast-free diet (negative control) exhibited 0% survivorship across all replicates (Figure 1-2), indicating that yeast is essential for *D. suzukii* development.

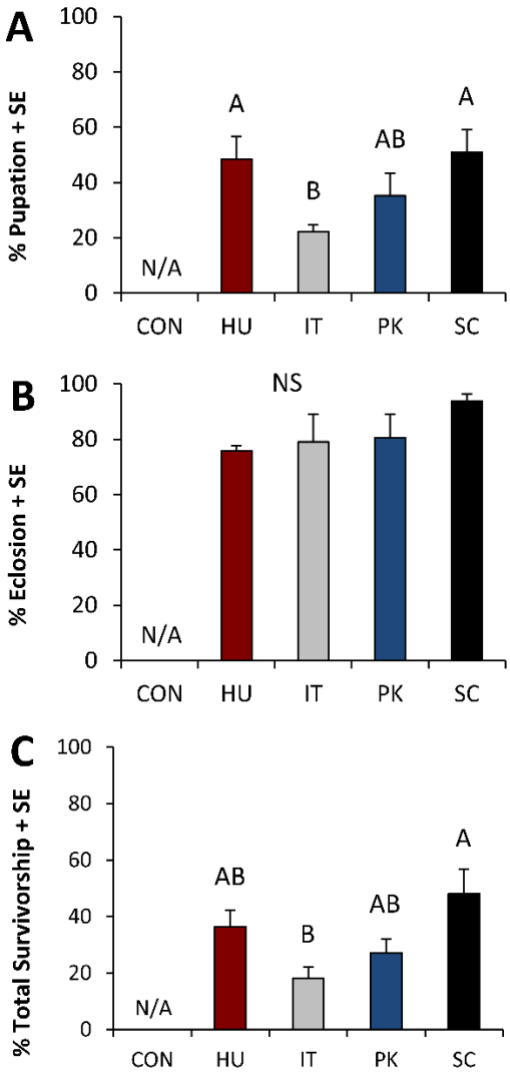

Figure 1-2. *D. suzukii* survivorship in response to experimental diets. Larvae were reared on diets containing either no yeast (CON), *Hanseniaspora uvarum* (HU), *Issatchenkia terricola* (IT), *Pichia kluyveri* (PK), or *Saccharomyces cerevisiae* (SC). Mean percent survivorship + standard error of (A) larvae that pupated (larval survivorship), (B) pupa that eclosed as adults (pupal survivorship) and (C) larvae that successfully eclosed as adults (total survivorship) (N=3 replicate experiments) are presented. Data were analyzed using a linear mixed model. Yeast species significantly impacted the larval ($F_{3,6}$ = 6.688, P = 0.024) and total ($F_{3,6}$ = 6.466, P = 0.026) survivorship but not pupal survivorship ($F_{3,6}$ = 1.857, P = 0.238). Within a graph, bars that do not share a letter are significantly different ($p < 0.05$) Control larvae were excluded from all analysis due to 0% survival.

Apart from the yeast-free control, the lowest rates of larval survivorship occurred on diets containing *I. terricola* (22.2 ± 2.5%; mean ± standard error), while there was higher larval survivorship on either *S. cerevisiae* (50.8 ± 8.2%) or *H. uvarum* (48.3 ± 8.4%)

(Figure 1-2a). Total survivorship patterns were similar (Figure 1-2c; $F_{3,6}$ = 6.466, P = 0.026), with the highest percentage of larvae sucessfully emerging as adults in response to either *S. cerevisiae* (48.1 ± 8.7%) or *H. uvarum* (36.4 ± 5.8%). In contrast, pupal survivorship (pupa to adult; $F_{3,6}$ = 1.857, P = 0.2377) was not impacted by diet treatments.

Development Time.

The fastest larval development period occurred on diets containing *S. cerevisiae* (1st instar larvae to pupa; $F_{3,8}$ = 13.418, P = 0.002), with larvae taking 11.7 ± 0.6 days to pupate. Larvae reared on *H. uvarum* took 14.3 ± 0.2 days to reach the pupal stage, and the slowest larval development times occurred in response to either *I. terricola* or *P. kluyveri* (Figure 1-3a, Supplementary Figure S1a). Patterns in the total development times were similar (Figure 1-3c; $F_{3,8}$ = 12.799, P = 0.002), with the fastest larval to adult development times again occuring in response to *S. cerevisiae* (see also Appendix B, Figure S1b). We observed no significant differences in pupal development time among treatments ($F_{3,6}$= 3.369, P = 0.096; Figure 1-3b).

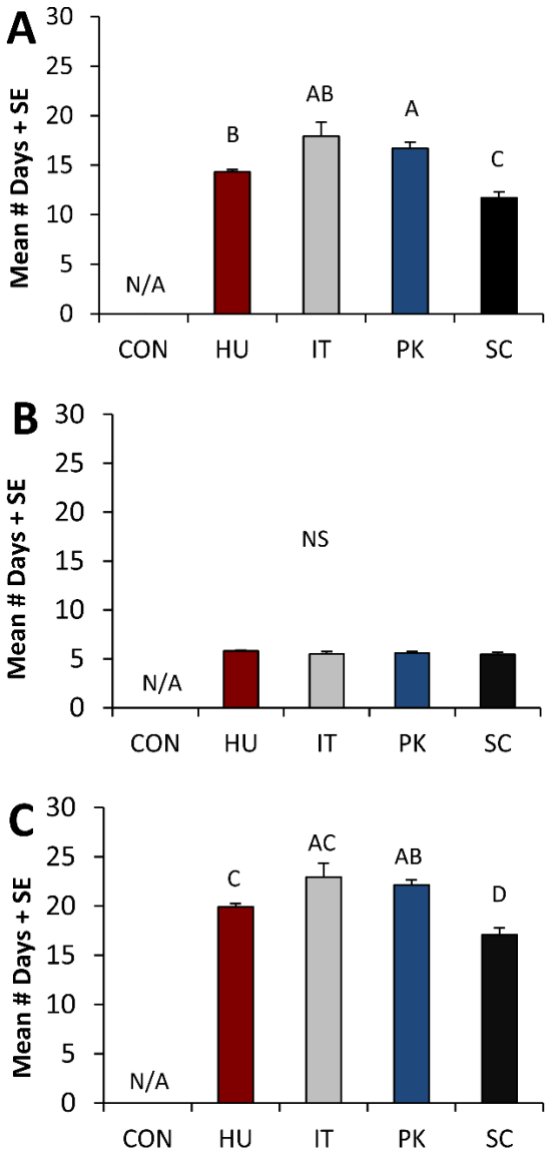

Figure 1-3. *D. suzukii* development period in response to experimental diets. Larvae were reared on diets containing either no yeast (CON), *Hanseniaspora uvarum* (HU), *Issatchenkia terricola* (IT), *Pichia kluyveri* (PK), or *Saccharomyces cerevisiae* (SC). Mean number of days for development + standard error for (A) larval development (1st instar to pupa), (B) pupal development (pupa to adult) and (C) total development (1st instar to adult) (N=3 replicate experiments) are presented. Larval and adult development data were analyzed using a mixed-model ANOVA with a (treatment residual variance)$^{-1}$ weighting factor. Pupal development times were analyzed without a weighting factor. Bars that share a letter were not significantly different (P>0.05).

Adult Body Size

The individual yeast diets significantly affected thorax length in both male ($F_{3,6}$ = 9.880, P = 0.010) and female ($F_{3,8}$ = 20.467, P < 0.001) *D. suzukii* (Table 1-1). The largest thorax lengths were observed in male (1.08 ± 0.01 mm) and female (1.24 ± 0.01 mm) flies reared on *S. cerevisiae*. In contrast, the smallest flies observed were those reared on a *H. uvarum* diet. Flies reared on either an *I. terricola* or a *P. kluyveri* based diet also exhibited a reduced body size relative to *S. cerevisiae* and were slightly larger than those observed from *H. uvarum*. Similar patterns emerged in the wing length of both male ($F_{3,6}$ = 6.983, P = 0.022) and female ($F_{3,8}$ = 17.462, P < 0.001) *D. suzukii*, with the largest wings occuring in flies reared on *S. cerevisiae* and the smallest wings in flies reared on *H. uvarum* (Table 1-1).

Table 1-1. Thorax and wing lengths of male and female *D. suzukii* reared on experimental diets. Wing and thorax length measurements were taken for adult *D. suzukii* that successfully emerged; therefore, the total number of flies measured within a given treatment varied. These subsamples were averaged by sex within each trial prior to analysis, and the mean wing length (millimeters) ± standard error (N=3) is presented. Statistical analyses were conducted separately for male and female flies and for wing length and thorax length. Within a given sex and measurement, values that do not share a letter are statistically different (P < 0.05).

Sex	Treatment	Total # Flies Measured	Average Wing Length (mm) ± SE	Average Thorax Length (mm) ± SE
Female	*H. uvarum*	105	2.14 ± 0.04 C	1.09 ± 0.02 C
	I. terricola	39	2.26 ± 0.01 B	1.17 ± 0.01 B
	P. kluyveri	61	2.21 ± 0.08 ABC	1.14 ± 0.05 ABC
	S. cerevisiae	100	2.35 ± 0.01 A	1.24 ± 0.01 A
Male	*H. uvarum*	24	1.83 ± 0.05 b	0.90 ± 0.03 b
	I. terricola	28	1.99 ± 0.02 ab	1.00 ± 0.01 ab
	P. kluyveri	36	1.96 ± 0.07 ab	1.00 ± 0.03 ab
	S. cerevisiae	72	2.06 ± 0.01 a	1.08 ± 0.01 a

Diet Nutritional Analysis.

To compare the nutritional content between our experimental diets and the standard *Drosophila* diet used to maintain our laboratory stocks, we conducted proximate nutritional analysis on all diets used in this study and a standard diet prepared using freeze-dried *S. cerevisiae* (Appendix A, Table S1). Nutrient analyses were repeated twice, using diets prepared on two separate dates.

We observed no major nutritional differences between any of our experimental diets, which were all prepared using yeast cells scraped from media plates. However, diets prepared without yeast (negative control) consistently had the lowest caloric content and fell below the detectable protein threshold in both replicates. In contrast, diets prepared using freeze-dried *S. cerevisiae* had higher caloric values (52.5 ± 0.5 calories per 100 grams diets; N=2 replicates) relative to any other treatment. For example, diets prepared using wet *H. uvarum* cultures had the second highest caloric value at 43.0 ± 1.0 calories per 100 grams. We also observed higher relative amounts of ash (inorganic residue), carbohydrates, and protein within diets prepared using freeze-dried yeast cultures (Appendix A, Table S4).

Larval Yeast Preference

We evaluated larval *D. suzukii* feeding preference for five species of yeast (*H. uvarum, P. kluyveri, I. terricola, W. pijperi,* and *S. cerevisiae*) through two-choice feeding assays. In each assay, larvae were placed on a large water-agar plate provisioned with two yeast options (colored red or blue) on opposite ends of the plate, and larval feeding preference were assessed after one hour based on the color of the alimentary canal. For each set of two-choice tests, we conducted 12 replicate assays.

Overall, *D. suzukii* larvae preferred *H. uvarum* (T_{11} = 7.214, P < 0.001) and *W. pijperi* (T_{11} = 2.286, P = 0.043) over *S. cerevisiae* (Figure 1-4). For example, 54.0% ± 2.3% (mean ± standard error) of the larvae assayed chose to feed on *H. uvarum*, compared with the 28.8% ± 2.3% that chose to feed on *S. cerevisiae*. Similarly, in comparisons between *W. pijperi* and *S. cerevisiae*, 46.0% ± 4.3% and 28.7% ± 3.6% of larvae assayed chose to feed on each yeast respectively (Figure 1-4). However, larvae exhibited no significant feeding preferences in pairwise comparisons between *S. cerevisiae* and either *P. kluyveri* or *I. terricola*. Larvae also demonstrated no significant feeding preferences in pairwise comparisons of *I. terricola*, *P. kluyveri*, and *W. pijperi* (Appendix B, Supplementary Tables S5-S7).

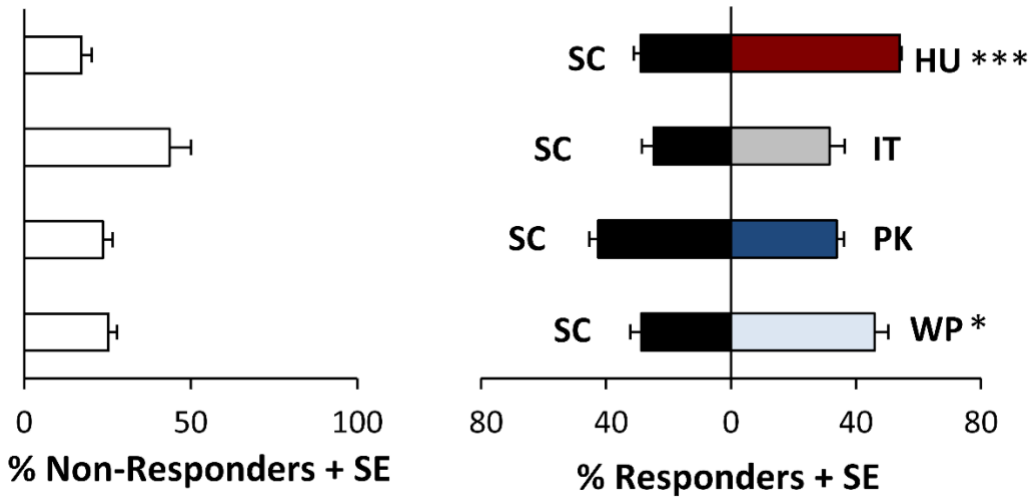

Figure 1-4. Larval *D. suzukii* feeding preferences for all comparisons involving *S. cerevisiae*. Mean percentage of larvae + standard error that responded to each yeast paired with the mean percentage of larvae + standard error that did not respond (N=12 replicate binary choice assays) are presented for HU = *Hanseniaspora uvarum*, IT = *Issatchenkia terricola*, PK = *Pichia kluyveri*, SC = *Saccharomyces cerevisiae*, WP = *Wickerhamomyces pijperi*. Responding larvae were analyzed using a paired t-test, with larvae demostrating significant preference for one yeast over another at the * = P<0.05, ** = P<0.01, or *** = P<0.001 level.

Across all binary comparisons of larval feeding preference, *H. uvarum* elicited the strongest feeding response in *D. suzukii* (Fig. 1-5). In addition to demonstrating significant

preferences for *H. uvarum* over *S. cerevisiae,* significantly more larvae chose to feed on *H. uvarum* over *P. kluyveri* (T_{11} = 7.468, P < 0.001), *I. terricola* (T_{11} = 8.601, P < 0.001), and *W. pijperi* (T_{11} = 3.042, P = 0.011).

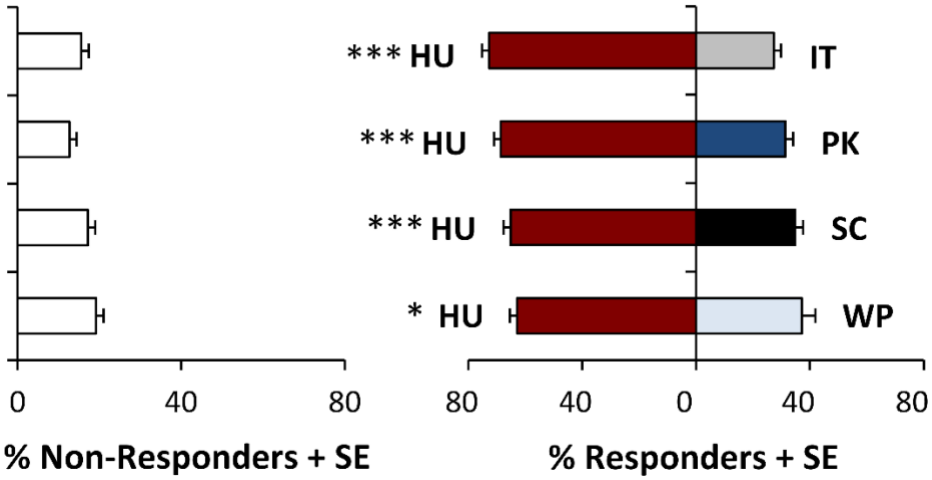

Figure 1-5. Larval *D. suzukii* feeding preferences for all comparisons involving *H. uvarum*. Mean percentage of larvae + standard error that responded to each yeast paired with the mean percentage of larvae + standard error that did not respond (N=12 replicate binary choice assays) are presented for HU = *Hanseniaspora uvarum,* IT = *Issatchenkia terricola*, PK = *Pichia kluyveri*, SC = *Saccharomyces cerevisiae,* WP = *Wickerhamomyces pijperi*. Responding larvae were analyzed using a paired t-test, with larvae demonstrating significant preference for one yeast over another at the * = P<0.05, ** = P<0.01, or *** = P<0.001 level.

Discussion

D. suzukii encounters a diverse microbial community within fruit that undergoes successional changes in both density and species composition (Duarte et al., 2009; Fleet, 2003; Morais et al., 1995), allowing them to selectively feed on a wide variety of yeast microbes that play a critical role in their life history. Larvae exhibit a strong attraction to live yeast cultures, and yeasts are important components of *D. suzukii*'s diet. Similar to previous work (Bellutti et al., 2017; Bing et al., 2017), we found that larvae reared in a completely yeast-free environment universally failed to pupate or eclose. Our ability to rescue larval development with heat-killed microbes confirms that yeasts provide *D. suzukii* larvae with a source of protein and essential nutrients not otherwise found in fruit

or fly diets (Bos et al., 1976; Fanson & Taylor, 2012; Skorupa et al., 2008). We initially hypothesized that larvae would preferentially feed on certain species of yeast based on perceived differences in resource quality. However, the mismatch between larval yeast preference and performance suggests that larvae do not discriminate between yeast species based on nutritional quality alone and instead there may be alternative mechanisms shaping *D. suzukii*'s yeast associations.

Results from this study suggest that *D. suzukii* larvae have developed a close association with *H. uvarum*. In binary laboratory choice assays, *D. suzukii* larvae preferentially fed on *H. uvarum* over alternative natural yeast associates as well as *S. cerevisiae.* This result is consistent with previous reports that *H. uvarum* predominates the culturable larval gut microbial community in geographically distant populations of *D. suzukii* (Hamby et al., 2012; Lewis et al., 2019) and to the best of our knowledge, is the first evidence that larval *D. suzukii* exhibit feeding preferences for specific yeast species. Our studies suggest that larval feeding is not random; despite being confined to a single fruit throughout development, larval *D. suzukii* appear to deliberately seek out and feed on *H. uvarum,* indicating that there may be an association between these two organisms.

We initially hypothesized that *D. suzukii* preferentially fed on *H. uvarum* because it was a higher quality yeast that better supported larvae. Instead, larvae exhibited reduced performance on diets containing *H. uvarum*. Larvae reared on *H. uvarum* developed more slowly relative to diets containing *S. cerevisiae,* and adult body size was smaller compared to individuals reared on diets prepared with *S. cerevisiae, P. kluyveri,* or *I. terricola*. Surprisingly, the most robust fitness occurred on diets containing *S. cerevisiae,* with higher rates of surviorship and shorter developmental times relative to flies reared on diets

containing natural yeast associates. Flies reared on *S. cerevisiae* based diets also had significantly larger adult body sizes, a trait that generally indicates higher levels of fecundity, survival, and mating success (Kingsolver & Huey, 2008). This higher performance on *S. cerevisiae* was surprising, because *Drosophila* rarely associate with this species of yeast in nature (Chandler et al., 2012; Hamby et al., 2012; Lewis et al., 2019) and larvae did not prefer *S. cerevisiae* in binary choice assays. It is possible that these results reflect phenotypic plasticity in resource use, with larvae able to exploit and perform well on diverse yeast resources despite specialization towards *H. uvarum* (Richards et al., 2006). Similar plastic behavior may occur in adult *D. suzukii* during winter and early spring when ripe fruit is scarce in temperate climates; under no-choice laboratory conditions female *D. suzukii* will accept and oviposit into less optimal resources including mushroom, apple, and chicken-manure based diets (Stockton et al., 2019). The enhanced performance we observed could also result from laboratory colony selection effects. All flies used in this study came from a *D. suzukii* colony reared for over 50 generations on a standard *S. cerevisiae* based diet. Alternatively, the benefits conferred from *S. cerevisiae* may reflect commercial selection effects, because the particular strain of yeast used in this study was originally selected for making bread, which could impact protein content and secondary metabolite production. Since *D. suzukii* larvae do not frequently encounter *S. cerevisiae* in nature, they may not have recognized this particular yeast as a superior food source.

This mismatch in larval yeast preference and performance suggests that *D. suzukii* larvae do not discriminate between individual species of yeast based solely on the nutritional quality of the yeast. Yeast quantity, rather than quality, may be a more important determinant of larval fitness. In this study, *D. suzukii* larvae developed under protein-

limited conditions. We prepared our fly diets following the standard recipe used to maintain our laboratory *Drosophila* stocks, changing only the species of yeast added. Instead of using dehydrated yeast, we harvested and weighed all our yeast directly from PDA plates, a step that likely reduced the nutritional value relative to the original, freeze-dried yeast recipe (Appendix A, Table S2). The protein content in our freeze-dried yeast diet averaged 2.42%, similar to protein concentrations (1.11% and 2.0%) in other published *Drosophila* diets (Bing et al., 2017; Hardin et al., 2015). In contrast, average protein concentration in our experimental diets ranged from 0.781% - 1.24%. Negative fitness impacts due to the limited protein conditions within our diets were likely compounded by the use of heat-killed microbes. By steam sterilizing our diets to avoid differences due to variable yeast growth rates (Williams et al., 2015) and contamination by other microbes, we stopped microbial growth. This may have created yeast shortages typically not observed in the field where natural yeast growth or larval niche construction and yeast seeding would increase yeast abundance over time (Stamps et al., 2012; Witzgall et al., 2012).

Yeast shortages and the associated low dietary protein may have detrimentally impacted larval fitness, especially prolonging development. In this study, the fastest development time occurred in larvae reared on *S. cerevisiae,* with first instar larva to pupa development taking an average of 11.7 ± 0.6 days and first instar larva to adult devleopment taking an average of 16.9 ± 0.8 days. In contrast, other studies have observed that *D. suzukii* development times on *S. cerevisiae*-based fly diets takes 6.0 – 7.1 days for pupation (Bellutti et al., 2017; Emiljanowicz et al., 2014) and 11.9 – 12.8 days for adult emergence (Bing et al., 2017; Emiljanowicz et al., 2014) at temperature levels comparable to our study conditions (Bellutti et al., 2017; Bing et al., 2017; Emiljanowicz et al., 2014). Beyond

development time, low protein conditions can negatively impact other aspects of larval fitness, including survivorship and adult body size (Keebaugh et al., 2018; Silva-Soares et al., 2017; Young et al., 2018). Within carbohydrate-rich ripening fruit (Hardin et al., 2015), larval *D. suzukii* likely rely on the yeast microbial community to obtain sufficient protein for development.

Given the importance of dietary protein for development (Bing et al., 2017), *D. suzukii* larvae may prioritize feeding on yeasts that are abundant and readily available over selectively seeking higher quality species. For example, *D. suzukii* larvae may preferentially feed on *H. uvarum* because it predominates yeast microbial communities during the early stages of fermentation, thus providing a more abundant source of protein. Previous studies have demonstrated that *D. suzukii* larvae provisioned with live cultures of *H. uvarum* generally experience a robust fitness phenotype relative to other yeast associates (Bellutti et al., 2017; Bing et al., 2017). The higher performance observed in these studies likely reflects a higher yeast abundance when using live cultures, because *Drosophila* spp. larval feeding increases yeast abundance within their host substrates in field (Hamby et al., 2012) and laboratory experiments (Stamps et al., 2012), and live yeasts are able to continually grow during development assays. Microbial abundance postively correlates with larval growth rates in *D. melanogaster*, suggesting that a microbe's ability to proliferate may be one of the most important predictors of its effect on larval fitness (Keebaugh et al., 2018). Therefore, *D. suzukii* larvae may benefit from *H. uvarum*'s widespread and competitive nature (Cadez & Smith, 2011; Morais et al., 1995), as it means that *H. uvarum* can quickly increase its density, providing larvae with a consistent and abundant source of protein.

Beyond its ability to proliferate, there are a number of other factors that could mediate larval attraction to *H. uvarum*. For example, *H. uvarum*'s attractiveness may reflect yeast adaptations that enhance its fitness. Adult *Drosophila* disperse yeasts (Buser et al., 2014; Lam & Howell, 2015), and more attractive yeast strains experience higher rates of dispersal (Christiaens et al., 2014). Larval feeding may also confer competitive advantages to yeast by promoting yeast growth or genetic diversity (Reuter et al., 2007; Stamps et al., 2012; Witzgall et al., 2012). Alternatively, some strains of *H. uvarum* produce "killer toxins" that may help larvae outcompete harmful plant pathogenic fungi (Magliani et al., 1997) or create an enemy-free space (Thompson, 1988), consequentially enhancing larval fitness through measures not quantified in this study. A similar competitive advantage has been proposed for *D. melanogaster,* with larvae parasitized by the wasp *Asobara tabida* preferentially feeding on yeast species that enhance their ability to melanotically encapsulate parasitic attacks (Anagnostou et al., 2010). It is also possible that *H. uvarum* confers additional fitness benefits during the adult life stage not quantified in this study such as adult survivorship (Grangeteau et al., 2018), adult cuticular pheromone production (Grangeteau et al., 2018), or reproductive outputs such as ovariole numbers (Silva-Soares et al., 2017).

Hanseniaspora uvarum is a widespread yeast species frequently isolated from fermenting fruits and insects (Cadez & Smith, 2011), including adult *Drosophila*. A survey of *Drosophila* spp. yeast associations found that with a few exceptions, the *H. uvarum* species complex was the most abundant OTU isolated from the gut of adult flies (Chandler et al., 2012), suggesting a general feeding association between *Drosophila* and *H. uvarum*. Volatiles associated with *H. uvarum* are also highly attractive to multiple adult species,

including *D. suzukii* and *D. melanogaster* (Batista et al., 2017; Scheidler et al., 2015). While further work is necessary to fully understand the mechanism and nature of *H. uvarum*'s association with *D. suzukii,* it is clear that *H. uvarum* strongly impacts *D. suzukii*'s ecology, similar to other *Drosophila*.

The extent to which adult yeast associations overlap with the larval life-stage remains unclear. Adult flies are highly mobile insects, capable of visiting a diverse community of host plants, which provides them a different, broader feeding niche than larvae (Morais et al., 1994). Field and laboratory surveys of cactophilic *Drosophila* yeast associations report differences between adult and larval yeast preferences (Cooper, 1960). For example, in laboratory assays, female *Drosophila buzzati* exhibited a significant preference for ovipositing and feeding on cactus innoculated with *Pichia cactophila* relative to *Clavispora opuntiae* (Vacek et al., 1985), while larvae exhibited high attraction to both yeast species (Barker et al., 1988). In addition, surveys of adult feeding behavior on decaying oranges found that *Drosophila* spp., including *D. melanogaster* and *D. pseudoobscura*, fed more frequently on yeasts available at the surface of necrotic tissue compared to yeasts colonizing the interior fruit rot, suggesting a spatial separation between adult and larval feeding niches (Vacek et al., 1979).

Yeast associations and preferences have been fairly well surveyed within adult frugivorous *Drosophila* (Chandler et al., 2012; Gilbert, 1980; Morais et al., 1992; Palanca et al., 2013; Quan & Eisen, 2018). However, records of natural yeast associations within frugivorous larvae are more limited. Previous laboratory studies using *D. melanogaster* and cactophilic *Drosophila* larvae demonstrate that larvae have specific yeast preferences (Anagnostou et al., 2010; Cooper, 1960; Hoang et al., 2015), and these preferences vary

between species. In pairwise yeast preference comparisons, *D. buzzati* and *Drosophila aldrichi* exhibited slight differences in their yeast preferences (Barker et al., 1988). Also, within decaying oranges, *D. arizonensi* and *D. melanogaster* consumed *H. uvarum* at lower frequencies than it occurred in the orange microbial community (Fogleman et al., 1982), a result that suggests larvae were avoiding *H. uvarum,* in contrast to the strong preference for *H. uvarum* we observed in *D. suzukii*. Within fermenting fruit, it therefore seems plausible that different species of *Drosophila* larvae develop different yeast preferences and associations, and that these associations shift across temporal niches within fermenting fruit. For example, *D. suzukii* larvae could develop closer associations with early stage fermentation communities compared to *D. melanogaster* and other late stage colonizers. Systematic comparisons of larval yeast preferences and surveys of larval yeast associations would be needed to test this hypothesis.

Conclusions

Because yeasts play such a critical role in *D. suzukii*'s ecology, there may be opportunities to exploit these interactions for more sustainable pest management (Hamby & Becher, 2016). Yeast associated volatiles could be integrated into monitoring programs for *D. suzukii*. Fermentation based-lures have already been developed, but current trapping systems remain difficult to use due to issues with trap selectivity and poor correlations between adult trap captures and larval infestation (Burrack et al., 2015; Hamby et al., 2014). It may be possible to use yeast volatile components specifically attractive to *D. suzukii* (Scheidler et al., 2015) to develop a more selective trapping system. Similarly, yeast-associated volatiles could also be incorporated into a push-pull system for *D. suzukii* (Wallingford et al., 2017).

Recent research efforts have also focused on incorporating yeasts into feeding baits or biopesticides specific to *D. suzukii*. In laboratory trials, adult and larval *D. suzukii* exhibited reduced fitness after ingesting *S. cerevisiae* that was genetically modified to express double-stranded RNA (Murphy et al., 2016). Yeasts have also been tested as potential phagostimulants for insecticide applications, with variable efficacy. Adding yeast to either spinosad or cyanotraniliprole increased adult mortality and decreased larval infestation compared to treating with the insecticide alone (Knight et al., 2015). However, efficacy varied between yeast species and insecticides, with highest efficacy observed when using *S. cerevisiae* and commercial formulations of the yeast *Aureobasidium pullulans* as phagostimulants (Knight et al., 2015). Similarly, laboratory assays also reported that combinations of spinosad and *H. uvarum* increased *D. suzukii* mortality relative to the insecticide alone (Mori et al., 2016). In contrast to these studies, recent field and laboratory assessments found that adding *S. cerevisiae* to various organic insecticides did not improve control of *D. suzukii* in either semi-field or laboratory assays, a difference that may reflect variation in *D. suzukii*'s physiological status between studies (Roubos et al., 2019) .

There appears to be considerable variation in how *D. suzukii* interacts with yeasts throughout its life history. Both adult and larval *D. suzukii* exhibit specific yeast preferences, and during the adult life stage, different species of *Drosophila* vary in their response to specific yeast volatile components (Scheidler et al., 2015). Furthermore, the physiological status of adult flies can also influence behavioral responses. For example, unmated or reproductively immature females exhibit a higher attraction towards yeast volatiles (Swoboda-Bhattarai et al., 2017; Wong et al., 2018), and winter and summer

morph *D. suzukii* vary in their responses to fungal-associated volatiles (Kirkpatrick et al., 2018). Deepening our understanding of this interspecific and intraspecific variation may provide opportunities to develop more targeted management programs specific to *D. suzukii*.

Chapter Two: Interactions Between *Drosophila suzukii* and Plant Pathogenic Fungi in Fall-Bearing Caneberries

Introduction

Within fruit production systems, plant disease outbreaks cause substantial reductions in fruit quality and yield. Specific damage and loss rates are dependent on myriad factors, including variation in cultivar susceptibility to disease, the density and strain of a particular disease agent, and environmental conditions such as temperature, humidity, light intensity, and plant density (Magarey et al., 2005; Mani et al., 2016). Herbivorous insects can also play an important role in disease epidemiology by acting as a vector (Hatcher, 1995; Heck, 2017). The majority of known insect-vectored plant diseases emerge as the result of specialized relationships between insects and disease agents that lack independent dispersal mechanisms, such as viruses and bacteria (Eigenbrode et al. 2018; Nault 1997). However, numerous surveys have also documented associations between adult frugivorous insects and fungal pathogens, suggesting that insects similarly can influence fungal diseases. For example, viable grey mold (*Botrytis cinerea*) propagules have been isolated from *Drosophila melanogaster*, the Mediterranean fruit fly (*Ceratitis capitata*), and the light brown apple moth (*Epiphyas postvittana*) (Bailey et al., 1997; Rene Engelbrecht & Gustav, 2002; Louis et al., 1996). Other plant pathogenic fungal genera, including *Alternaria* spp., *Penicillium* spp., *Rhizopus* spp., and *Mucor* spp., have also been isolated from *C. capitata* (Engelbrecht et al. 2004). Although common, these insect-fungal interactions are generally considered passive, with insects unintentionally acquiring fungal propagules on their exterior body.

Passive interactions between herbivorous insects and pathogenic fungi may still play an important role in disease epidemiology. Insect feeding and egg laying can create

opportunities for fungal colonization by providing an entry route for epiphytic propagules (Rombaut et al., 2017). For example, infestation by both the raspberry beetle (*Byturus tomentosus*) and the grape berry moth (*Lobesia botrana*) increases incidence of grey mold (*Botrytis cinerea*) in raspberries and grapes respectively (Fermaud and Giboulot 1992; Woodford et al. 2002). Beyond wound facilitation of new infections, insects may also play an important role in fungal disease dispersal (Golan & Pringle, 2017). Light brown apple moth (*Epiphyas postvittana*) larvae are capable of vectoring *B. cinerea* to sterile media under lab conditions (Bailey et al., 1997), while navel orangeworm larvae transmit *Aspergillus flavus* to almonds (Palumbo et al., 2014). Laboratory vectoring assays have demonstrated that the tortoise thistle beetle and two aphid species (*Aphis fabeae* and *Uroleucon cirsii*) act as competent vectors for *Puccina punctiformis*, a rust fungus that infects creeping thistle plants (Kluth et al. 2002). Additionally, loose vectoring relationships likely exist between the rice stink bug and various fungi, including *Alternaria alternata* and *Fusarium oxysporum*. Fungal propagules were isolated from both the stylet and saliva of stink bugs, and field-assessments of both insect and fungal damage to rice kernels positively correlated with stink bug abundance (Lee & Tugwell, 1993). Understanding interactions between herbivorous insects and pathogenic fungi may advance disease management programs, particularly in systems with overlapping periods of heavy insect and disease pressure.

Primocane (fall-bearing) caneberries are vulnerable to pre- and post-harvest damage from both insects and fungal pathogens. In particular, the invasive vinegar fly *Drosophila suzukii* Matsumura (spotted-wing drosophila) has emerged as a primary pest of soft-skinned fruit and is a major contributor to pre-harvest caneberry loss (Farnsworth

et al., 2017; Goodhue et al., 2011). Adult and larval populations generally increase as the growing season progresses (Hamby et al., 2014) so that peak activity usually overlaps with primocane fruit harvest as well as several primary and secondary caneberry pathogens, most notably Botrytis and Cladosporium fruit rot. Botrytis fruit rot (caused by *Botrytis cinerea*) is a widespread disease that infects over 200 host plants, including raspberries and blackberries (Elad et al., 2007; Williamson et al., 2007). In raspberries, post-harvest losses due to *B. cinerea* may be as high as 85% when fruit is stored at room temperature for 8 days (Ellis et al., 2008). Cladosporium fruit rot, caused by the *Cladosporium cladosporioides* species complex (Swett et al. 2019), was traditionally considered a minor, post-harvest pathogen of raspberries. However, pre-harvest *Cladosporium* infection rates as high as 32% have been found in mid-Atlantic raspberries (Swett et al. 2019), suggesting its prevalence has been underestimated historically. More recent sampling efforts in primocane raspberries have also reported pre-harvest anthracnose fruit rot (caused by *Colletotrichum fiorinae*) at rates as high as 40% (Schoeneberg & Hu, 2020). Beyond these pre-harvest pathogens, both raspberries and blackberries are also susceptible to damage caused by a suite a post-harvest fungi, including *Penicillium* spp., *Rhizopus* spp., and *Mucor* spp. (Martins et al. 2017). Previous surveys indicate that *D. suzukii* may interact with at least a subset of this caneberry fungal community.

As larvae, *D. suzukii* co-occur with and feed on fungal pathogens at low rates. Viable *Botrytis*, *Cladosporium*, and *Penicillium* propagules have been isolated from the frass of field-collected larvae that were sampled from apparently healthy raspberries. This suggests that larvae encounter and feed on fungi, and some of the ingested spores survive passage through the digestive tract (Lewis et al., 2019). Additional field scouting efforts

have also found low numbers of raspberries simultaneously infested with *D. suzukii* larvae and infected with visible Botrytis or Cladosporium fruit rot (Lewis et al. 2019). Although the extent to which these tripartite interactions extend to the adult life stage remains unclear, it is plausible that adult flies may interact with and/or impact disease epidemiology.

In this study, our primary objective is to advance our understanding of these epidemiological consequences by assessing adult *D. suzukii*'s role as a potential vector of fruit rot fungi. We first surveyed adult *D. suzukii*'s fungal associations over a three-year period. Repeating these sampling efforts over multiple field sites and time points allowed us to characterize the breadth of *D. suzukii*'s fungal associations and to infer how frequently adult flies encounter and interact with caneberry pathogens. To assess whether *D. suzukii* can transmit these potential fungal associates, we also conducted laboratory vectoring assays; these experiments were conducted under worst-case scenario, no-choice conditions and designed to serve as a proof of concept for vectoring that will justify future field studies. Given the diffuse associations documented between plant pathogenic fungi and other frugivorous insect taxa, we hypothesized that adult *D. suzukii* are carrying and transmitting fungal pathogens at low rates.

Materials and Methods

Survey of Fungal Associations in Adult Drosophila suzukii

We surveyed fungal associations in field-collected adult *D. suzukii* annually from 2018 – 2020 at two field sites, the Western Maryland Research and Education Center in Keedysville, MD and the Wye Research and Education Center in Queenstown, MD (Table

2-1; Figure 2-1). In 2018 and 2019, flies were collected from a mixed raspberry/blackberry planting that was occasionally sprayed with insecticides and fungicides. In 2020, flies at Queenstown were collected from unsprayed, floricane blackberries, while at Keedysville, flies were collected from a large raspberry planting that was also used for a separate insecticide spray trial in which small plots were sprayed with either Delegate WG, a proprietary insecticide at various concentrations, or left unsprayed. No fungicides were applied, and flies were only collected from unsprayed rows at the field margin.

Table 2-1. Number of adult *D. suzukii* sampled for each sampling date at Keedysville, MD and Queenstown, MD field sites 2018 – 2019.

Year	Keedysville		Queenstown		Sampled Annually
2018	12-Sep 8	26-Sep 7	24-Aug 4	18-Sep 6	25
2019	13-Aug 4	N/A	14-Aug 3	N/A	7
2020	2-Sep 10	6-Oct 10	26-Aug 9	23-Sep 10	39
Total SWD Sampled					**71**

On each collection date, adult *D. suzukii* were hand-collected using an ethanol sterilized aspirator and immediately transferred into an individual 1.5 mL microcentrifuge tube containing 1 mL of sterile phosphate buffer solution (PBS; 2018 collections) or 1 mL of PBS with 0.1% Tween 20 (2019 and 2020 collections). Tubes were vigorously shaken to drown the flies and minimize fungal spore degradation. All samples were stored on ice until processing.

It is possible that by using PBS alone in 2018 underestimated the external fungal community. Many filamentous fungi, including various *Aspergillus* spp., *Cladosporium* spp., and *Schizophyllum* spp., produce hydrophobins, a structural protein with at least eight cysteine residues, in the cell wall of airborne hyphae and conidia. Hydrophobins have

diverse functions and often render conidia and other fungal propagules hydrophobic (Sunde & Latge, 2012; Whiteford & Spanu, 2002), potentially reducing the efficiency with which a neutral buffer could extract their propagules. Across years, the total number of unique isolates per fly was similar. For example, we isolated 2.68 strains per fly in 2018 and 2.67 strains per fly in 2020. However adding 0.1% Tween solution to our external rinse in 2020 (Swett et al. 2019) may have helped to disrupt hydrophobic fungal propagules for genera that were not dislodged by a PBS buffer alone, potentially producing a richer representation of the *D. suzukii* – associated fungal community.

Figure 2-1. Approximate location of the field sites used for sampling adult *D. suzukii* in 2018, 2019, and 2020 at (A) Keedysville and (B) Queenstown. Images were captured using Google Earth.

Two types of fungal isolations were performed on each fly collected (Figure 2-2). First, each fly was individually vortexed in PBS (2018) or PBS + 0.1% Tween 20 (2019 and 2020), which dislodged fungal propagules from their cuticle or exterior body surface (exterior fungi). To determine if *D. suzukii* ingests fungal propagules, we then assessed the interior microbial community using methods adapted from Hamby et al. 2012. After the exterior fungal isolation, we surface sterilized the flies. Each fly was transferred into a microcentrifuge tube containing 400 uL of 95% ethanol, vortexed for one minute, and left

soak to for an additional five minutes. Then, the fly was removed and serially transferred into two additional microcentrifuge tubes containing sterile PBS or PBS + 0.1% Tween 20 solution. Surface sterilized flies were finally placed in fresh tubes containing sterile buffer (PBS or PBS + 0.1% Tween 20) solution and homogenized with a sterile pestle. To confirm that all surface-dwelling microbes were killed, the second sterile buffer rinse was plated on potato dextrose agar (PDA); if no microbial growth occurred on the sterile rinse plate, we could assume that the remaining microbe cultures from the homogenized body came from *D. suzukii*'s alimentary canal, indicating feeding (interior fungi). If a fly's sterile rinse was contaminated with any microbial growth, all associated plates (exterior and interior) were discarded.

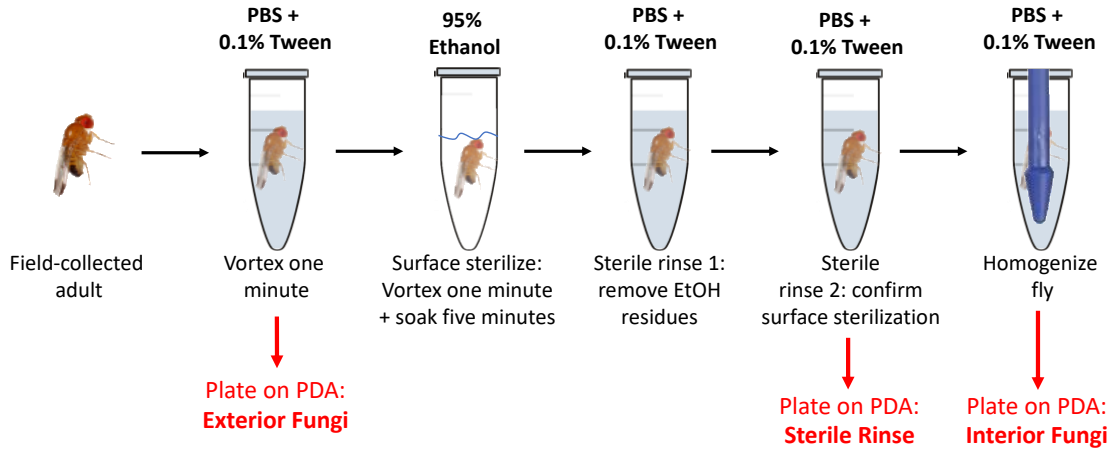

Figure 2-2. Visual summary of methods for interior and exterior fungal isolations.

Two hundred uL serial dilutions (1X, 0.1X, and 0.01X) of both the exterior and interior fungal suspensions were plated on PDA and incubated at room temperature for 1-2 weeks. Plating dilutions helped reduce the loss of isolates due to fungal competition. Each fungal morphospecies was isolated and purified through single hyphal tipping following methods adapted from Swett et al. 2019. Nine of the fungal strains initially isolated in 2018 and 29 of the fungal strains initially isolated in 2020 could not be purified

due to contamination by yeasts or fungal taxa with aggressive growth (e.g., *Aspergillus* spp.) or died before we could successfully perform DNA extraction. In 2019, we also discarded the majority of our fungal strains due to cross-contamination from an unknown mite species, ultimately only identifying fungi from the first sampling period and only using isolates that had successfully been archived prior to the mite contamination. Fungal isolates that were successfully purified were identified to the genus level through polymerase chain reaction (PCR) and sequencing of the internal transcriber (ITS) region of the fungal genome.

In 2018, DNA was extracted from fungal isolates following the methods in Chi et al. 2009. Briefly, 10-20 mg of 3-7 day old hyphal tissue was suspended in an extraction buffer (1M Tris-HCl, 2M KCl, 0.5M EDTA) and homogenized using a sterile pestle. Samples were centrifuged at 5,000 rpm for 10 minutes and the resulting supernatant decanted into 2-propanol. The samples were then centrifuged at 12,000 rpm for 10 minutes, and the resulting supernatant was discarded. The remaining cell lysate was rinsed in 70% molecular grade ethanol and suspended in DEPC treated water. DNA concentration were estimated using a Nanodrop 2000 and adjusted as necessary for subsequent PCR reactions (to a concentration less than 250 nmol/uL). Due to issues consistently obtaining quality DNA through the Chi et al. protocol, DNA extractions for the 2019 and 2020 fungal samples were performed using the Quick-DNA Fungal/Bacterial Miniprep Kit (Zymo Research, Irvine CA) following the manufacturer's instructions.

Twenty five uL polymerase chain reactions were carried out using Promega GoTaq Master Mix (2018) and Apex Red Master Mix (2019 and 2020) and 0.1 uM ITS1/ITS4 primer (White et al., 1990). DNA was amplified using a C1000 Touch Thermocycler

(Biorad Laboratories Inc., Hercules, CA) following the amplification procedures described in White et al. 1990. PCR products were purified using the ExoSap-IT™ PCR Product Cleanup Reagent (Applied Biosystems ™, Foster City, CA) following the manufacturer's instructions. PCR samples were sequenced using Sanger sequencing (Genewiz LLC, South Plainfield, NJ), and DNA chromatograms were examined for quality and trimmed using FinchTV 1.4.0 (Geospiza Inc., Seattle, WA) or 4Peaks (Nucleobytes B.V, The Netherlands). The resulting sequences were compared against the NCBI database with the basal local alignment search tool (BLAST, National Library of Medicine, Bethesda, MD). Using only the ITS region of the fungal genome, we were unable to obtain reliable species level identifications. However, genus level identifications were made using NCBI entries previously published in peer-reviewed journals with > 98% of the nucleotides matching.

Pre- and Post-Harvest Fruit Rot Evaluation

To generate baseline data about the prevalence of primary fungal pathogens within our field site and to understand whether *D. suzukii* acquires fungal propagules from caneberry plants or other habitats, we surveyed raspberry and blackberry fruit rot incidence in 2020, using the same field sites where flies were collected. At Queenstown, post-harvest evaluations of blackberries were performed on one sampling date (8/25/20) due to limited fruit availability (fruit at the Queenstown field site were harvested weekly and destructively sampled for larval *D. suzukii* infestation measurements as part of a separate field study evaluating caneberry trellising systems). In this evaluation, 60 marketable blackberry fruit were randomly selected and incubated in humid, bleach-sterilized crisper boxes for one week, at which point we assessed the presence or absence of fungi on each berry. Any fungi detected were cultured and purified for subsequent identification. At Keedysville, fruit

availability was not a limiting factor, allowing us to conduct visual surveys of pre-harvest fruit rot infections on both sampling dates (9/2/20 and 10/6/20). In each row that flies were collected (two rows during the first collection and three rows during the second collection), we randomly selected and assessed 40 berries for fruit rot. Berries were selected from both sides of the row as well as from varying canopy heights and locations (interior and exterior canopy). Fruit that had a visible fungal infection were harvested and returned to lab, where fungi were isolated and purified for subsequent identification. Fungi were identified molecularly to genus and/or species as previous described.

Complementing the sampling conducted in our field sites, separate post-harvest raspberry fruit rot evaluations were at Queenstown in 2020 by the Hu lab (UMD Department of Plant Sciences and Landscape Architecture). This work was completed for a separate fungicide efficacy trial but represents a much more comprehensive sampling effort. In this trial, small raspberry plots were artificially inoculated with *Botrytis* on 7/16/20 and 8/5/20 and treated with either Pristine, a proprietary fungicide at varying concentrations, or left unsprayed as an untreated control. Applications were applied on five separate dates between 7/9/20 and 9/5/20, and post-harvest fruit evaluations were conducted three times from August - September. Harvested fruit were held in lab in humidified crisper boxes for 3-5 days, at which point any visible fungi were isolated and purified on PDA for identification.

Species-Level Identifications

To understand whether *D. suzukii'*s fungal associates are potentially pathogenic to caneberry fruit, species-level identifications were performed for isolates within select fungal genera (Table 2-2). These efforts focused on genera that had previously published

robust PCR protocols and that were likely to contain fruit rot pathogens. Previous surveys of caneberries have identified pre-harvest *Cladosporium, Botrytis*, and *Colletotrichum* infections (Swett et al. 2019; Hu and Schoeneberg 2020), and *Fusarium* spp. was commonly isolated from raspberry fruit sampled at Keedysville in 2020 as part of this study as well as raspberries sampled at Queenstown in 2020 (Hu, *Unpublished Data*).

Table 2-2. Summary of gene regions and primers used to isolate *Cladosporium, Botrytis, Colletotrichum,* and *Fusarium* isolates to species level.

Genus	Target Locus	Direction	Primers	Original Reference
Botrytis	HSP60	F	hsp60for+	Staats et al. 2005
		R	hsp60rev+	Staats et al. 2005
	RPB2	F	rpb2for+	Staats et al. 2005
		R	rpb2rev+	Staats et al. 2005
Cladosporium	ACT	F	ACT-512F	Carbone and Kohn 1999
		R	ACT- 783R	Carbone and Kohn 1999
	EF1	F	EF1-728F	Carbone and Kohn 1999
		R	EF1-986R	Carbone and Kohn 1999
Colletotrichum	G3PDH	F	Bo_G3PDHfor+	Staats et al. 2005
		R	Bo_G3PDHrev+	Staats et al. 2005
	G3PDH	F	GDF1	Hu et al. 2015
		R	Gdr	Templeton et al. 205
Fusarium	EF1	F	EF1-728F	Carbone and Kohn 1999
		R	EF1-986R	Carbone and Kohn 1999

PCR reactions, DNA cleanup, and DNA sequencing were performed as previously described using species-specific primers. Due to the short length of the actin (ACT) and elongation factor (EF1) target loci, DNA samples for these regions were sequenced in both directions (Swett et al. 2019); forward and reverse sequences were trimmed and checked for quality, and cleaned sequences were merged with Benchling (Benchling [Biology Software] 2021). All sequences were compared against the NCBI database, and species level identifications were performed using the same criteria as previously described.

Statistical Analysis of Fungal Association Data

Data analyses were performed with genus level identifications, because only a subset of the fungal isolates were identified to the species level. Any isolates that were identified as yeast through PCR (e.g., *Saccharomycopsis* sp.), too contaminated with yeast to purify, or that died in culture prior to DNA extractions were excluded from analysis. Data are presented as the incidence for each fungal associate, with summary tables showing the number of flies and/or berries collected with each genera of fungi detected. Histograms were also generated using ggplot2 (Wickham, 2016) to visualize how frequently individual fungal genera were isolated. Histograms were subset by year and sampling type. Each graph shows the number of flies sampled on the x-axis and the number of fungal genera isolated from a given quantity of flies on the y-axis.

Variation Across Field Sites and Sampling Dates

Canonical correspondence analysis (CCA) was conducted using Canoco 5 (Microcomputing Power) to evaluate how field site and sampling date impacted the overall *D. suzukii* fungal community. Because sampling efforts were inconsistent, with generally lower numbers of flies collected during an individual sampling event (Table 2-1), 2018 and 2019 data were excluded from this analysis. We selected a unimodal model, because data were entered as the presence or absence of each fungal genera identified on each fly sampled (pooled across interior and exterior samples), and this method was recommended by the Canoco Analysis advisor. Genera that were detected on only one fly were removed from the dataset, and rare genera were downweighted in the analysis. Two factors were included as explanatory variables: site (Queenstown and Keedysville), and sampling date (coded as "Early" for the 8/26 and 9/2 sampling events and "Late" for the 9/23 and 10/6

sampling event). Analysis results are presented in a biplot, which shows the relationship between each taxon score and the centroids for each explanatory variable, and the significance of the first and all ordination axes were tested using a Monte Carlo permutation test (N=499 permutations).

Comparison of the Interior and Exterior Fungal Community

A separate CCA was conducted to determine whether the fungal community varied between the interior and exterior regions of the flies. Data were pooled across all sampling years, and all fungal genera (included rare taxa only isolated one time) were included in this analysis; to aid in visualization of the data, only the top fifteen genera by weight were included in the ordination biplot. Fly location (e.g., interior vs. exterior) was included as an explanatory variable, and sampling year and field site were added as covariates. We tested the significance of all ordination axes with Monte Carlo permutation tests (N=499 permutations), and analyses results were again graphically displayed on a biplot that showed the relationship between each taxon score and the explanatory variable centroids.

To compare the incidence of fungi between the interior and exterior regions, we developed contingency tables; by year, each table shows the total number of flies with one or more genera of fungi isolated on the exterior, on the interior, on both the exterior and interior, or in neither location. We tested for significant differences in interior and exterior accumulation using McNemar's chi-square test. This is a nonparametric test that evaluates differences in two sample proportions that were collected from the same individual; in contrast, standard chi-square tests whether two factors are independent (Morrison, 2010).

Contingency graphs were also used to visualize the interior and exterior incidence of key fungal taxa across sampling years; this included the top four most commonly

isolated genera (*Cladosporium, Penicillium, Mucor,* and *Aspergillus*) as well as any genera for which species-level identifications were performed (*Fusarium* and *Colletotrichum*). Subset by year, each graph shows the cumulative number of flies with a given fungal genera present on the exterior, present on the interior, present on both the exterior and the interior, or not present in either location. McNemar's chi-square was again used to compare fungal incidence between the interior and exterior regions of the flys' body.

To evaluate how many genera of fungi adult *D. suzukii* carry, we calculated genus richness as the number of unique fungal genera isolated on the exterior and interior region of each fly. Genus richness was analyzed using a mixed model ANOVA in the nlme package in R (Pinheiro et al., 2017), with data subset between sampling years. Each model included the number of genera isolated as the response variable and sampling type (interior versus exterior fungi), site (Queenstown or Keedysville), and the type by site interaction as explanatory variables. Because data were paired (with both the interior exterior isolations coming from the same fly), we also added a random term to account for each fly sampled and specified that samples from the same fly were autocorrelated using a compound symmetry correlation matrix structure. All models were checked for the assumptions of homogeneity of variance and normality of the residuals. Data from each year were summarized for the significant effect (mean + standard error) and graphed using bar plots.

Laboratory Evaluation of Vectoring by Drosophila suzukii

Laboratory vectoring assays were conducted to correlate *D. suzukii*'s fungal associations with vectoring. Assays were conducted using the two species of primary fruit rot fungi that had previously been isolated from *D. suzukii* larvae, *Botrytis cinerea* and

Cladosporium cladosporioides. Including both species allowed us to test for potential differences in how flies transmit different fungal taxa.

Assays were conducted under worst-case scenario, no-choice laboratory conditions. Thirty male and 30 female *D. suzukii* were placed directly onto sterile PDA plates that were either (1) inoculated with 200 uL of a 1.5 x 10^4 *Botrytis cinerea* spore suspension, (2) inoculated with 200 uL of a 1.5 x 10^4 *Cladosporium cladosporioides* spore suspension, or (3) not inoculated with any fungal pathogens (untreated control). At these concentrations, plates were completely overgrown with fungi within 7 days. Plates were held for 7-14 days at room temperature until sporulation before use in experiments. *D suzukii* were confined on these fungal exposure plates for five hours, ensuring a high likelihood of interaction with the fungi. At the end of this exposure period, all flies were removed from the media, and two sets of measurements were performed. We evaluated *D. suzukii*'s ability to transmit fungal pathogens to sterile media over time and quantified the incidence and density of interior and exterior fungal propagules, a measurement that allowed us to correlate vectoring with fungal propagule acquisition on the fly's body. Six replicates were performed for each experiment.

Vectoring Competency Over Time

Vectoring assays were conducted to evaluate *D. suzukii*'s ability to transmit *Botrytis* and *Cladosporium* to sterile PDA over time. For each replicate, four *D. suzukii* (2 males and 2 females) were removed from the fungal culture plates. Each fly was placed onto a small individual PDA dish and held for 24 hours, at which point it was serially transferred onto fresh PDA plates at 24, 48, and 72 hours after exposure. If the flies were capable of vectoring pathogens, we expected to see fungal colonies grow on the PDA.

Plates were incubated for two weeks and assessed for the presence or absence of *Botrytis* and *Cladosporium*. Fungal identifications were morphologically confirmed using characteristics such as spore ontogeny and color as described in Barnett and Hunter 1981.

Comparison of External and Internal Fungal Accumulation

To correlate laboratory vectoring with interior and/or exterior fungal accumulation, we simultaneously quantified the density of *Botrytis* and *Cladosporium* propagules accumulated by *D. suzukii* at the same time points used for the vectoring evaluation, 0, 24, 48, and 72 hours after exposure to fungal cultures. At each time point, a total of four flies (two male and two female *D. suzukii*) were used to quantify exterior and interior fungal presence and abundance. Individual flies within a time point were treated as subsamples and pooled for data analysis.

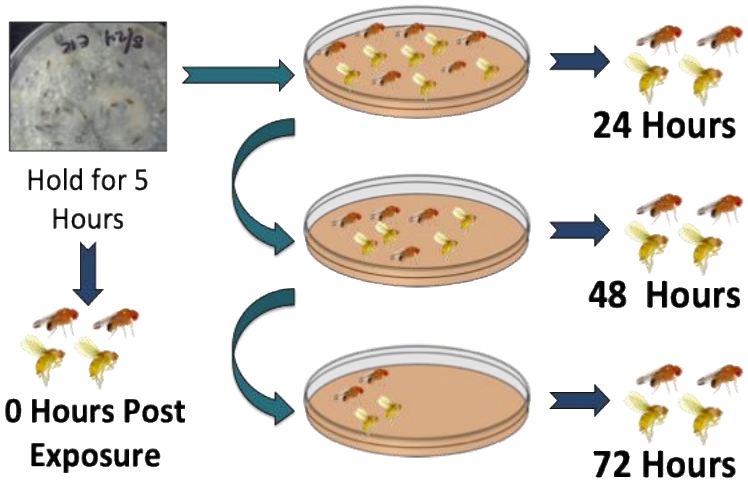

Figure 2-3. Visual summary of methods to quantify fungal propagule accumulation over time.

For the 0-hour post exposure time point, flies were directly removed from the fungal culture plate and transferred into sterile microcentrifuge tubes for immediate analysis of exterior and interior fungi following the methods described above for field-collected flies (Figure 2-2). The remaining *D. suzukii* were removed from the fungal culture

plate at the same time, transferred onto fresh PDA, and held for another 24 hours. At the end of 24 hours, we removed an additional four *D. suzukii* for the 24-hour time point and again transferred the remaining flies to fresh PDA. This process was repeated two more times to generate the 48 and 72 hour time points (Figure 2-3).

Both the interior and exterior fungal solutions were serially diluted, plated on PDA, and incubated at room temperature for 1 – 2 weeks, at which point plates were assessed for the presence or absence of *Botrytis* and *Cladosporium.* We also calculated the resulting colony forming units / mL (CFUs) per fly based on the average number of colonies in the serial dilution containing a countable number of colonies (2 – 80 colonies). CFU data were subset by the type of fungi (*Botrytis* and *Cladosporium*) as well as sampling type and analyzed with a mixed model ANOVA using the lme4 (Bates et al., 2015) and lmerTest (Kuznetsova et al., 2016) packages in R. In each model, CFUs / mL was included as a continuous response variable; explanatory variables included time point (modeled as a fixed effect) and experimental replicate (modeled as a random effect). Model residuals were checked for the assumption of homogeneity of variance and normality, and data were transformed as necessary to satisfy them. Meanwise comparisons using the emmeans package (Lenth, 2018) were performed for significant effects, and data are presented in bar plots as the mean number of CFU's + standard error (SE) observed at each time point.

Results

Survey of Fungal Associations in Adult Drosophila suzukii

In total, we isolated 37 unique genera of fungi from 71 field-collected *D. suzukii* adults across all three years of the study (Table 2-3). Among these genera, *Cladosporium* was isolated most frequently, occurring on the exterior surface of 33 out the 71 flies that we sampled. Other commonly identified genera included *Penicillium, Mucor, Aspergillus,* and *Fusarium,* with many of these taxa occurring repeatedly across sampling years and at different field sites (Appendix B, Table S1).

We observed some overlap between the fungi isolated from *D. suzukii* and fungi isolated from fruit in 2020 (Table 2-4). For example, five of the fungal genera isolated from raspberry fruit at Keedysville in 2020 (*Botrytis, Fusarium, Geotrichum* and *Mucor*) were also found on *D. suzukii.* Similarly, *Gilbertella,* the only fungi isolated post-harvest in the Queenstown blackberries, was also fairly predominant in our fly-associated samples. Post-harvest sampling of raspberries at Queenstown

Table 2-3. Total number of adult *D. suzukii* carrying each fungal genus internally or externally. Data were pooled across years and sampling sites (N=71 adults sampled total) and only includes genera that were isolated at least two times (18 genera were only isolated in one instance); a full list of isolated genera can be found in supplementary Appendix A.

Genus	Exterior	Interior
Cladosporium	33	5
Penicillium	13	8
Mucor	10	9
Aspergillus	12	6
Fusarium	12	4
Gilbertella	7	4
Colletotrichum	5	3
Geotrichum	2	6
Botrytis	5	1
Alternaria	3	1
Bjerkandera	2	2
Curvularia	3	1
Gonatobotryum	2	2
Sarocladium	3	1
Stereum	3	1
Epicoccum	2	1
Pilidium	1	2
Talaromyces	0	3
Trametes	2	1

(Hu, *Unpublished Data*) revealed a broader fungal community that overlapped with and expanded upon our survey results, with *Botrytis, Colletotrichum,* and *Fusarium* detected at high frequencies (Figure 2-4).

Table 2-4. Incidence of fungi isolated pre-harvest from raspberry fruit at Keedysville in 2020 and post-harvest from blackberries at Queenstown in 2020. Columns show the total number of berries at each site and sampling data that found to be infected with each fungal taxon.

	Keedysville		**Queenstown**
	8/26/20 N=80	**10/6/20 N=120**	**9/2/20 N=60**
Botrytis cinerea	1	2	0
Fusarium fujikuroi	5	0	0
Fusarium proliferatum	2	0	0
Fusarium sp. cf *bullatum*	3	0	0
Geotrichum sp.	4	0	0
Gilbertella sp.	0	0	1
Mucor sp.	2	1	0
Pestalotiopsis sp.	0	1	0
Neopestalotiopsis sp.	1	0	0

Subsequent species-level identifications focused on the four target genera (*Botrytis, Cladosporium, Fusarium,* and *Colletotrichum*) that contain known pathogens of raspberry and/or that were isolated from raspberry fruit in 2020 (Table 2-4). In total, we identified 12 unique species or species complexes; this includes several known pre-harvest raspberry pathogens as well as pathogens known to cause disease in other species (Table 2-5). In 2020, we did not isolate *Botrytis cinerea* from any field-collected adults, despite finding it in the raspberry field we used for sampling at Keedysville (Table 2-4). However, *B. cinerea* was detected externally from one fly sampled in 2018. Many of the species identified, including *Cladosporium cladosporioides, Cladosporium pseudocladsporoides,* and *Colletotrichum fioriniae,* were isolated from multiple flies and across multiple sampling dates and field sites (Table 2-5). Two of the fly-associated species, *Fusarium fujikuroi* and

Fusarium proliferatum, were also identified from raspberries that were sampled pre-harvest at Keedysville (Table 2-4).

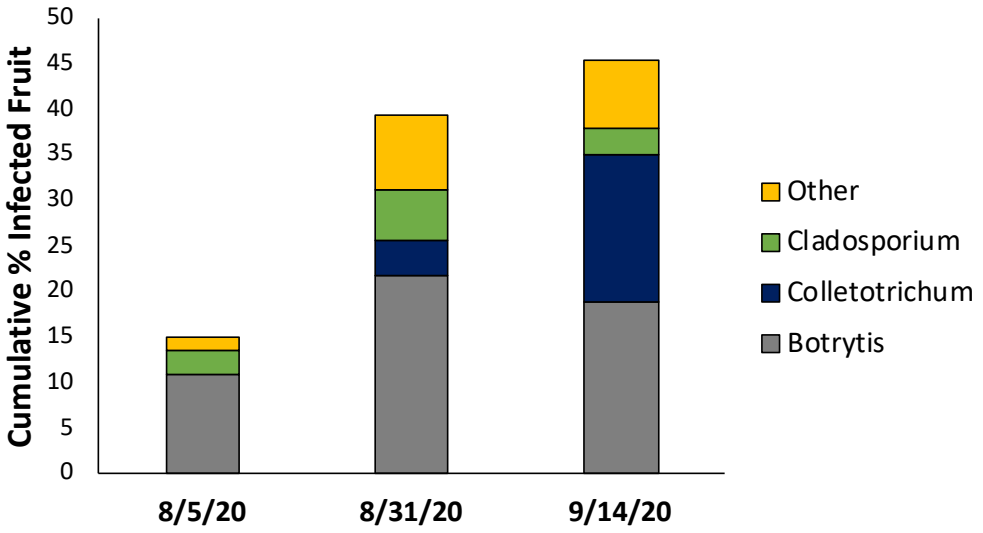

Figure 2-4. Summary of post-harvest fruit rot incidence in a separate raspberry field at Queenstown in 2020. Data were collected by Dr. Mengjun Hu (UMD Department of Plant Sciences at Landscape Architecture) as part of a fungicide efficacy trial. For this trial, the raspberry field was artificially inoculated with *Botrytis cinerea*. They y-axis shows the cumulative percentage of raspberry fruit sampled from untreated control plots with various post-harvest pathogens across different dates: 8/5/20 (N=214 berries sample), 8/31/20 (N=205 berries sampled), and 9/14/20 (N=203 berries sampled). The category "Other" included less frequently isolated fungi: *Alternaria, Aspergillus, Fusarium, Rhizopus, Penicillium,* and *Pestalotiopsis.*

Table 2-5. Summary of species-level identifications for fungi isolated from *Drosophila suzukii* in 2020. Each column shows the number of flies with each species of fungi present internally and externally across sites and sampling dates. 10 flies were sampled on each day, except for Queenstown 8/26, where only 9 flies were sampled. Total shows the sum unique isolations for each fungal genera. Rows that are highlighted in blue indicate taxa that are known raspberry pathogens, and rows highlighted in yellow indicate taxa that are known to cause fruit rot in other crops.

	Keedysville 9/2		**Keedysville 10/6**		**Queenstown 8/26**		**Queenstown 9/23**		**Total**
	Int.	**Ext.**	**Int.**	**Ext.**	**Int.**	**Ext.**	**Int.**	**Ext.**	
Botrytis fabiopsis	1	1	0	1	0	0	0	0	**3**
Botrytis cinerea	0	0	0	0	0	0	0	0	**0**
Cladosporium sp.	1	6	0	2	0	2	1	7	**19**
Cladosporium pseudocladosporioides	0	1	0	1	1	1	0	4	**8**
Cladosporium cladosporioides	0	0	0	2	0	2	0	3	**7**
Cladosporium tenuissimum	0	0	0	0	1	2	0	0	**3**
Colletotrichum fioriniae	0	1	0	0	0	0	0	2	**3**
Colletotrichum nymphaeae	0	0	0	0	1	0	0	0	**1**
Fusarium fujikuroi sp. c.	1	0	1	0	1	2	1	1	**7**
Fusarium proliferatum	0	0	0	0	0	0	0	2	**2**
Fusarium avenaceum	0	0	0	0	0	0	0	1	**1**
Fusarium incarnatum-equiseti sp. c.	0	0	0	0	0	1	0	0	**1**
Fusarium lateritium	0	0	0	0	0	0	0	1	**1**

In total, 96% of field-collected adults (N=71 *D. suzukii* pooled across 2018, 2019, and 2020) carried one or more genera of fungi on their body, and many flies (60%, 85%, and 44% in 2018, 2019, and 2020 respectively) had fungal propagules present in both the interior and exterior measurements. Most fungal genera were infrequently detected (Figure 2-5). For example, out of the 30 genera that we identified in 2020, 18 were only isolated from a single fly (this includes 11 genera that were each isolated once from the exterior of a fly, 4 genera that were each isolated once from the interior a fly, and 4 genera that were simultaneously isolated from both the interior and exterior of a fly one time).

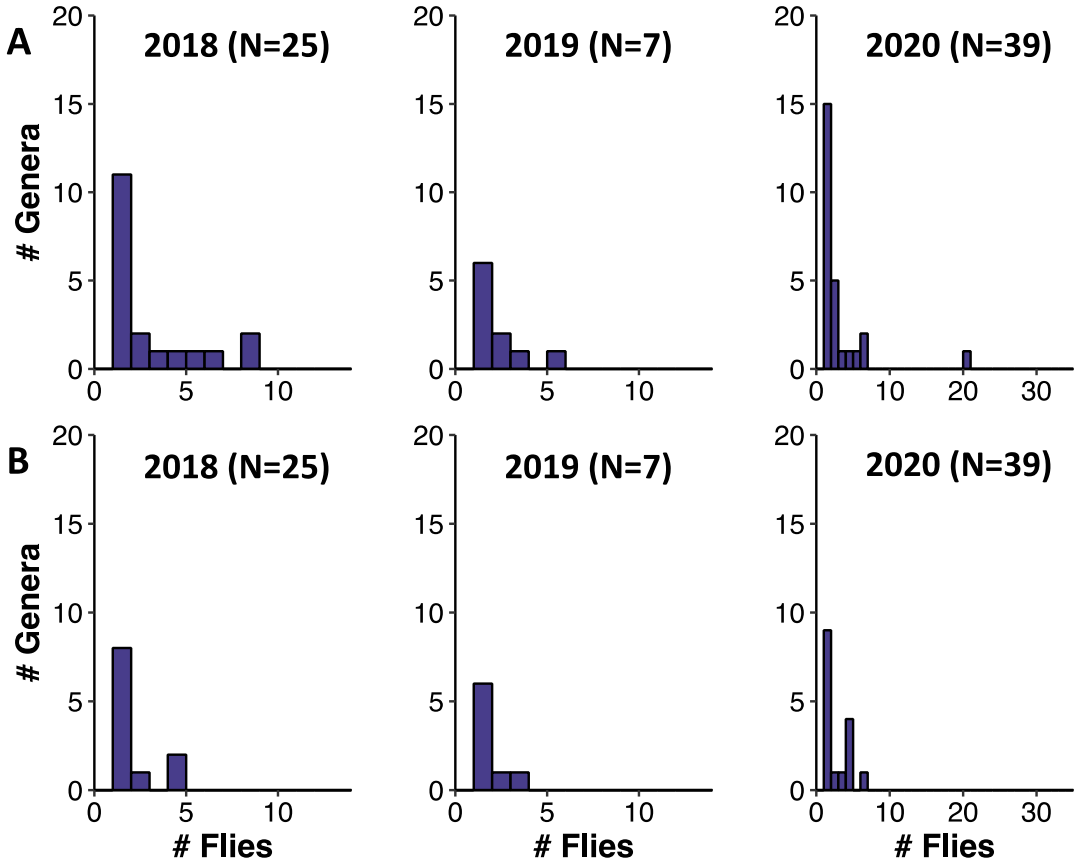

Figure 2-5. Frequency at which fungal genera were isolated from the (A) exterior or (B) interior of adult *D. suzukii* for each year. The y-axis shows the total number of genera that were isolated from a given number of flies (x-axis). Numbers on the x-axis are scaled differently based on the number of flies sampled annually and the frequency distribution to improve visualization.

Variation Across Field Sites and Sampling Dates

The 2020 fungal community was not strongly influenced by either the field site or the sampling date. In the CCA, the first ordination axis primarily captured variation due to sampling site, while the second ordination axis captured variation due to the sampling period (Figure 2-6). Together these axes captured 8.63% of the variation in the genus data and 100% of the fitted variation due to the two explanatory variables (Table 2-6). However, neither the first ordination axis (Pseudo-F = 0.8, P = 0.182) nor all ordination axes (Pseudo-F = 1.5, P = 0.054) significantly explained the variation observed. Indeed, most fungal taxa, including predominant genera such as *Cladosporium, Penicillium, Fusarium,* and *Mucor,* did not cluster around any explanatory variable (Figure 6). However, a few taxa seemed to strongly associate with specific explanatory variables; for example, *Pilidium* was most commonly isolated during the later sampling period, and *Botrytis* was most commonly associated with Keedysville. These relationships were likely driven by small sample numbers. *Botrytis* was only isolated from three *D. suzukii,* all of which were collected at Keedysville. Similarly, *Pilidium* was only isolated from two *D. suzukii* in total, both of which were collected at Queenstown during the second sampling period.

Table 2-6. Summary statistics for CCA examining the effect of site and date on the 2020 fungal community. Eigenvalues measure the relative explanatory power of each ordination axis. The explained variation is the cumulative proportion of variability in the genus data explained by each ordination axis Pseudo-canonical correlation measures the correlation between the response axis and axis fit by the environmental variables. Explained fitted variation is the cumulative proportion of variability each ordination axis explains in the fitted genus data due to field site and sampling date.

Statistic	Axis 1	Axis 2	Axis 3	Axis 4
Eigenvalues	0.1968	0.1393	0.5979	0.5212
Explained variation (cumulative)	5.06	8.63	24.00	37.39
Pseudo-canonical correlation	0.6341	0.5891	0.0000	0.0000
Explained fitted variation (cumulative)	58.56	100.00		

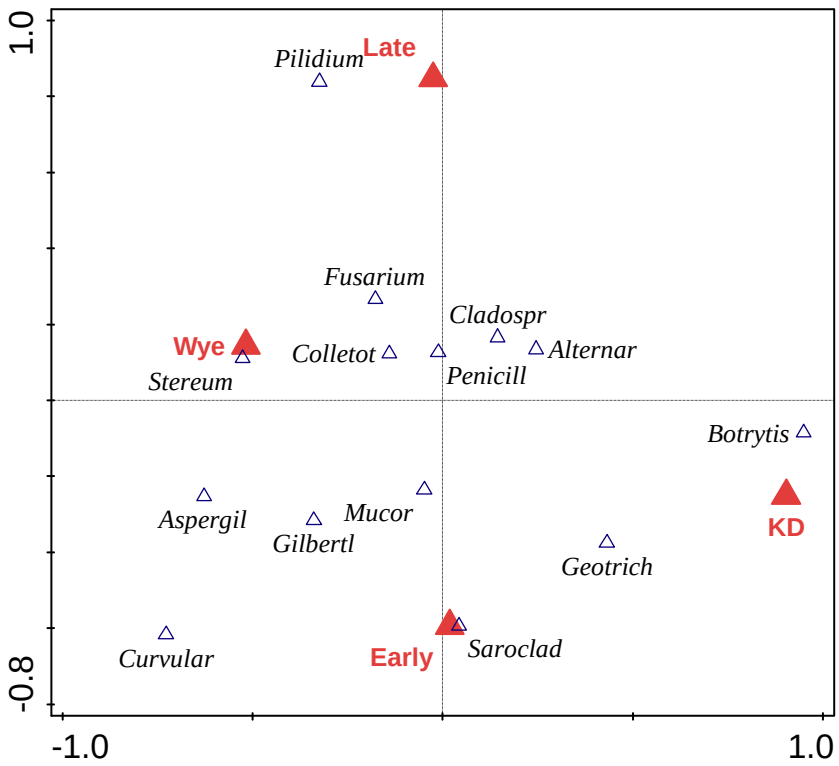

Figure 2-6. CCA biplot displaying the relationship between explanatory variables and the prevalence of fungal genera in 2020. The first two axis explained a cumulative 8.63% of the variance, and neither the first axis nor all ordination axes significantly explained variation (P > 0.05 using Monte Carlo permutations). Taxa that were detected more than one time are included in this figure: KD = Keedysville field site, Wye = Queenstown field site, Early = early sampling period (8/26 and 9/2), Late = later sampling period (9/23 and 10/6), *Alternar = Alternaria, Aspergil = Aspergillus, Cladospor = Cladosporium, Colletot = Colletotrichum, Curvular = Curvularia, Geotrich = Geotrichum, Gilbertl = Gilbertella, Penicill = Penicillium, Saroclad = Sarocladium.*

Comparison of the Interior versus Exterior Fungal Community

Multivariate analyses also detected no significant differences between the interior and exterior fungal community. Fly location only accounted for 1.43% of the total variation in the fly fungal community and Monte-Carlo permutation tests found all ordination axes to be non-significant (Pseudo-F = 1.48, P = 0.058). In the resulting biplot, we also observed no strong clustering of fungal genera across fly location (Figure 2-7; Table 2-7).

Table 2-7. Summary statistics for CCA examining the effect of sampling type on the fungal community. Eigenvalues and explained variation measure the relative explanatory power of each ordination axis. Pseudo-canonical correlation measures the correlation between the response axis and axis fit by the environmental variables. The explained fitted variation shows the relative proportion of the model variation explained by each axis.

Statistic	Axis 1	Axis 2	Axis 3	Axis 4
Eigenvalues	0.1377	0.6788	0.6420	0.5854
Explained variation (cumulative)	1.43	8.49	15.16	21.24
Pseudo-canonical correlation	0.5080	0.0000	0.0000	0.0000
Explained fitted variation (cumulative)	100.00			

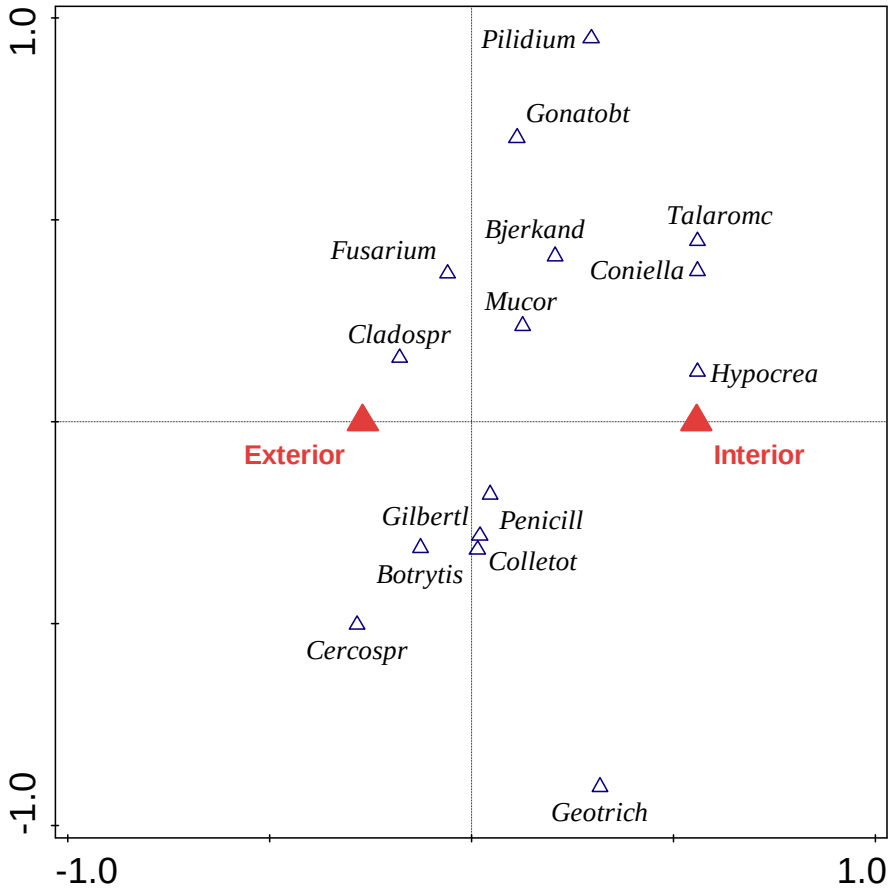

Figure 2-7. CCA biplot displaying the relationship between sampling type and the prevalence of fungal genera in all sampling years. The first axis explained 1.43% of the variation, and all ordination axes were non-significant (P > 0.05 Monte Carlo permutations). The type 15 taxa by weight are included in this picture. *Bjerkand = Bjerkandera, Cercospr= Cercospora, Cladospor = Cladosporium, Colletot = Colletotrichum, Curvular = Curvularia, Geotrich = Geotrichum, Gilbertl = Gilbertella, Gonatobt = Gonatobotryum. Hypocrea = Hypocreales, Penicill = Penicillium, Talaromc = Talaromyces.*

Despite the lack of community level effects, the overall incidence and richness of fungi varied significantly between the interior and exterior communities (Figure 2-8). Overall, a significantly higher proportion of flies carried fungi externally in 2020 ($\chi^2_{(1)}$ = 4.2, P = 0.002), and a similar trend was present in 2018 ($\chi^2_{(1)}$ = 3.1, P = 0.07).

2018: $\chi^2_{(1)}$ = 3.1, P = 0.07

Exterior \ Interior	Absent	Present	**Total**
Absent	2	1	**3**
Present	7	15	**22**
Total	**9**	**17**	**25**

2019: 2019: $\chi^2_{(1)}$ = 0, P = 1

Exterior \ Interior	Absent	Present	**Total**
Absent	0	0	**0**
Present	1	6	**7**
Total	**1**	**6**	**7**

2020: $\chi^2_{(1)}$ = 4.2, P = 0.02

Exterior \ Interior	Absent	Present	**Total**
Absent	5	3	**8**
Present	14	17	**31**
Total	**19**	**20**	**39**

Figure 2-8. Contingency tables depicting the number of field collected adult *D. suzukii* in each sampling year with no fungi detected on their bodies, one or more genera of fungi present externally only, one or more genera of fungi present internally only, and fungi present both internally and externally. The numbers in bold in the table margin are the sums of the rows and/or columns. Differences in the proportion of flies carrying fungi internally and externally were tested with a McNemar's chi-square test.

We also observed some differences in the incidence of some key fungal taxa between the interior and exterior region. Across all three years of the study, *Cladosporium* predominated the culturable fungal community, occurring on 8 out of 25, 5 out of 7, and 20 out of 39 *D. suzukii* sampled in 2018, 2019, and 2020, respectively. Accumulation on the fly exterior drove this prevalence; significantly higher proportions of flies carried *Cladosporium* externally in 2018 ($\chi^2_{(1)}$ = 6.13, P = 0.013) and 2020 ($\chi^2_{(1)}$ = 12.50, P < 0.001), and exterior *Cladosporium* abundance trended higher in 2019 although differences

were non-significant (Figure 2-9). Contrasting the results for *Cladosporium*, incidences for the next most commonly isolated taxa were low and not significantly different between sampling locations (Figure 2-10).

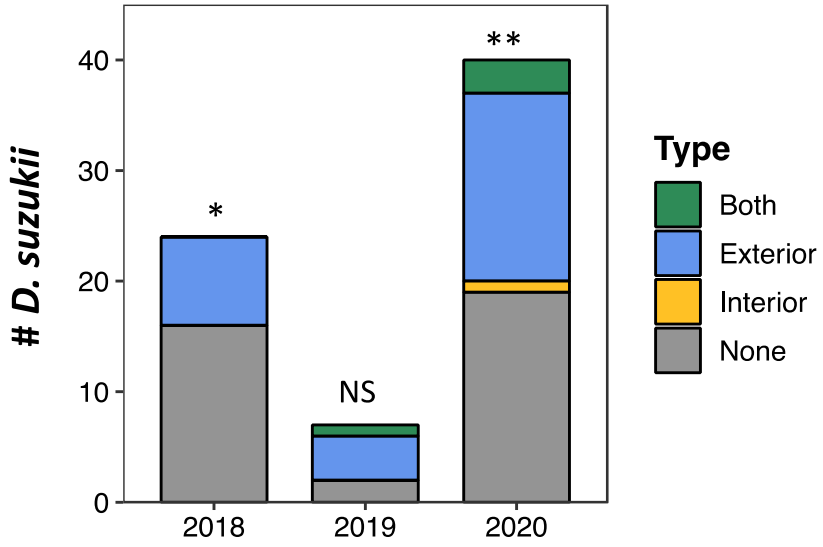

Figure 2-9. Incidence of *Cladosporium* in field-collected *D. suzukii* in 2018, 2019, and 2020, showing the cumulative number of flies with no *Cladosporium* detected, *Cladosporium* only detected internally, *Cladosporium* detected only externally, and *Cladosporium* detected both internally and externally. The proportion of flies with fungi present externally and internally was compared using McNemar's chi-square test, with separate analyses conducted by year. * $P < 0.05$, ** $P < 0.001$.

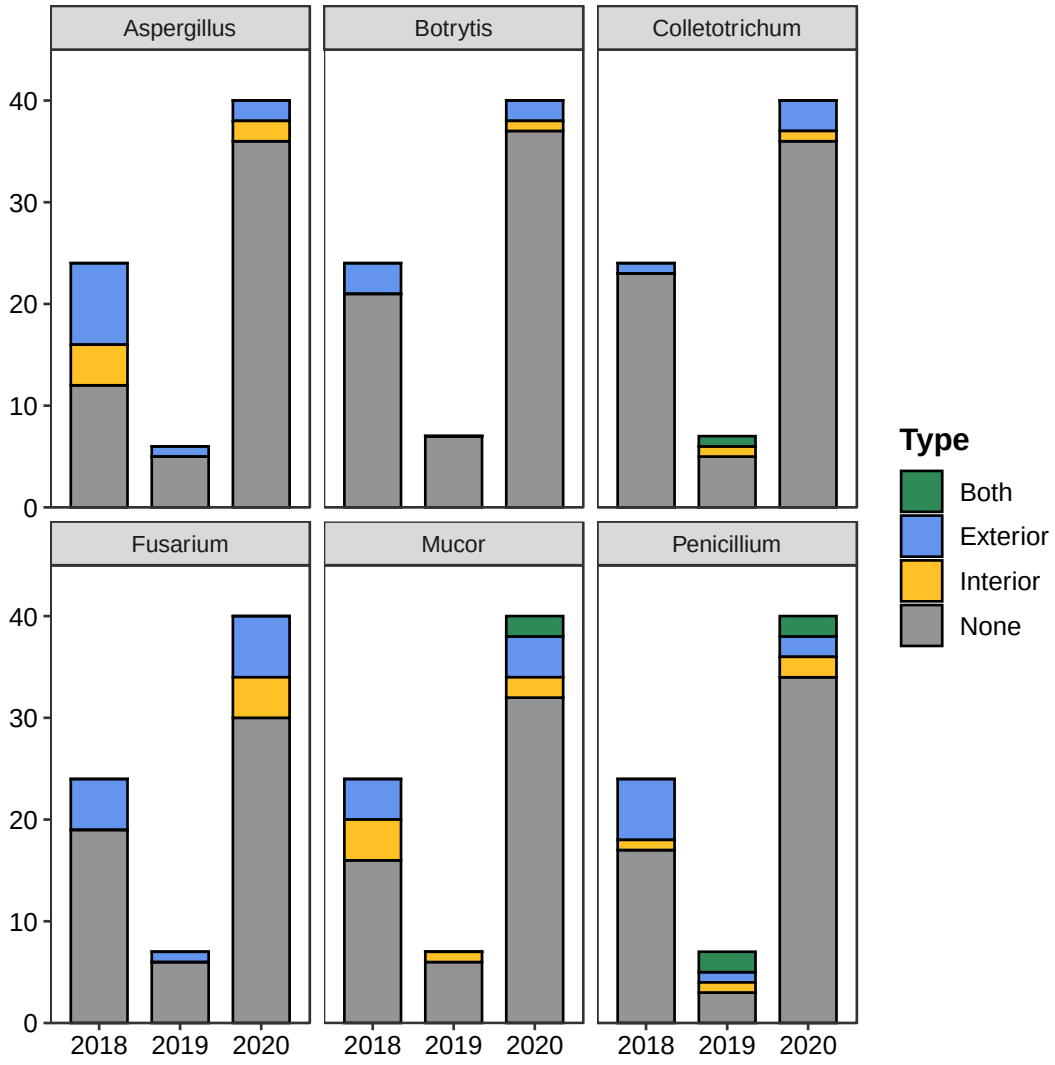

Figure 2-10. Cumulative number of *D. suzukii* found to be carrying different fungal taxa internally (yellow), externally (blue), in both locations (green), or in neither location (gray) in 2018, 2019, and 2020. The proportion of flies carrying each fungi externally and internally were compared for each year with a McNemara chi-square test; separate analyses were conducted for each fungal genera by year combination. No significant differences ($P > 0.05$) were found in any analyses.

Many flies carried multiple fungal genera on their bodies simultaneously; across all sampling years, external genus richness ranged from 0 to 6 genera isolated per fly and internal richness ranged 0 to 3 genera per fly. In 2018, genus richness increased by 65% between the internal and external sampling locations ($F_{1,23} = 25.81$, $P < 0.001$). No significant differences in richness were observed in 2019 ($F_{1,5} = 1.12$, $P = 0.326$), and

significant increases in external genus richness only occurred at Queenstown in 2020 ($F_{1,37}$ = 5.31, P = 0.027; Figure 2-11).

This difference in 2020 may reflect the overall genera richness observed on flies collect at each site; at Queenstown, we isolated 29 unique genera of fungi total across both sampling dates, with a higher number of genera found exclusively in the external fungal isolations. Additionally, the majority of exclusively external fungal genera (13 out of 15 total) were only isolated one time each from a fly (Appendix B, Figure S1). In contrast, only 10 genera were isolated from Keedysville in 2020; while fewer total genera were collected, the taxa observed occurred more frequently and many were found both internally and externally within flies. Fewer fungal genera were detected overall the 2018 sampling (Appendix B, Figure S1), although this may be an artifact of reducing sample size compared to 2020.

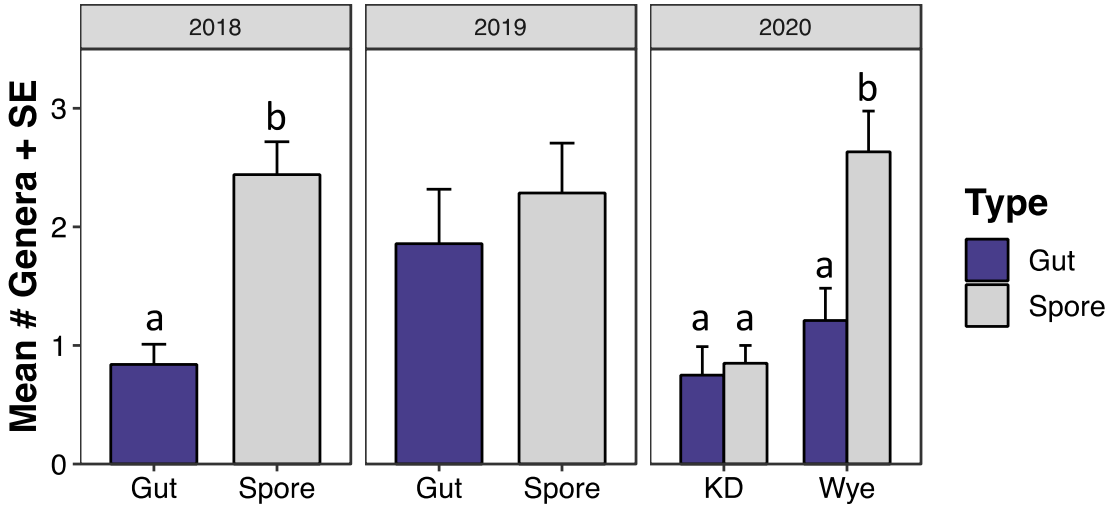

Figure 2-11. Mean number of genera + standard error (SE) isolated internally (gut) and externally (spore) from *D. suzukii* in 2018, 2019, and 2020. Data were analyzed separately by year with a mixed model ANOVA that included sampling site, sampling type, and the site by type interaction; significant effects are presented graphically. Within a year, bars that do not share a letter are significantly different by Tukey's HSD ($p<0.05$).

Laboratory Evaluation of Vectoring by Drosophila suzukii

Vectoring Competency Over Time

Under no-choice laboratory conditions, adult *D. suzukii* exposed to sporulating *Botrytis* or *Cladosporium* cultures vectored both *Botrytis* and *Cladosporium* to sterile PDA through 72 hours (Table 2-8). In contrast, *D. suzukii* from the untreated control did not acquire or vector *Botrytis* or *Cladosporium* at any time point, confirming that our colony was not infected with either species of fungi during these experiments.

Table 2-8. Mean percentage and standard error of *D. suzukii* that scored positive for vectoring *Botrytis* or *Cladosporium* to sterile media at 0, 24, 48, and 72 hours post exposure.

	0 Hrs	**24 Hrs**	**48 Hrs**	**72 Hrs**
Control	0% ± 0%	0% ± 0%	0% ± 0%	0% ± 0%
Botrytis	100% ± 0%	100% ± 0%	100% ± 0%	93.3 ± 6.7%
Cladosporium	100% ± 0%	100% ± 0%	100% ± 0%	100% ± 0%

Comparison of Interior versus Exterior Accumulation

We observed no difference in fungal propagule acquisition between male and female *D. suzukii*, so data on fungal propagules incidence and density were pooled across sex. 100% of flies exposed to *Cladosporium* scored positive for carrying fungal propagules on their cuticle through the 48 hours post exposure time point; by 72 hours post exposure, that number only dropped to 87.5% ± 8.5% of flies sampled. Similarly, for *Botrytis*, 100% of all flies tested scored positive for carrying fungal propagules on their cuticle through the 72 hours. We observed significant reductions in the density of externally accumulated *Botrytis* ($F_{3,15} = 18.145$, $P < 0.01$) and *Cladosporium* ($F_{3,15} = 9.215$, $P < 0.01$) over time.

Overall, rates of fungal acquisition and persistence were lower on the interior compared to the exterior, suggesting that the cuticle may be the primary mechanism by which flies transmit fungal propagules. For example, 0 hours after exposure to *C.*

cladosporioides, we found that flies carried an average of 4,502.1 ± 1,967.1 *Cladosporium* CFUs/mL on their cuticle, compared with 734.2 ± 192.1 CFUs/mL within their gut (Figure 2-12). We also observed significant decreases in density of both *Botrytis* (Figure 2-13; $F_{3,15} = 15.779$, $P < 0.001$) and *Cladosporium* (Figure 2-12; $F_{3,15} = 16.896$, $P < 0.001$) over time, which likely reflects spores degrading within the midgut or passage through the digestive tract.

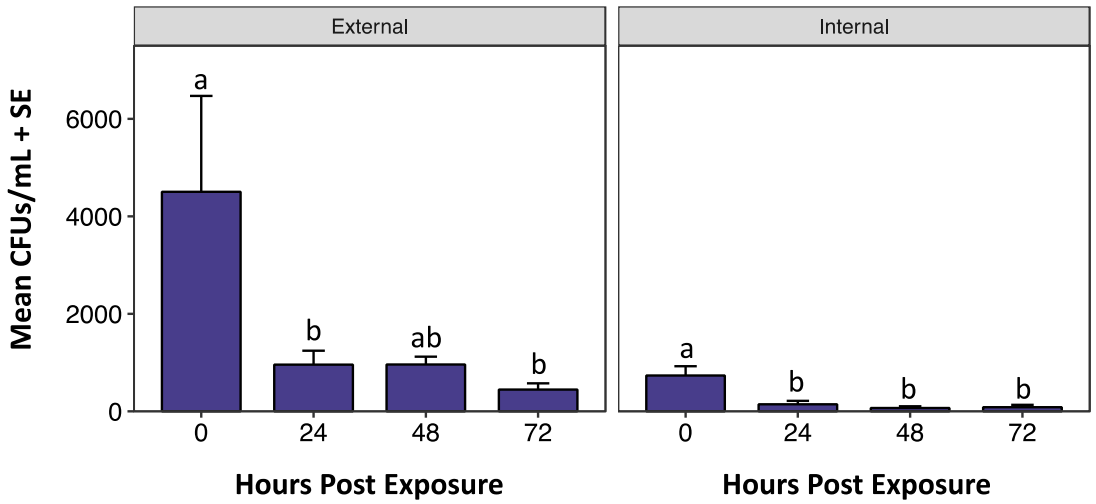

Figure 2-12. Mean *Cladosporium* CFUs / mL isolated externally and internally from *D. suzukii* in no-choice laboratory assays. CFU abundance over time was analyzed separately or the internal and external accumulations using a mixed-model ANOVA and pairwise comparisons with Tukey's HSD. For each sampling type, bars that do not share a letter are significantly different (P > 0.05).

Total ingested *Cladosporium* incidence decreased from 91.6% ± 5.3% of *D. suzukii* surveyed 0 hours post exposure to 70.8% ± 16.4% at the 72 hour time point. In contrast, 87.5% ± 5.6% of flies surveyed 0 hours post exposure were found to carry some amount of *Botrytis* within their alimentary canal. However, that number decreased to 8.3% ± 5.3% of flies by the 24 hour post exposure time point and remained low for the remained of the study. This difference most likely stems from differences in fungal density, as flies appeared to consume greater quantities of *Cladosporium* relative to *Botrytis*.

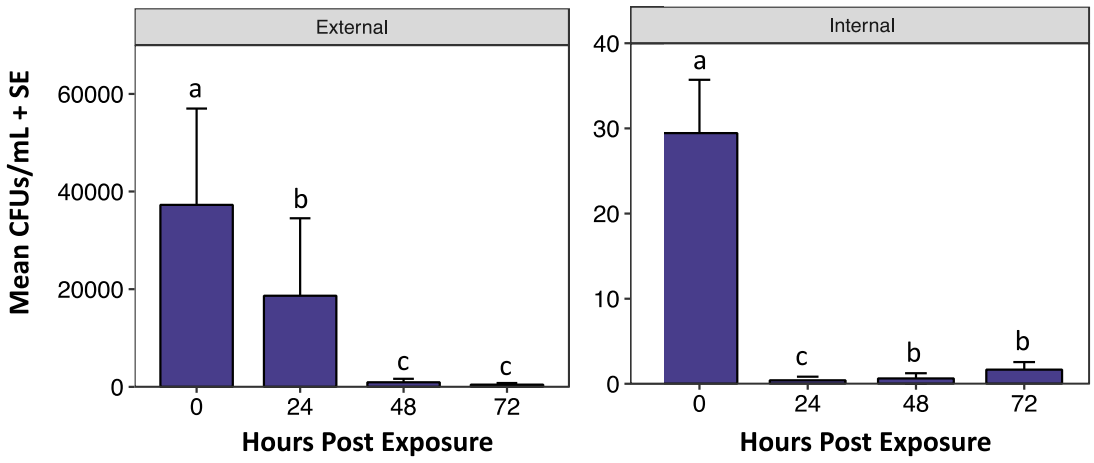

Figure 2-13. Mean *Botrytis* CFUs / mL isolated externally and internally from *D. suzukii* in no-choice laboratory assays. CFU abundance over time was analyzed separately or the internal and external accumulations using a mixed-model ANOVA and pairwise comparisons with Tukey's HSD. For each sampling type, bars that do not share a letter are significantly different ($P > 0.05$).

Discussion

Drosophila suzukii associates with a rich hyphal fungal community. Over three years of sampling, we isolated 37 unique fungal genera from the interior and exterior of field-collected adults; many of these genera are highly speciose and contain species that occupy varying habitats. For example, the five most commonly occurring genera, *Cladosporium, Penicillium, Mucor, Aspergillus,* and *Fusarium*, contain known pre- and/or post-harvest fruit pathogens (López et al., 2016; Palmer et al., 2019; Swett et al., 2019; Thomidis & Exadaktylou, 2012; Hu, *Unpublished Data*) as well as species capable of colonizing non-crop spaces (e.g. soil, decaying plant material, or indoor habitats). Some species within these genera are also known opportunistic pathogens of humans (Al-Hatmi et al., 2016; Bensch et al., 2015; Paulussen et al., 2017).

Species-level identifications of *Cladosporium, Fusarium, Colletotrichum,* and *Botrytis* confirmed that a subset of *D. suzukii*'s fungal isolates are fruit-associated and

pathogenic to caneberries. In 2020, we identified three known raspberry pathogens from *D. suzukii*: *Cladosporium cladosporioides, Cladosporioium pseudocladosporioides,* and *Colletotrichum fioriniae* (Schoeneberg & Hu, 2020; Swett et al., 2019). All three species were present across both field sites and/or across multiple sampling dates at low rates, indicating a consistent, albeit diffuse association. Additionally, two *Fusarium* species with unconfirmed pathogenicity to caneberries, *Fusarium fujikuroi* and *Fusarium proliferatum*, were isolated from both *D. suzukii* and from raspberry fruit sampled at Keedysville (Table 7). Beyond these species-level identifications, we also observed overlap between the culturable raspberry and fly fungal community at the genus level, a trend that further suggests *D. suzukii* are carrying fungal propagules acquired while visiting raspberry fruit.

In addition to these suspected and known plant pathogens, we also isolated some fungal genera that were more specialized, making it easier to speculate on their ecological role. Seven of the genera that we detected (*Bjerkandera, Stereum, Irpex, Ceriporia, Trametes, Schizophyllum,* and *Tyromyces*) primarily colonize live or decaying wood, while three (*Trichoderma, Isaria,* and *Lecanicillium*) contain known entomopathogens. Many of the other genera isolated have not been well studied or include both endophytic and parasitic species of other, non-crop plants. Together, this broad spectrum of fungi suggests that *D. suzukii* visits and/or utilizes non-crop hosts, which is supported by their movement patterns and laboratory resource use (Klick et al., 2016; Stockton et al., 2019). For example, daily adult movement in and out of the crop field (Evans et al., 2017; Swoboda-Bhattarai & Burrack, 2020) as well as the variety of non-crop resources available at both field sites could increase fungal encounters.

Both Queenstown and Keedysville are best characterized as diverse, heterogenous landscapes; both farms produce various fruit (particularly apples, pears, and peaches), vegetable and field crops and contain small, unmanaged forested tracts proximal to our field sites that serve as potential sources of non-crop fungi (Figure 2-1). For example, *D. suzukii* may acquire wood-associated fungi while moving in and out of the forested plots on the farm. The presence of post-harvest pathogens on the fly body may also reflect feeding on cull fruit that was either left on the caneberry plants or was available in nearby fields. Adult *D. suzukii* are known to feed on a variety of damaged fruits, including less preferred hosts such as apples, oranges, grapes, or peaches (Walsh et al., 2011).

Influence of Site and Sampling Date on the Fungal Community

We observed no meaningful variation in the composition of the 2020 *D. suzukii*-associated fungal community across field sites or sampling dates, which contrasts *Drosophila* spp. results for the overall (e.g., exterior and interior) and interior bacterial microbiome. Such surveys have found significant variation in the microbiome of *Drosophila* that were collected from different field sites or collected at the same field site on different sampling dates approximately 15 days apart (Adair et al., 2018; Staubach et al., 2013; Wong et al., 2013). This suggests that the *Drosophila* bacterial microbiome is driven by stochastic processes such chance encounters with different bacterial taxa as well as ecological drift in the bacterial microbiota that is available over time (Adair et al., 2018; Douglas, 2018). If this stochastic model also applies to *D. suzukii*'s fungal associations, we would expect to see changes in the fly fungal community over time and across sites in response to fungal community dynamics. Indeed, post-harvest raspberry sampling at from Queenstown (Hu, *Unpublished Data*) highlights the distinct shifts that may occur within

the fruit fungal community over time. During early season sampling periods (August 2020), *Botrytis* was the most commonly isolated post-harvest pathogen. As the season progressed (mid-September and late October sampling periods), the relative abundance of *Cladosporium*, *Colletotrichum*, and "Other" fungal pathogens (a grouped category that includes *Fusarium, Aspergillus, Penicillium, Alternaria,* and *Pestalotiopsis*) increased (Figure 2-4), which was not reflected in the fungi isolated from *D. suzukii*.

Differences in how flies interact with bacteria and hyphal fungi likely underlie our results. Whereas the interior bacterial microbiome (the focal region for most surveys) is primarily acquired by feeding on fruit or other host substrates (Wong et al., 2015), the majority of fungi isolated in this study occurred externally and were not associated with fruit. Additionally, sampling effort may also account for these differences, because overall genus richness varied between years and corresponded with the number of flies sampled. When only seven flies were sampled 2019, we identified 11 unique genera of fungi. In contrast, higher sampling efforts in 2020 (39 flies) corresponded with higher total genus richness (29 genera detected total). Species accumulation curves for both pooled data and the 2018 and 2020 sampling efforts separately failed to reach a plateau (Appendix B, Figures S2-S4), indicating that additional sampling may be necessary to estimate the full richness of *D. suzukii*'s fungal associations and to detect sources of variability in the community structure. Individual fungal taxa may vary in terms of how well and how long they adhere to the fly cuticle or remain viable within the alimentary canal (Sunde & Latge, 2012; Whiteford & Spanu, 2002), which could potentially lead us to underestimate the prevalence of some fungal taxa.

Contrasting most studies of the *Drosophila* bacterial microbiome (Adair et al., 2018; Staubach et al., 2013), we only quantified *D. suzukii*'s fungal associations as the presence of absence of fungi on each fly sampled; using culture-based methods made it unfeasible to accurately estimate the relative density of individual fungal taxa. However, DNA barcoding surveys have demonstrated that different yeast and bacterial operational taxonomic units (OTUs) vary in their abundance within the *Drosophila* microbiome, and several key taxa typically predominate the microbial community (Chandler et al., 2011, 2012; Fountain et al., 2018; Nin et al., 2011). Although these barcoding methods have not yet been applied to hyphal fungal associations in *Drosophila* spp., surveys of the total fungal community (interior and exterior fungi) associated with the olive fruit fly demonstrate a similar, uneven abundance distribution in which *Cladosporium* spp. account for the majority fungal OTUs (Malacrinò et al., 2015, 2017). Given these trends, it seems likely that the abundance of individual fungal taxa on adult *D. suzukii* also varies, and it is possible that incorporating this variation in abundance into our multivariate analyses would have allowed us to discern more meaningful patterns over time or space.

However, we did observe differences in the richness of genera isolated between Queenstown and Keedysville in 2020. Overall genus richness was higher in Queenstown, which was primarily driven by non-fruit associated fungi that were infrequently detected on the exterior surface of the fly's body. The difference between these sites may be due to on-farm sampling location. The 2020 Keedysville samples were collected in a field approximately 100 m away from the nearest forested tract that was surrounded by empty grass fields and one corn field (Figure 2-1). It is possible that flies at the 2020 Keedysville site were still able to move in and out of crop and non-crop habitat; under calm wind

conditions, marked adult *D. suzukii* have been captured as far as 250 m away from their release point within a 48 hour period, though the majority of individuals tested exhibited a shorter flight range that was less than 100 m (Vacas et al., 2019). Given the distance the flies would need to travel to visit non-crop resources, we can assume a longer duration of travel time in and out of the raspberry field, and fungal propagules may have fallen off their cuticle or broken down within the alimentary canal during flight. Alternatively, the large size of the 2020 Keedysville raspberry planting, combined with its relatively isolated location, may have resulted in higher retention of flies within the raspberry field, consequentially reducing the number of non-crop resources they visited.

Influence of Sampling Type on the Fungal Community

Similar to the trends across sampling sites and dates, we failed to detect meaningful variation in the composition of the interior and exterior fungal community; the same sampling issues previously described may have affected the results of this analysis. In contrast to these community level patterns, total fungal abundance (the presence of one of more genera of fungi) and genus richness varied significantly between samples types. Fungi were detected more frequently and at higher richness on the exterior fly surface. This result matches previous surveys of *Drosophila* spp. bacterial associations, in which the gut microbiome of field-collected adult flies represented a subset of the externally isolated bacteria (Chandler et al., 2011). Several mechanisms may account for this pattern. Ingested fungal propagules may pass through the digestive tract and/or break down under acidic midgut conditions, precluding their detection (Douglas, 2015; Shanbhag & Tripathi, 2009). Alternatively, flies may selectively avoid feeding on hyphal fungi. Whereas yeasts and bacteria positively benefit and attract both adult and larval *D. suzukii* (Batista et al., 2017;

Keesey et al., 2015; Lewis & Hamby, 2019; Mori et al., 2016; Scheidler et al., 2015), interactions with hyphal fungi may be more antagonistic. Hyphal fungi generally compete with frugivorous insects from water and nutrients (Cha et al., 2019; Rizvi et al., 2016; Rohlfs, 2005; Tasin et al., 2012; Trienens et al., 2017; Trienens & Rohlfs, 2020; Wallingford et al., 2016), and at least one species of fruit rot fungi, *Botrytis cinerea*, is known to cause an aversive behavior in adult *D. suzukii* (Cha et al. 2019).

The intensity and nature of these interactions likely varies across fungal taxa. We observed higher rates of *Cladosporium* incidence on the exterior fly surface compared to the interior, a result that suggests flies will walk over, but avoid feeding on *Cladosporium* infected fruits. In contrast, sample type did not impact incidence for other predominant taxa, suggesting a milder, less aversive response. Further laboratory experiments are necessary to confirm this hypothesis, as several other confounding factors may also account for this trend. The lack of a difference in other predominate taxa may be a result of sample size, as fewer unique isolation events occurred for these taxa relative to *Cladosporium*. Alternatively, the reduced incidence of *Cladosporium* internally may again reflect spore degradation during passage through the digestive tract, leading us to underestimate its true internal prevalence.

Laboratory Fungal Vectoring

Our vectoring assays demonstrate that adult *D. suzukii* can transmit both *Botrytis* and *Cladosporium* to new substrates and can potentially do so for a prolonged period of time. Although we could not directly compare results from the *Botrytis* and *Cladosporium* vectoring assays, we did observe variability in the interior incidence and density of both species. In general, flies fed on *Cladosporium* at a higher initial density and retained fungal

propagules within their digestive tract at higher rates over time. This difference further suggests that flies differ in their interactions with individual fungal taxa, with *Botrytis* seeming to induce a stronger aversive response than *Cladosporium.* Indeed, *Botrytis* was detected very infrequently in our fungal association surveys, despite occurring at relatively high frequencies in 2020, particularly in the Queenstown raspberries (Figure 2-4). Although further behavioral work will be necessary to understand these relationships, such aversive behavior may limit *D. suzukii*'s influence on disease epidemiology.

For both species of fungi, interior and exterior CFU density declined significantly over time, with the largest drop in CFUs occurring between the 0 and 24 hour time points. In the fly interior, fungal propagules likely break down and/or pass through the digestive tract (Douglas, 2015; Shanbhag & Tripathi, 2009), while on the exterior fungal propagules naturally dislodge from the cuticle over time. Grooming behaviors commonly observed in *D. suzukii* and other *Drosophila* spp. (Yanagawa et al., 2017) may also dislodge propagules. Therefore, fungal propagules may not remain at detectable levels for prolonged periods of time under field conditions. In these laboratory assays, flies were confined to densely sporulating fungal cultures for five hours in a proof-of-concept scenario. Under field conditions, flies encounter infected fruit in varying stages of disease progression and may not spend prolonged periods of time feeding on sporulating fungal infections.

Multiple environmental factors affect the rate of fungal disease transmission in the field. If *D. suzukii*'s interactions with fungi are antagonistic, flies may avoid and/or limit the time spent visiting diseased fruit, which could reduce the density of fungal inoculum initially acquired and the likelihood of a fly initiating new disease (López-Escudero & Blanco-López, 2007; Stahr & Quesada-Ocampo, 2020). The microbiome present within a

fruit may also affect disease development; yeasts, bacteria, and hyphal fungi engage in competitive interactions (Deveau et al., 2018), and pre-existing microbes might inhibit new infections. Additionally, many of these fungal pathogens have developed independent mechanisms for long-distance spore dispersal (Rieux et al., 2014), so it possible that *D. suzukii* plays a minor role in vectoring. Given these potential sources of variability, additional laboratory vectoring assays with fruit and larger scale field studies will be necessary to understand how well laboratory vectoring result translates to *D. suzukii*'s ability to initiate infections within caneberry fruit.

Conclusions

Despite hosting a wide breadth of fungi, *D. suzukii* does not appear to strongly associate with any particular taxa of hyphal fungi. The majority of its culturable fungal associates were infrequently detected, with 49% occurring on only one of the 71 flies sampled. Even the most commonly occurring genera, *Cladosporium, Fusarium*, and *Mucor,* were found on less than 50% of the flies surveyed. In contrast, *D. suzukii* appears to have developed close associations with various yeast taxa, particularly one species, *Hanseniaspora uvarum*. Nearly 100% of field-collected adult and larval *D. suzukii* carry *H. uvarum* within their digestive tract (Hamby et al., 2012; Lewis et al., 2019), an association that is considerably stronger than what we observed with fungi.

Together, these survey results indicate that *D. suzukii*'s acquisition of hyphal fungi is largely unintentional, with flies picking up fungal propagules on their exterior as they move between crop and non-crop resources. This acquisition may occur when *D. suzukii* land on infected substrates to rest between flights or while flies are assessing fruit quality; female *Drosophila* spp. also rely on a combination of olfactory, tactile, and gustatory cues

to select appropriate oviposition sites (Dweck et al., 2013; Yang et al., 2008; Zhang et al., 2020), necessitating direct contact with their host plant.

These unintentional associations still have the potential to affect the raspberry fruit rot community. Under no-choice, laboratory conditions, we have demonstrated that *D. suzukii* are capable of vectoring fungi to sterile media and that once acquired, both interior and exterior fungal propagules can persist on their body for a prolonged period of time. Species-level identifications demonstrate that some of *D. suzukii*'s fungal associates are capable of facilitating pre-harvest disease. Although they associate with *D. suzukii* at a low incidence, these pathogenic species were repeatedly detected across multiple field sites and sampling periods. Together, these results point to a persistent diffuse, association between *D. suzukii* and plant pathogenic fungi and a potential vectoring relationship.

Beyond vectoring, *D. suzukii* oviposition wounds and feeding also can impact disease (Fermaud & Giboulot, 1992; Rombaut et al., 2017; Woodford et al., 2002). Indeed, pathogenicity assays with *Cladosporium* have demonstrated that wounding significantly enhances disease incidence (Swett et al. 2019), suggesting that *D. suzukii* may play a role in Cladosporium fruit rot's epidemiology. Similarly, flies may also facilitate some of the other fungal taxa isolated in this study, including taxa that may otherwise not be considered serious pre-harvest caneberry pathogens.

Long term, understanding these interactions may help guide the development of more targeted pest management tactics, as has been seen in other systems. For example, a recent study indicates that grape sour rot disease is most effectively controlled through spray programs that combine antimicrobial products with insecticides that target *Drosophila* spp. (Hall et. al, 2018). If a similar relationship exists in raspberries, growers

may be able to minimize fungal disease through more careful control of insect vectors. This may include integrating pesticide and fungicide applications or adopting strategies to optimize pesticide spray coverage (Lewis and Hamby 2020). In organic production (where pesticide options are more limited), the integration of cultural control tactics, including exclusion netting, sanitation, and habitat manipulation could also reduce *D. suzukii* populations, consequentially contributing to pathogen management. Adopting these tactics may be especially important for controlling fungal pathogens with limited effective fungicide options, such as *Colletotrichum* or *Cladosporium.* In addition to increasing profits, such integrated management programs can also reduce reliance on fungicide applications improving sustainability.

Chapter Three: Optimizing Caneberry Spray Coverage for *Drosophila suzukii* (Diptera: Drosophilidae) Management on Diversified Fruit Farms

Abstract

Spray coverage may influence the efficacy of insecticides targeting the invasive vinegar fly *Drosophila suzukii* (Matsumura), a primary pest of raspberries and blackberries. In commercially managed caneberries, spray coverage is typically lowest in the inner and lower plant canopy, regions that overlap with higher levels of adult *D. suzukii* activity. To understand how spray coverage of fruit impacts efficacy against *D. suzukii*, laboratory bioassays were conducted using raspberries. In laboratory bioassays, higher spray coverage did not impact larval infestation rates but did increase adult mortality, indicating that flies can avoid a lethal dose of insecticide when applications do not achieve adequate coverage. We also evaluated how carrier water volume impacts spray coverage patterns throughout the canopy of raspberry and blackberry plants using both airblast and CO_2 backpack sprayers. Increasing carrier water volume generally improved spray coverage in the lower plant canopy. However, effects in the upper plant canopy were inconsistent and varied between sprayer types. In addition to carrier water volume, other approaches, including adjusting the pesticide sprayer equipment used and/or sprayer calibration, should also be explored to improve coverage. Growers should evaluate spray coverage in their caneberries to identify and troubleshoot coverage issues. Results from this study indicate that taking the time to optimize this aspect of pesticide application may improve chemical management of *D. suzukii* and will likely also improve control of other important caneberry pests.

Introduction.

Since its initial introduction to the continental United States in 2008, *Drosophila suzukii* (Matsumura), spotted-wing drosophila, has become a key insect pest of soft skinned fruit (Asplen et al. 2015; Walsh et al. 2011; Farnsworth et al. 2017, Digiacomo et al. 2019), particularly primocane (fall-fruiting) raspberries and blackberries (Bellamy et al. 2013; Burrack et al. 2013). Management options for *D. suzukii* are limited, and growers often rely on broad-spectrum insecticides as their primary management tool in both conventional and organic production (Haye et al., 2016; Sial et al., 2019). Adult flies are susceptible to multiple classes of insecticides (Beers et al., 2011; Bruck et al., 2011), though most products have short windows of residual activity (Beers et al., 2011; Van Timmeren & Isaacs, 2013), necessitating frequent applications. Additionally, spraying insecticides does not guarantee consistent *D. suzukii* control, particularly on small-scale farming operations. Recent surveys from diversified Maryland fruit farms detected larval *D. suzukii* in market-ripe raspberries, with populations increasing through early fall despite regular insecticide sprays (Lewis et al. 2019; Schöneberg, unpublished data). These trends highlight the challenges of effectively managing *D. suzukii* as well as the need to optimize pesticide spray programs on smaller farms.

Managing *D. suzukii* can be especially challenging on small (less than 80 hectare), diversified farms with multiple *D. suzukii* susceptible crops. These farms often plant multiple cultivars that ripen at different times, lengthening the harvest season (Keep, 1988; Pritts, 2008) and possibly exacerbating on-farm *D. suzukii* populations. The “you-pick” farm models common to the mid-Atlantic region face additional challenges, including crop sanitation (Haye et al., 2016) and timing pesticide sprays around specific opening dates; to

ensure that fields are open, farms often rely on pesticide products with shorter pre-harvest intervals. Many diversified farms also use the same equipment and sprayer settings (e.g. tractor speed or pressure) to treat crops with varying canopy heights and structures. This practice may decrease the amount of spray reaching a target plant canopy, reducing spray coverage, or the surface area of fruit and foliage within a target plant covered by a spray application.

Within *D. suzukii*'s preferred host crops, it may be difficult to achieve consistent spray coverage throughout the entire plant canopy. In many crops, including strawberries, soybeans, and tree fruit, dense foliage blocks pesticide dispersion, resulting in higher spray coverage and/or deposition in the outer region of the plant canopy (Hutchins and Pitre 1984; Cunningham and Harden 1998; Latheef et al. 2008; Austin et al. 2011; Sharpe et al. 2017). Multiple application parameters, including spraying with adjuvants, using protective shields, and adjusting nozzle size can influence spray coverage patterns (Wolf et al. 1993; VanEe et al. 2000; Latheef et al. 2008; van Zyl et al. 2010; Wise et al. 2010; Nansen et al. 2011; Ferguson et al. 2016). However, tactics that raise production costs through significant investments in specialized spray equipment or increased labor may not be feasible for smaller-scale operations. Optimizing carrier water volume, or the volume of water a pesticide is suspended in, to improve spray coverage is one application factor small fruit growers can easily adjust. There are several approaches that growers can take to change carrier water volume, including altering the speed of the tractor, the sprayer pressure, and/or the type of nozzle used. Spraying with a higher carrier water volume improves spray coverage in multiple systems, particularly in the inner plant canopy (Wise et al. 2010; Nansen et al. 2015; Ferguson 2016; Sharpe et al. 2017). However, higher carrier

water volumes also increase the risk of pesticide run-off (Wise et al., 2010) and may lengthen the application time, potentially deterring adoption by small-scale fruit producers.

While insecticide spray coverage has not been examined within caneberry production, previous work in other systems suggests that it variably impacts efficacy against insect pests. In soybeans, higher methyl parathion spray coverage corresponded with lower populations of various lepidopteran pests (Hutchins & Pitre, 1984), while thiodicarb spray coverage had no effect on soybean looper mortality (Grymes et al., 1999). Higher chlorpyrifos-methyl spray coverage and deposition increased mortality rates for California red scales during the pre-pupal stage but did not impact nymph survivorship (Garcerá et al. 2011). Multiple factors may underlie this variability, including the target pest mobility and/or susceptibility to the insecticide product sprayed. Currently, it is not known if spray coverage impacts control of *D. suzukii.*

In this study, our primary objectives were to evaluate how spray coverage impacts insecticide efficacy against *D. suzukii* and to determine if increasing the carrier water volume improves caneberry spray coverage. To increase the applicability of our results across different fruit production systems, trials were repeated in both primocane blackberries and raspberries using two types of spraying equipment. In the mid-Atlantic region, where this study was conducted, most fruit growers operate airblast sprayers, which are used to spray both their small fruit plantings and trellised tree fruit orchards. However, beginning farmers and smaller, organic operations often cannot afford commercial sprayers and are restricted to Solo® backpack sprayers (Bryan Butler, Personal Communication). With their small tank size and simpler design, backpack sprayers require different spray strategies. We hypothesized that increasing spray coverage would correlate with improved

control of *D. suzukii* and that increasing carrier water volume would improve spray coverage rates throughout the inner and outer caneberry canopy.

Materials and Methods.

Impact of Spray Coverage on Efficacy Against Drosophila suzukii.

Laboratory bioassays were conducted to determine if higher percent spray coverage improves *D. suzukii* control. Individual raspberry fruit were treated with zeta-cypermethrin (Mustang® Maxx, FMC Agricultural Solutions) or water (untreated control), and spray coverage levels were adjusted to either a low coverage or high coverage treatment (approximately 22% and 85% of a spray card's surface covered respectively; see Appendix C, Supplemental Methods for further details). Bioassays were replicated twenty times per treatment over four separate dates, with five replicates conducted on each day. To account for variation in insecticide droplet concentration across different carrier water volumes, two sets of bioassays were conducted over separate periods of time. One experiment used zeta-cypermethrin concentrations equivalent to 468 liters per hectare (L/ha; 118.3 mL of zeta cypermethrin per 189 L of water) while the other used zeta-cypermethrin concentrations equivalent to 935 L/ha (118.3 mL of zeta cypermethrin per 378 L of water).

Store-bought raspberry fruit were washed with a 10% bleach solution followed by cold water to remove any pesticide residues. Raspberries were sprayed inside a laboratory fume hood using a refillable paint canister sprayer (Preval®, Nakoma Products LLC) that was moved along a 22.7 centimeter (cm) track past the raspberry (Appendix C, Figure S1), simulating field airblast sprayer spray card coverage patterns. These canisters had a refillable reservoir that attached to a disposable aerosol sprayer unit. The sprayer used a

proprietary propellant system to expel liquid with an output pressure of 482.6 kilopascals (kPa). To create variation in spray-coverage rates, we adjusted the speed at which the canister moved along the track (Appendix C, Supplemental Methods). Within a given bioassay date (five experimental replicates) two aerosol units were used, one to spray the zeta-cypermethrin treated raspberries and one to spray the water treated raspberries. The same aerosol unit and insecticide solution was used for both spray coverage treatments; to account for potential confounding effects due to decreased propellant levels over time, high coverage and low coverage applications were alternated.

Protocol for Quantifying Percent Spray Coverage.

We estimated the percent spray coverage that each bioassayed raspberry received following methods similar to the approach described in Legleiter et al. (2018). One 10.8 cm x 14.0 cm white paper spray card (Kromekote Card Stock with a glossy finish and C/2S coating) was deployed approximately 4 cm behind the raspberry for each pass of the sprayer. Prior to starting the bioassays, 2-butoxyethanol pink dye (Vision Pink ™ Foam Marker Dye; Garrco Products Inc., Converse, IN, USA) was added to the insecticide or water solution at a rate of 940 mL per 378 L water. When the dye droplets came in contact with the spray cards, they stained the cards pink. Spray cards were left to air dry in the lab for at least one hour, at which point they were stored in plastic Ziploc bags for later processing.

Each card was individually scanned using an Epson scanner at a resolution of 600 dots per inch (DPI). A cardboard sheet was placed above each card while scanning to ensure that the card remained flat. Due to variation in spray coverage patterns along the vertical and horizontal gradients within each spray card, scanned images were cropped

prior to analysis to capture an approximately 10 cm^2 area directly right of the raspberry (Appendix C, Figure S2). To calculate the percent coverage, each spray card was converted to a binary image (highlighting only the areas of the card stained pink) using the color thresholding function in ImageJ (Schneider et al. 2012). Image thresholding was conducted using the full range of values for hue (0-255) and brightness (0-255). Saturation levels were adjusted as necessary to ensure that only the pink dye spots on the spray card were included and generally ranged between 0 and 25; all spray cards within a given trial were processed by the same individual to minimize measurement bias. Once cards had been converted to a binary image, ImageJ was used to calculate the proportion of the card stained pink, providing a quantitative measure of percent coverage. Across all replicate bioassays, the low and high spray coverage treatments respectively produced 21.8 ± 1.4% and 84.6 ± 1.5% coverage on average (pooled data from Appendix C, Table S1), rates that are comparable to spray coverage levels obtained on commercial farms (Figure 3-3, see below for details).

Impact on Adult Mortality and Larval Infestation Rates.

Once spray treatments were applied, raspberries were dried for 24 hours inside a fume hood and subsequently transferred into individual bioassay arenas. Bioassays arenas were constructed using 59 mL plastic soufflé cups (Comfy Package ©, Brooklyn, NY) with a thin layer of 0.1% water agar (Apex Bioresearch Products) at the bottom to provide moisture. 6.5 cm^2 holes were cut into each deli cup lid and covered with mesh fabric to allow air circulation. Individual bioassay arenas consisted of a single treated raspberry and five female *D. suzukii* between four and seven days old from our laboratory colony.

Prior to loading the bioassays, female *D. suzukii* were lightly anesthetized with CO_2, sorted into groups of five, transferred into vials of Jazz Mix ™ *Drosophila* diet (Fisher Scientific), and held overnight. On the day of the bioassay, flies were re-anesthetized with CO_2 and transferred into the bioassay cup using a paintbrush. Once loaded, flies were monitored to ensure that they fully revived from the CO_2. Flies that did not revive were not replaced but were recorded so that they could be excluded from percent mortality and larval infestation calculations. Flies were held in the bioassay arenas for 24 hours in a temperature controlled growth chamber (22°C, 16:8 L:D cycle), after which we assessed mortality following methods adapted from Van Timmeren et al. (2019). Each fly was classified as either alive, dead, heavily moribund (on their back or side with one or more legs twitching, but unable to walk or stand), or lightly moribund (showing effects due to the insecticide but still able to move around and self-right; behaviors include a slow or staggering walk and wing/leg grooming behavior). Flies classified as either dead or heavily moribund were counted as dead for percent mortality calculations.

We also quantified larval infestation within treated raspberry fruits using methods adapted from Hamby et al. (2014). After 24 hours of exposure to treated fruit, all flies were removed from bioassay arenas, and raspberries were incubated at 22°C for an additional 4-5 days to allow eggs to hatch and larvae to develop into later larval instars. Then, raspberries were gently crushed, submerged, and agitated in a sugar water solution (~ 60 mL sugar mixed with 950 mL of water) for 12 minutes to encourage larvae to exit the fruit. In several experimental replicates, one or more of the flies loaded into a bioassay arena did not revive from the CO_2. To account for this variation, larval infestation density was quantified as the mean number of larvae counted per the starting number of female *D.*

suzukii. Incidence of larvae was calculated as the mean proportion of raspberry fruit with one or more larvae detected.

Statistical Analyses

Because the 468 and 935 L/ha bioassays were conducted over separate periods of time, data from each carrier water volume delivery rate were treated as separate experiments and analyzed separately. All data analyses were conducted in R Studio V. 3.4.1 (R Core Team 2017). Adult mortality data were analyzed using a mixed model binomial logistic regression with the lme4 and car packages (Bates et al. 2015, Fox and Weisberg 2019), which modeled the number of dead versus alive *D. suzukii* against spray coverage treatment as a fixed effect with trial date as a blocking factor. Control bioassay data were excluded from this analysis due to low (<3%) mortality rates across all experimental replicates. Similarly, larval incidence data were also analyzed using a binomial model. Data were pooled by trials (N=4 replicates) with the incidence (presence versus absence within a raspberry fruit) of larvae serving as the response variable. Main effects in the model included insecticide treatment (zeta-cypermethrin or control), sprayer speed, and the insecticide by speed interaction, with experimental date included as a random effect. All binomial models were checked for overdispersion by checking the ratio of the Pearson chi-square statistic to the residual degrees of freedom, and models were not overdispersed.

Due to high variability in female oviposition rates, larval density (mean number of larvae counted per female fly) data were analyzed using a non-parametric Kruskal-Wallis test in the R base package. Data were subset by insecticide treatment prior to analysis, and

models included larval counts as a response variable and spray coverage treatment as a fixed effect.

Field Spray Coverage Trials

Spray coverage trials were conducted in primocane red raspberries and blackberries from 2016 – 2019 on two commercial fruit farms as well as two research farms managed by the University of Maryland: the Western Maryland Research and Education Center, Keedysville, MD (Washington Co., MD) and the Wye Research and Education Center, Queenstown, MD (Queen Anne's Co., MD). Field trials were conducted using various types of equipment and sprayer application settings (Appendix C, Table S2). However, the same procedure was used to quantify percent spray coverage across all trials.

Protocol for Evaluating Spray Coverage.

To account for spatial variation in spray coverage throughout the plant canopy, 10.8 cm x 14.0 cm white paper spray cards (Kromekote Card Stock with a glossy finish and C/2S coating) were deployed at multiple locations within each replicate (Appendix C, Figures S3-S4). All cards were oriented horizontally and perpendicular to the ground, and percent spray coverage was only quantified on the side of the card facing outwards towards the nearest row middle (Appendix C, Figure S4). In raspberries, spray cards were placed in both the inner and outer plant canopy at three heights (six canopy locations total): 0.46 meters (m), 0.91 m, and 1.22 m above ground. Growth from adjacent raspberry plants overlapped to form a continuous canopy; instead of deploying spray cards in individual plants, we spaced replicates at least 0.5 meters apart from one another throughout the row, placing inner and outer canopy spray cards for a given replicate within approximately 0.3 meters of one another. Plant growth in blackberries was more discrete, so we were able to

assign replicates to individual plants. In blackberries, which have a taller canopy, spray cards were deployed at four heights in the inner and outer canopy (eight canopy locations total): 0.46 m, 0.91 m, 1.22 m, and 1.68 m above ground. Spray cards were positioned within the canopy using wooden dowels, which mimicked caneberry cane movement. We used large binder clips to attach spray cards onto two wooden dowels (0.32 cm diameter) that were taped together.

Prior to pesticide application, 2-butoxyethanol pink dye (Vision Pink ™ Foam Marker Dye; Garrco Products Inc., Converse, IN, USA) was added to the tank mix at a rate of 940 mL per 378 L water. We used dye in lieu of water sensitive paper to enable quantification of coverage despite the wet, humid climate. Spray cards were left to dry in the field for up to 24 hours before collection. Cards were individually scanned using an Epson scanner at 600 DPI. A thin outer margin of the card was cropped to ensure that shadows from the card edges did not interfere with analysis. Once cropped, images were analyzed with ImageJ as described previously.

Grower Standard Reference Trials.

Spray coverage trials were conducted in 2016 and 2017 at two commercial diversified fruit farms in Maryland that manage a mixture of caneberries and tree fruit. Through these trials, we were able to quantify typical spray coverage in Maryland commercial caneberry production, which provided a reference point for the spray coverage rates observed in our carrier water volume spray trial; data from these trials were also used to determine bioassay spray coverage treatments. Data were summarized individually for each farm as the mean percent spray coverage ± standard error observed in each of the six spray card canopy locations.

At Site One (Montgomery Co., MD), we quantified spray coverage in primocane raspberries in 2016 and 2017, using different fields each year of the study. At Site Two (Howard Co., MD), spray coverage was quantified in 2017 in primocane raspberries. All spray trials were conducted using airblast sprayers with each farm's standard pesticide application practices (Appendix C, Table S3). Six replicate sets of spray cards were deployed in 2016 and eight in 2017 (inner and outer canopy at varying heights; Appendix C, Figure S3). In all spray trials, replicates were distributed between three rows of plants, with spray cards facing in alternating directions between replicate plants and oriented as previously described (Appendix C, Figure S4).

Effect of Carrier Water Volume on Raspberry and Blackberry Spray Coverage.

Carrier water volume spray coverage trials were conducted from 2016 – 2019 at the Washington Co., MD and the Queen Anne's Co., MD field sites. In all spray trials, we evaluated how two carrier water volume treatments, 468 and 935 liters per hectare (L/ha), impacted spray coverage throughout the canopy of primocane raspberries and blackberries. Spray trials were repeated using four replicate sets of spray cards per carrier water volume treatment for the 31 August (2016) trial and six replicate sets per treatment for all other spray trials. Spray trials were conducted using both a CO_2 powered backpack sprayer and an airblast sprayer with varying configurations.

Airblast spray coverage trials were conducted only at Washington Co. using a Durand Wayland 150 gallon airblast sprayer (#CDP20P150P). The sprayer had a 81.3 cm fan with an orchard head and twelve double rollover nozzles supplied by the tractor manufacturer, of which the bottom three were turned on for each side. Sprays were applied to both sides of the row. To change carrier water volume, we used two sets of hollow cone

nozzles that had differently sized discs and swirl plates, resulting in different discharge rates. Each set of nozzles was calibrated to spray at either 468 or 935 L/ha. For both carrier water volume treatments, the tractor operated at a speed of 5 kilometers per hour (km h^{-1}) and a pressure of 1,034 kPa.

From 2016 – 2017, airblast spray coverage trials were conducted in primocane red raspberries (see Appendix C, Supplemental Methods for further details). During the first trial (31 August 2016), spray coverage was very low in the bottom half of the raspberry plant (Appendix C, Figure S5). In order to correct this issue, the sprayer was set to its lowest height and the nozzle angles were adjusted, turning the two lowest sets of nozzles downward. These sprayer modifications improved lower canopy spray coverage for the remaining raspberry airblast spray trials on 21 September (2016) and 22 October (2017).

For the airblast spray trials conducted in 2018 and 2019, a two-way row crop head (Durand Wayland; #16-4935; Appendix C, Figure S6) was attached to the sprayer. This equipment directs the pesticide spray downwards and minimizes overhead drift.

Additionally, spray trials using a CO_2 powered backpack sprayer were conducted in 2017 at both the Washington Co. and Queen Anne's Co sites. A vertically oriented, 120 cm spray boom with four evenly spaced anti-drip nozzles (TeeJet; #8005VS) was attached to the backpack sprayer and sprays were applied to both sides of the row. At Queen Anne's Co., primocane red raspberries and blackberries were sprayed at 468 and 935 L/ha; at Washington Co., backpack spray trials were only conducted in raspberries due to issues with the sprayer malfunctioning during the blackberry spray trials. To change carrier water volume, we adjusted the walking pace; 468 L/ha treatments were applied at a pace of 100

steps per minute while 935 L/ha treatments were applied using a pace of 50 steps per minute which was standardized using a metronome.

Statistical Analyses.

Separate analyses were conducted for each spray trial using ANOVA in the R base package (R Version 3.4.1). Models for the grower standard reference trials included canopy location, height, and the location by height interaction as fixed effects. Data from the carrier water volume trials were subset by height within a trial for analysis; each model included carrier water volume treatment, canopy location, and the treatment by location interaction as fixed effects. Model residuals were assessed for normality using a Shapiro-Wilk test, and data were transformed as necessary. Residual homogeneity of variance was tested using a Levene's test and weighted least squares were applied as needed with a weighting factor of the $(\text{residual variance})^{-1}$ for the fixed effect that deviated from homogeneity. Pairwise comparisons for the significant fixed effect were conducted using the emmeans package (Lenth, 2018). When overall ANOVA models were non-significant, model statistics are reported instead of treatment effects.

Results

Impact of Spray Coverage on Efficacy Against Drosophila suzukii

When raspberries were treated with zeta-cypermethrin in the laboratory, percent adult mortality increased significantly with spray coverage at both concentrations (Figure 3-1). In the 935 L/ha bioassays, average percent mortality increased by 108% at the high spray coverage rate ($\chi^2_{1,37} = 33.81$, $P < 0.001$). In the 468 L/ha bioassays, average percent mortality increased by 29% between the low and high spray coverage rates ($\chi^2_{1,37} = 11.90$,

P < 0.001). In the water untreated control, mortality rates remained low. In the 935 L/ha bioassays, control mortality was 0% across all experimental replicates. In the 468 L/ha bioassays, control mortality was 0% in the low spray coverage treatment and 2.25 ± 1.56% in the high spray coverage treatment.

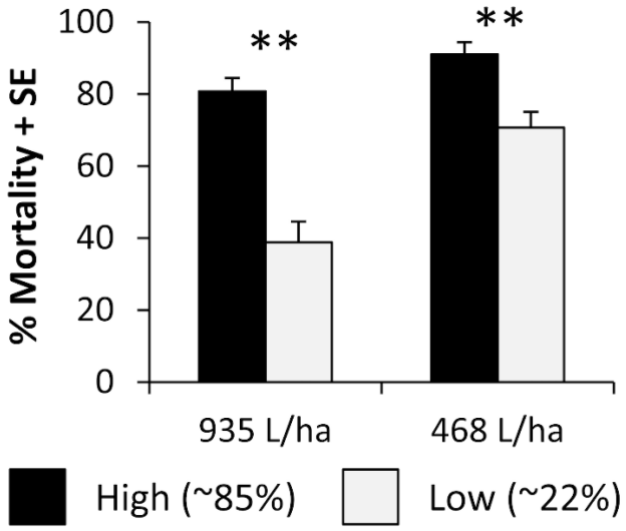

Figure 3-1. Mean percent *D. suzukii* mortality plus standard error (SE) in response to raspberries treated with zeta cypermethrin at high (~85%) and low (~22%) spray coverage rates in laboratory bioassays. Bioassays were conducted using insecticide concentrations equivalent to two carrier water volume application rates: 468 and 935 liters per hectare (L/ha). In the 935 L/ha (low droplet concentration) bioassays, control mortality was 0% across all experimental replicates. In the 468 L/ha (high droplet concentration) bioassays, control mortality was 0% in the low spray coverage treatment and 2.25 ± 1.56% in the high spray coverage treatment. Data were only analyzed for zeta-cypermethrin treated berries. Separate analyses were conducted for each carrier water volume rate using mixed model binomial logistic regression. Percent mortality significantly decreased in the low spray coverage treatments at both 935 L/ha ($\chi^2_{1,37}$ = 33.81, P < 0.001) and 468 L/ha ($\chi^2_{1,37}$ = 11.90, P < 0.001).

Larval densities were highly variable and not significantly affected by spray coverage in either the control [(935 L/ha: χ^2_1 = 0.02, P = 0.876), (468 L/ha: χ^2_1 = 0.51; P = 0.473)] or zeta-cypermethrin treated raspberries [(935 L/ha: χ^2_1 = 0.87, P = 0.351), (468 L/ha: χ^2_1 = 0.92, P = 0.336)]. When raspberry fruit were sprayed with water, the number of *D. suzukii* larvae counted ranged from 0 to 23.4 per starting female *D. suzukii* across the low and high concentration bioassays. In contrast, infestation rates remained consistently

low in fruit treated with zeta-cypermethrin, regardless of droplet concentration or spray coverage treatment (Appendix C, Table S4).

Significantly higher incidences of larval infestation were observed in raspberry fruit sprayed with water compared to zeta-cypermethrin (Figure 3-2) for both the 935 L/ha ($\chi^2_{1,11}$ = 18.12, P < 0.001) and the 468 L/ha ($\chi^2_{1,11}$ = 22.91, P < 0.001) bioassays. In contrast, spray coverage treatment did not affect incidence of infestation in either bioassay [Figure 3-2; (935 L/ha: $\chi^2_{1,11}$ = 1.96, P = 0.162); (468 L/ha: $\chi^2_{1,11}$ = 0.47, P = 0.493)].

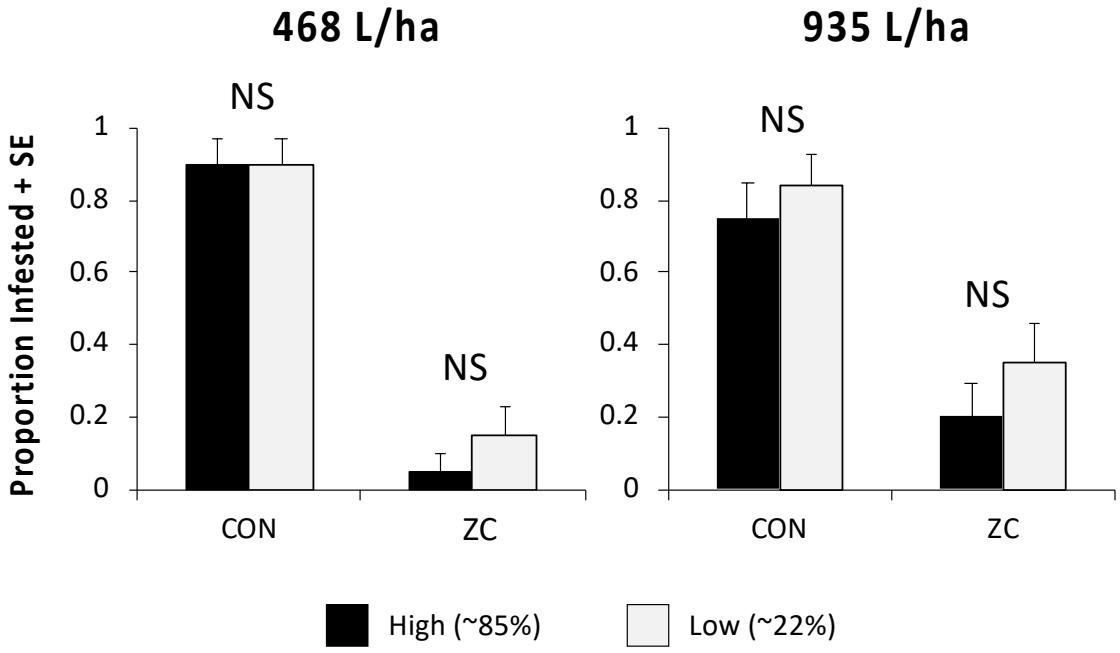

Figure 3-2. Proportion of raspberries plus standard error infested with one or more *D. suzukii* larvae after 24 hours of exposure to five adult female *D. suzukii*. Raspberries were sprayed with either water (untreated control; CON) or zeta-cypermethrin (ZC) at a high (85%) and low (22%) spray coverage rates, and bioassays were repeated using insecticide concentrations equivalent to carrier water volume rates of 468 and 935 liters per hectare (L/ha). Data were analyzed separately for each carrier water volume rate using mixed model binomial logistic regression. The proportion of infested fruit did not differ as a result of percent spray coverage in either the 468 L/ha ($\chi^2_{1,11}$ = 0.47, *P* = 0.493) or 935 L/ha ($\chi^2_{1,11}$ = 1.96, *P* = 0.162) experiments.

Grower Standard Reference Trials

Spray coverage rates were variable. For example, percent spray coverage across all canopy locations ranged from less than 1% to 99% coverage at Site One in both the 2016 and 2017 spray trials. However, spray coverage also tended to vary spatially between different regions of the canopy. Average percent spray coverage varied by canopy height at Site One in 2017 ($F_{2,42} = 14.74$, $P < 0.001$) and Site Two in 2017 ($F_{2,42} = 24.93$, $P < 0.001$), with the lowest coverage occurring 0.46 meters above ground (Figure 3-3). Similar trends in coverage also occurred at Site One in 2016, but the overall model was non-significant (Model $F_{5,29} = 1.99$, $P = 0.110$). At Site One in 2017, canopy location also significantly influenced percent spray coverage; averaged across all heights, mean percent coverage increased from 31.81 ± 6.73% in the inner canopy to 67.41 ± 7.42% (112% increase) in the outer plant canopy ($F_{1,42} = 25.38$, $P < 0.001$). In contrast, there were no significant effects due to canopy location at Site One in 2016 (Model $F_{5,29} = 1.99$, $P = 0.110$) or Site Two in 2017 ($F_{1,42} = 2.09$, $P = 0.156$).

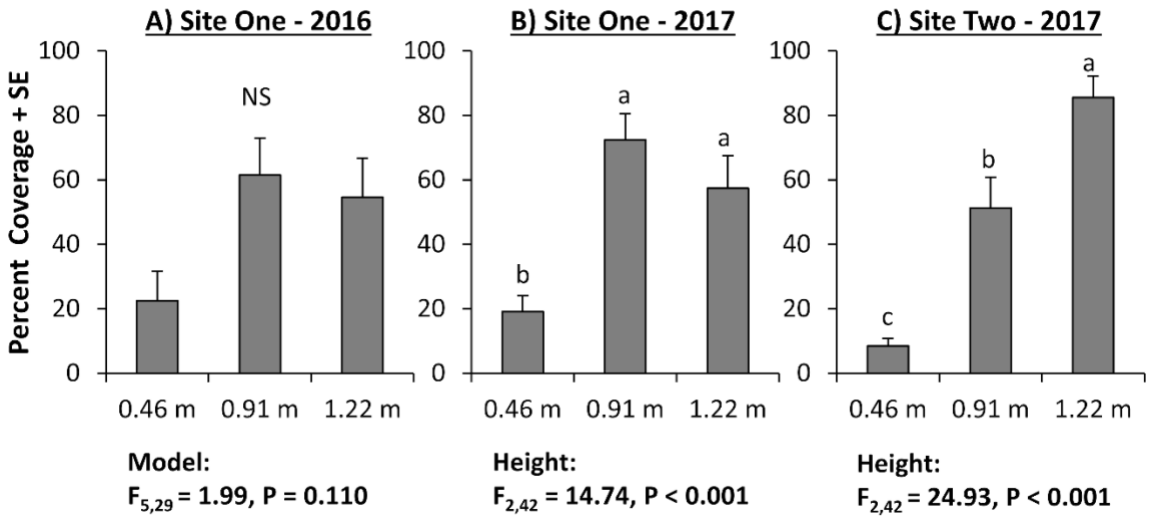

Figure 3-3. Mean percent coverage plus standard error observed at three card heights in commercially managed primocane raspberries at A) Site One in 2016, B) Site One in 2017, and C) Site Two in 2017. Data were analyzed separately for each site and year using an ANOVA with canopy location, canopy height, and the location by height interaction as main effects. Tukey's HSD comparisons were conducted for significant effects (denoted below each graph) for each model, and bars that do not share a letter are significantly different (α=0.05).

Airblast Sprayer Field Trials

During the first airblast sprayer trial (31 August 2016), percent spray coverage at 0.46 m was low compared to the upper canopy heights, ranging from 0.10% to 5.74% coverage (Appendix C, Figure S5). There were no significant treatment effects due to carrier water volume at any height [(0.46 m: Model $F_{3,12}$ = 1.16, P = 0.365), (0.91 m: Model $F_{3,12}$ = 1.59, P = 0.243), (1.22 m: Treatment $F_{1,12}$ = 3.51, P = 0.086)]. However, at 1.22 m, we observed significant reductions in inner canopy spray coverage ($F_{1,12}$ = 10.18, P=0.008) relative to the outer canopy (Appendix C, Figure S5).

In subsequent airblast sprayer trials (21 September 2016 and 22 August 2017), modifications to the sprayer configuration corresponded with increased coverage in the lower canopy; across all 0.46 m cards percent coverage averaged 42.49 ± 4.50% in the 21 August 2016 trial and 47.74 ± 5.76% in the 22 August 2017 trial. Both years, carrier water

volume significantly impacted spray coverage, but effects were not consistent between canopy heights (Figure 3-4). At 1.22 m above the ground, increasing carrier water volume from 468 L/h to 935 L/ha had no impact on spray coverage in 2016 (Model $F_{3,20} = 0.31$, $P = 0.816$), but increased coverage by 265% in 2017 ($F_{1,20} = 13.20$, $P = 0.002$). At 0.91 m, no significant effects due to carrier water volume occurred in 2016 (Model $F_{3,20} = 2.99$, $P = 0.056$) or 2017 (Model $F_{3,20} = 2.25$, $P = 0.114$). Increasing carrier water volume significantly improved spray coverage at 0.46 m in both years [Figure 3-4; (2016: $F_{1,20} = 9.29$, $P = 0.006$), (2017: $F_{1,20} = 7.13$, $P = 0.015$)].

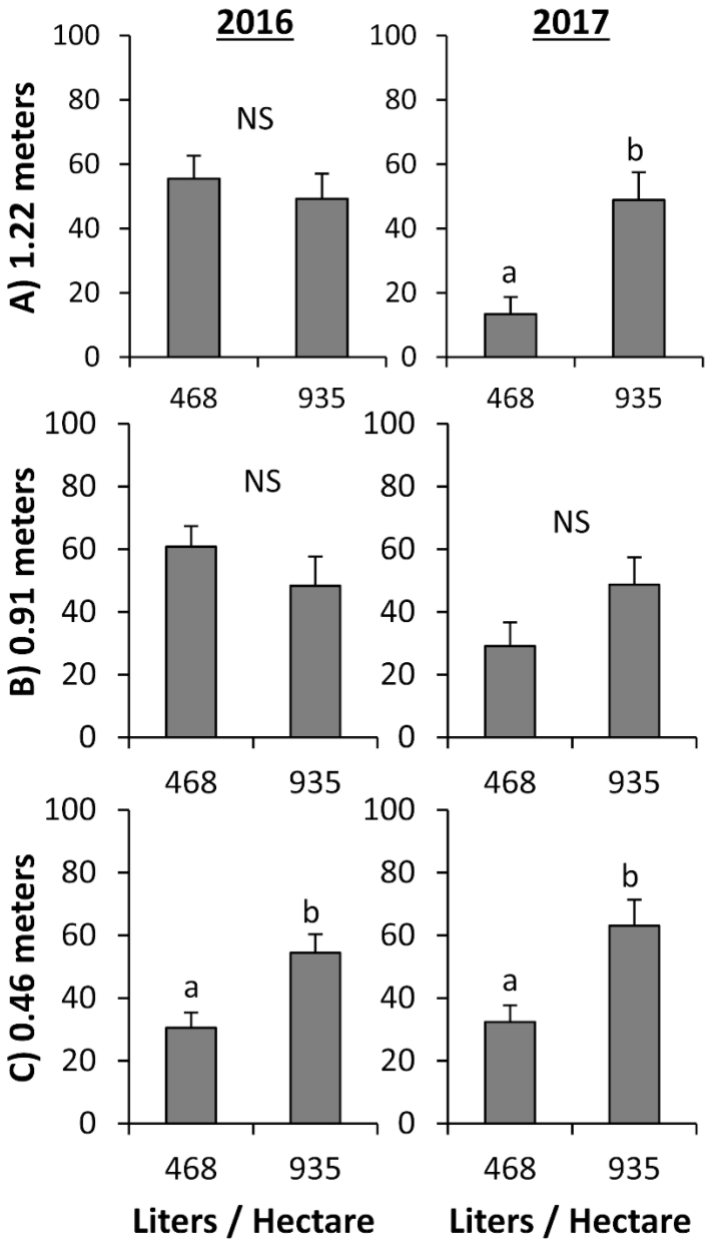

Figure 3-4. Mean percent spray coverage plus standard error observed in raspberry trials that were conducted using an airblast sprayer at a Washington Co. site in 2016 and 2017. Plants were sprayed using two carrier water volumes, 468 and 935 liters per hectare (L/ha) and spray cards were deployed in the inner and outer canopy at three heights: (A) 1.22 meters above ground, (B) 0.91 meters above ground, and (C) 0.46 meters above ground. Data were subset by canopy height and spray trial year prior to analysis, with each model including carrier water volume treatment, canopy location (inner versus outer), and the treatment by location interaction. Percent spray coverage only varied significantly due to carrier water volume treatment, so data were pooled across canopy locations. Within a graph, bars that do not share a letter are significantly different by Tukey's HSD (α=0.05).

Airblast Sprayer with Two-Sided Row Crop Head Field Trials

Using the two-sided row crop head in 2018 corresponded with considerable improvements in blackberry spray coverage, particularly at 0.46 m (Table 3-1); at this height, spray coverage averaged 50.51% ± 6.50%. Spray coverage ranged from 46.65% to 99.99% across the top three canopy heights (0.91 m, 1.21 m, and 1.67 m). There were no differences in coverage due to canopy location or carrier water volume treatment at any height (Table 3-1).

Table 3-1. Mean percent spray coverage ± standard error observed in primocane blackberries treated at 468 and 935 liters per hectare (L/ha) using an airblast sprayer with a two-sided row crop head in 2018. Spray cards were deployed in the inner and outer plant canopy at four heights: 0.46, 0.91, 1.22, and 1.68 meters (m) above the ground. Data were subset by canopy height and analyzed using an ANOVA. At each spray card height, no differences in percent spray coverage were detected due to carrier water volume or canopy location.

Height	Location	468 L/ha	935 L/ha
0.46 m [a]	Inner	70.19 ± 12.41	47.34 ± 11.93
	Outer	51.24 ± 12.51	33.27 ± 13.58
0.91 m [b]	Inner	90.85 ± 6.26	96.86 ± 0.85
	Outer	98.28 ± 0.46	95.20 ± 3.86
1.22 m [c]	Inner	99.68 ± 0.18	93.18 ± 2.86
	Outer	99.62 ± 0.13	99.78 ± 0.19
1.68m [d]	Inner	98.76 ± 0.39	98.46 ± 0.71
	Outer	91.08 ± 8.89	99.99 ± 0.003

[a] Model $F_{3,20} = 1.53$, $P = 0.238$
[b] Model $F_{3,20} = 0.47$, $P = 0.708$
[c] Model $F_{3,20} = 2.04$, $P = 0.141$
[d] Model $F_{3,20} = 0.75$, $P = 0.535$

In contrast to 2018, when we repeated the row crop head spray trials in raspberries in 2019, we found that spray coverage tended to be lower, particularly in the bottom half of the canopy (Figure 3-5). At 0.46 m, percent spray coverage increased by 92% between the 468 and 935 L/ha treatments ($F_{1,20} = 4.57$, $P = 0.045$). We observed no effect of carrier water volume at 0.91 m ($F_{1,20} < 0.01$, $P = 0.951$), but spray coverage rates were significantly

higher in the outer plant canopy relative to the inner ($F_{1,20}$ = 32.65, P < 0.001). At 1.22 m, carrier water volume interacted with canopy location ($F_{1,20}$ = 7.32, P = 0.014); outer canopy spray coverage was significantly higher than inner coverage rates only at 468 L/ha (Figure 3-5).

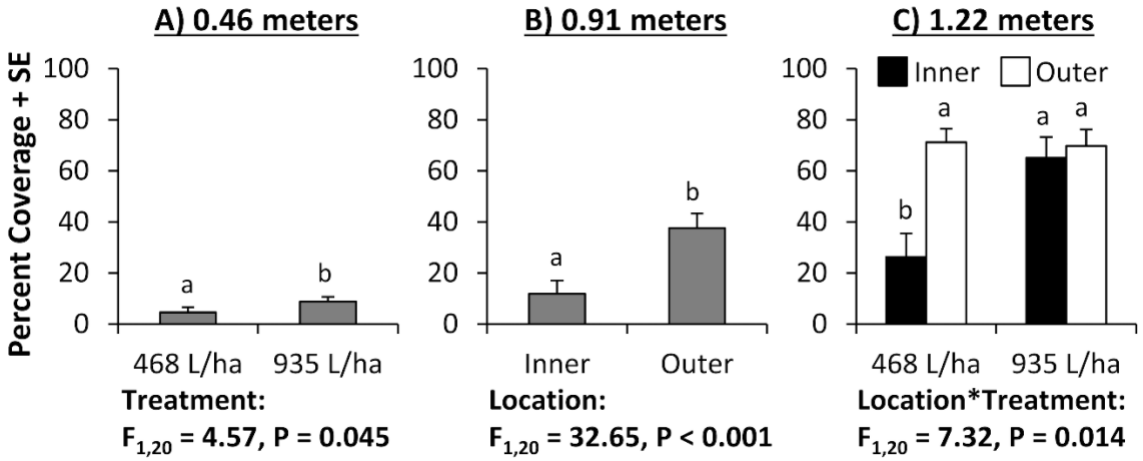

Figure 3-5. Mean percent spray coverage plus standard error in Washington Co. raspberries sprayed at 468 and 935 liters per hectare (L/ha) using an airblast sprayer with a two-sided row crop head attached in 2019. Data were subset and analyzed separately by spray card height: A) 0.46 meters above ground, B) 0.91 meters above ground, and C) 1.22 meters above ground. Each analysis included carrier water volume treatment, canopy location, and the location by treatment interaction as fixed effects. Tukey's HSD comparisons were conducted for significant fixed effects (denoted below each graph) for each model, and bars that do not share a letter are significantly different (α=0.05).

Backpack Sprayer Field Trials

Percent spray coverage in raspberries treated with a CO_2 powered backpack sprayer varied due to both canopy location and carrier water volume treatment; no interactions occurred between canopy location and carrier water volume (Table 3-2). Raspberry spray coverage rates were significantly higher in the outer canopy relative to the inner, and increasing carrier water volume to 935 L/ha increased spray coverage at both field sites and across all canopy heights (Table 3-2).

Table 3-2. Mean percent spray coverage ± standard error observed in the canopy of raspberry plants sprayed using a CO_2 powered backpack sprayer at two different carrier water volume treatments (Trt): 468 and 935 liters per hectare (L/ha). Spray trials were conducted once each at Queen Anne's Co. on 16 August (2017) and in Washington Co. on 22 August (2017). In each trial, spray cards were deployed in the inner and outer plant canopy at three different heights (0.46, 0.91, and 1.22 meters above the ground). Percent spray coverage data were analyzed separately for each spray coverage trial, with data subset by spray card height. Within a given canopy height, numbers within a row that do not share a lower case letter significantly differ due to carrier water volume treatment using Tukey's HSD. Numbers within a column that do not share an upper case letter significantly differ due to canopy location.

Site	Height	Location	468 L/ha	935 L/ha
Queen Anne's Co.	0.46 m[a]	Inner	11.96 ± 1.77 Aa	31.95 ± 8.67 Ab
		Outer	32.44 ± 8.16 Ba	54.96 ± 15.11 Bb
	0.91 m[b]	Inner	22.61 ± 4.96 Aa	52.88 ± 5.45 Ab
		Outer	61.12 ± 2.18 Ba	82.45 ± 5.00 Bb
	1.22 m[c]	Inner	43.85 ± 7.07 Aa	71.69 ± 3.90 Ab
		Outer	63.43 ± 3.10 Ba	79.82 ± 5.11 Bb
Washington Co.	0.46 m[d]	Inner	15.92 ± 4.87 Aa	34.14 ± 5.92 Ab
		Outer	33.40 ± 8.84 Ba	58.25 ± 11.50 Bb
	0.91 m[e]	Inner	10.17 ± 3.53 Aa	18.11 ± 5.19 Ab
		Outer	33.45 ± 7.56 Ba	70.52 ± 11.76 Bb
	1.22 m[f]	Inner	20.22 ± 5.14 Aa	40.73 ± 9.90 Ab
		Outer	35.37 ± 3.89 Ba	70.20 ± 12.06 Bb

[a] Trt $F_{1,20} = 4.54$, $P = 0.046$; Location $F_{1,20} = 10.43$, $P = 0.004$; Trt *Location $F_{1,20} = 0.55$, $P = 0.467$
[b] Trt $F_{1,20} = 31.70$, $P < 0.001$; Location $F_{1,20} = 55.17$, $P < 0.001$; Trt *Location $F_{1,20} = 0.95$, $P = 0.341$
[c] Trt $F_{1,20} = 18.00$, $P < 0.001$; Location $F_{1,20} = 7.74$, $P = 0.011$; Trt *Location $F_{1,20} = 2.06$, $P = 0.166$
[d] Trt $F_{1,20} = 7.69$, $P = 0.012$; Location $F_{1,20} = 5.61$, $P = 0.028$; Trt *Location $F_{1,20} < 0.01$, $P = 0.962$
[e] Trt $F_{1,20} = 6.10$, $P = 0.023$; Location $F_{1,20} = 21.01$, $P < 0.001$; Trt *Location $F_{1,20} < 0.01$, $P = 0.931$
[f] Trt $F_{1,20} = 8.06$, $P = 0.010$; Location $F_{1,20} = 6.34$, $P = 0.020$, Trt *Location $F_{1,20} = 0.04$, $P = 0.836$

In the blackberry spray trial conducted at the Queen Anne's Co. site, canopy location did not interact with carrier water volume at any height (Table 3). Spray coverage rates were higher in the outer canopy relative to the inner at the bottom three canopy heights, while treatment effects due to canopy location were not significant at 1.67 m (Table 3-3). Increased carrier water volume improved spray coverage for cards positioned

0.91 m above the ground. No differences due to carrier water volume were observed at the other three card heights (Table 3-3).

Table 3-3. Mean percent spray coverage ± standard error observed in the canopy of blackberry plants sprayed using a CO_2 powered backpack sprayer at 468 and 935 liters per hectare (L/ha). Spray trials were conducted once each at Queen Anne's Co. on 16 August (2017) and in Washington Co. on 22 August (2017). In each trial, spray cards were deployed in the inner and outer plant canopy at four different heights (0.46, 0.91, 1.22, and 1.67 meters above the ground). Percent spray coverage data were analyzed separately for each spray coverage trial, with data subset by spray card height. Within a given canopy height, numbers within a row that do not share a lower case letter significantly differ due to carrier water volume using Tukey's HSD. Numbers within a column that do not share an upper case letter significantly differ due to canopy location. The treatment by location interaction term was non-significant for all analyses.

Site	Height	Location	468 L/ha	935 L/ha
Queen Anne's Co.	0.46 m [a]	Inner	4.48 ± 2.34 Aa	7.56 ± 6.29 Aa
		Outer	17.50 ± 4.95 Ba	45.07 ± 8.82 Ba
	0.91 m [b]	Inner	2.82 ± 1.03 Aa	4.31 ± 1.52 Ab
		Outer	45.89 ± 7.88 Ba	78.09 ± 6.07 Bb
	1.22 m [c]	Inner	19.20 ± 7.14 Aa	14.79 ± 2.31 Aa
		Outer	45.07 ± 6.07 Ba	69.71 ± 12.87 Ba
	1.67 m [d]	Inner	36.00 ± 8.42 Aa	54.01 ± 13.98 Aa
		Outer	57.59 ± 6.01 Aa	68.05 ± 8.97 Aa

[a] Trt $F_{1,20} = 3.96$, $P = 0.060$; Location $F_{1,20} = 21.60$, $P < 0.001$; Trt *Location $F_{1,20} = 2.71$, $P = 0.115$

[b] Trt $F_{1,20} = 7.27$, $P = 0.014$; Location $F_{1,20} = 76.05$, $P < 0.001$; Trt *Location $F_{1,20} = 0.30$ $P = 0.592$

[c] Trt $F_{1,20} = 0.78$, $P = 0.388$; Location $F_{1,20} = 23.62$, $P < 0.001$; Trt *Location $F_{1,15} = 1.34$, $P = 0.261$

[d] Model $F_{3,20} = 1.33$, $P = 0.293$

Discussion

Spray Coverage Impacts Efficacy Against Drosophila suzukii

Under laboratory conditions, we observed significantly higher adult *D. suzukii* mortality in response to increased zeta-cypermethrin spray coverage. The higher coverage observed may have increased the dose of active ingredient adult flies received. Though we did not quantify pesticide deposition in this study, spray trials in apples, cotton, and soybeans have reported positive associations between pesticide spray coverage and

deposition (Womac et al. 1992; Garcerá et al. 2011; Graziano et al. 2017; Witton et al. 2018), and increasing zeta-cypermethrin deposition positively correlates with *D. suzukii* mortality in vial bioassays (Mermer et al., 2020). Additionally, higher spray coverage may limit *D. suzukii*'s ability to avoid pesticide residues, consequentially increasing exposure.

Adult *D. suzukii* are highly mobile and believed to move between crop and non-crop hosts (Klick et al., 2016). While moving in and out of treated plants, adult flies may encounter lethal doses of insecticide regardless of spray coverage levels because their movement increases contact. Alternatively, zeta-cypermethrin may induce aversive behaviors in adult *D. suzukii*, as has been seen with pyrethroid insecticides in other systems (Quisenberry et al. 1984; Penman et al. 1986; Siegert et al. 2009; Bowman et al. 2018). Laboratory bioassays conducted with spider mites indicate that low spray coverage combined with behavioral avoidance can reduce pesticide efficacy (Martini et al. 2012). Similarly, adult *D. suzukii* may not receive lethal insecticide doses if they are able to avoid landing on treated portions of fruit and foliage at lower spray coverage levels (Kennedy, 1947). However, more detailed behavioral studies would be needed to determine the extent to which spray coverage affects these behavioral responses.

Raspberries sprayed with zeta-cypermethrin were significantly less infested both in incidence and density compared to fruit sprayed with only water. However, we observed no differences in larval infestation due to spray coverage, despite differences in adult mortality. Flies may be able to oviposit before receiving a lethal dose despite variation in coverage and/or even small amounts of zeta-cypermethrin may reduce oviposition (Andreazza et al. 2017; Bezzar-Bendjazia et al. 2016). Exposure to sub-lethal doses of zeta-cypermethrin could also reduce fecundity (Buczek et al. 2013; Bibbs et al. 2018); in

laboratory bioassays, directly spraying female *D. suzukii* with sub-lethal concentrations of lambda-cyhalothrin significantly reduced oviposition rates for up to 168 hours after exposure (Shaw et al., 2019). Landing on insecticide-treated raspberry fruit could therefore preclude *D. suzukii* oviposition, introducing a potential mechanism for insecticides to protect fruit even if adult flies are not killed. Zeta-cypermethrin residues on the fruit tissue could also impact the immature life stages, with insecticide exposure in adult females reducing egg and larval survivorship in the subsequent generation (Costa et al., 2014; Shaw et al., 2019) or insecticide residues penetrating the raspberry fruit tissue to kill the immature stages (Hoffmann et al. 2009; Wise et al. 2009;Wise et al. 2014). Regardless of the mechanism, these results suggest that zeta-cypermethrin can protect fruit from *D. suzukii* infestation despite poor adult mortality.

Without additional testing, we cannot assume that the trends observed in this study consistently occur across all insecticide application scenarios. Here, bioassays were conducted under controlled laboratory conditions; flies were confined to bioassay arenas and we only used relatively fresh (24 hours old) zeta-cypermethrin residues. The age of the insecticide residues may impact residual efficacy against the adult and immature stages (Bellows et al. 1985; Van Timmeren and Isaacs 2013; Disi et al. 2020). Different classes of insecticides would be expected to cause alternate behavioral responses (Perez et al. 2007; Martini et al. 2012), potentially affecting adult mortality and/or larval infestation. Determining how adult and larval responses vary over time and across insecticides will be crucial for understanding how spray coverage impacts *D. suzukii* population dynamics.

Optimizing Caneberry Spray Coverage.

To the best of our knowledge, little work has been done to systemically evaluate spray coverage in caneberries. Results from this study suggest that spray coverage patterns within this system can be highly variable and influenced by multiple factors, including carrier water volume, the type of sprayer used, its configuration, and the environmental conditions during application. Beyond pesticide application parameters, natural fluctuations in plant canopy density may also impact coverage. In this study, we did not measure plant canopy, though this confounding factor did vary across sites and years. Despite variability, spray coverage was generally lower in the inner plant canopy of caneberries relative to the outer, a trend that matches previous assessments in other fruit crops (Cunningham and Harden 1998; Wise et al. 2010; Sharpe et al. 2017) and likely occurs because dense foliage blocks pesticide dispersion. We also observed reduced spray coverage in the lower plant canopy. Contrasting our results, previous assessments in blueberries reported higher rates of pesticide spray coverage and deposition in the lower plant canopy (VanEe et al. 2000; Mermer et al. 2020). This discrepancy may reflect variation between different styles and/or brands of airblast sprayers used in specific studies and/or variation in plant canopy architecture between crops; compared to blueberries, caneberries tend to have denser vegetation in the lower canopy.

These trends have important implications for *D. suzukii* management, given that adult flies exhibit higher activity and oviposition in the inner and lower plant canopy (Diepenbrock & Burrack, 2017; Rice et al., 2017). Uneven spray coverage patterns may create refuges within the plant canopy where adult flies are sub-lethally exposed. In addition to increasing survivorship, uneven insecticide distribution could also accelerate

the development of insecticide resistance (Olson et al. 2004; Shi et al. 2013). Insecticide resistance in *D. suzukii* has not yet been reported outside of California (Gress and Zalom 2019; Van Timmeren et al. 2019), but it is possible that reduced coverage within the inner and lower regions of caneberry canopies could allow reproduction of insecticide tolerant individuals. Strategies to optimize spray coverage within these regions may delay the development of resistance while also improving insecticide efficacy.

Increasing carrier water volume inconsistently improved spray coverage, which may result from differences in sprayer equipment as sprayer design drives droplet dispersal patterns (VanEe et al. 2000; Mermer et al. 2020). In our airblast sprayer trials, using a higher carrier water volume generally improved spray coverage in the lower plant canopy only. This may reflect the fact that baseline spray coverage tended to be sparse in the lower canopy, therefore leaving more room for improvement compared to the upper canopy regions. In other crops, including cotton, soybeans and bell peppers, using air-assisting sprayer technology improves pesticide spray penetration into the target plant canopy over hydraulic sprayer options (Womac et al. 1992; Derksen et al. 2007, 2008). Within this study, we cannot directly compare different sprayer types, because individual spray trials were conducted on separate dates and using different fields. However, this difference between air-assisted and hydraulic sprayer technologies could account for the variable impact that carrier water volume had on inner canopy coverage between the airblast and backpack sprayer trials. The methods used to adjust carrier water volume with each type of equipment may also have affected our results. In the airblast sprayer trials, we utilized two different sets of nozzles that had different delivery rates and drove the tractor at a constant speed, potentially changing droplet size. We were unable to quantify droplet size

due to runoff obscuring individual droplets in many of our spray cards. Anecdotally, spray droplet patterns appeared similar between the two sets of nozzles, though the smaller nozzle outlets used for the lower carrier water volume rate may have increased drift. The same set of nozzles were used for both treatments in the backpack sprayer trials, with carrier water volume instead adjusting by changing the walking pace. Though further testing would be needed for confirmation, our results suggest that the strategy used to adjust carrier water volume may impact coverage patterns.

In addition to increasing carrier water volume, minor sprayer adjustments may also increase the amount of spray reaching the target plant canopy. Anecdotally, we noticed that adjusting the height and angle of our airblast sprayer improved spray coverage in the lower canopy of raspberries. Adding a two-sided row crop head to the airblast sprayer also corresponded with spray coverage improvements in the inner and lower canopy of blackberries in 2018, suggesting that the row crop head helped direct the pesticide spray downwards and into the target plant canopy. Unfortunately, we did not see similar improvements using the row crop head in raspberries in 2019, despite using the same sprayer settings. Some of these differences may reflect the shorter canopy height in raspberries. Abiotic conditions may have also played a role. In 2019, average hourly wind speeds at a local weather station during the pesticide application period ranged from 12.8 – 20.9 kilometers per hour (km h^{-1}), compared with 8.0 – 11.3 km h^{-1} in 2018 (NOAA Land-Based Weather Station; ID 72406693706; Menne et al. 2012). The higher wind speeds in 2019 may have increased pesticide drift (Ucar and Hall 2001; Duga et al. 2015; Nansen et al. 2015), consequentially reducing the amount of spray that hit the target plant canopy. Given the role that abiotic factors play in pesticide drift, monitoring environmental

conditions during pesticide application and sprayer calibration is important to optimize spray coverage and minimize off-target drift.

Several other factors not explored in this study also impact spray coverage. In other systems, settings such as the angle of the airblast deflector plate (Pai et al. 2009), the nozzle style and orifice size (Zhu et al. 2004; Nuyttens and Foqu 2011; Nansen et al. 2015; Ferguson et al. 2016), and nozzle spacing on a boom sprayer (Nuyttens et al. 2004) influence pesticide coverage. Plant canopy management and architecture may also be an important factor (Müller et al., 2018). Tactics that open the plant canopy, such as increased pruning or trellising, may have significant effects on spray coverage patterns (Hall 1991; Austin et al. 2011), though these tactics have not yet been examined within caneberries. Surprisingly few studies have explored approaches to improve spray coverage in caneberry production; solutions that enable growers to quickly optimize application parameters for the crops grown on their farms would improve application efficiency and pest management on diversified fruit farms.

In the absence of more extensive comparisons of the various pesticide application parameters, developing universal management recommendations to improve spray coverage is not feasible. In addition, coverage patterns result from the interaction of specific sprayers with plant architecture, which varies by farm and field. This was highlighted in our airblast sprayer carrier water volume trials. For these reasons, it is commonly recommended that individual farms quantify spray coverage as a best management practice. In addition to the marker dye approach taken in this study, water sensitive paper (WSP) spray cards are available commercially. Because WSP cards change in response to ambient moisture such as rainfall or humidity, they need to be deployed

under dry conditions and analyzed immediately after spraying to generate accurate spray coverage measurements. Despite this, WSP cards may be a more feasible option for commercial growers, particularly in dryer production regions. WSP cards allow growers to evaluate coverage by only spraying water, and cell phone applications and computer software are available to assist with spray card analysis (Zhu et al. 2011; Nansen et al. 2015). Identifying gaps in coverage will help growers decide what adjustments may be appropriate.

In summary, optimizing spray coverage is an important component of caneberry pest management. Higher spray coverage levels throughout the plant canopy can improve the efficacy of a single pesticide application, potentially reducing the number of insecticide sprays needed during the season. Spray coverage could also advance management of other pestiferous insects and plant pathogens (Brink et al. 2006; Van Zyl et al. 2010, 2013) as well as non-insecticidal control alternatives. For example, crop sanitizers may be a potential approach to disrupt *D. suzukii* egg laying behavior (Van Timmeren et al. 2020). Given that adult flies have higher egg laying rates in the inner and lower plant canopy (Diepenbrock & Burrack, 2017; Rice et al., 2017), ensuring that crop sanitizers fully penetrate the canopy may increase their efficacy. Beyond enhancing caneberry pest management, it will be important to understand if spray coverage impacts outputs such as marketable yield or the amount of pesticide sprayed over the course of the growing season. Quantifying these benefits will encourage grower adoption of strategies to optimize caneberry spray coverage.

Conclusions – Working Towards an Integrated Insect and Pathogen Management Approach in Caneberries

Successful integrated pest management (IPM) programs draw upon a variety of chemical, cultural, and biocontrol tactics to manage pest populations and keep damage below economically harmful levels, balancing profitability with environmental and human health risks (Dara, 2019). IPM programs can vary in complexity, with many focusing control tactics on a single pest species. However, IPM programs that target multiple pests and/or multiple cropping systems are more synergistic and provide value (Kogan, 1998; Peterson et al., 2018). Agricultural production systems often face challenges from multiple pests simultaneously, and a unilateral focus on one organism could amplify damage from other pests or lead to missed opportunities to develop management tactics that simultaneously target an entire pest complex (Leach et al., 2020; Norris & Kogan, 2000).

This multifaced IPM model may be particularly important for caneberry production. *Drosophila suzukii* and pathogenic fungi (e.g., Botrytis or Cladosporium fruit rots) continue to constrain raspberry production, with both groups of pests capable of causing major reductions in fruit quality particularly important for caneberry production. *Drosophila suzukii* and plant and yield. Although they overlap temporally, *D. suzukii* and fungal pathogens are frequently treated as separate management issues. However, the work presented in this dissertation collectively highlights the potential breadth of tripartite interactions that occurs between caneberry fruit, *D. suzukii,* and fungal microbes; these interactions may impact insect and pathogen pressure in multiple ways. For example, *D. suzukii* may facilitate new fungal disease (Chapter 2), and management interventions that directly target *D. suzukii,* such as disrupting their interactions with yeast (a potential

downstream application of Chapter 1) or optimizing spray coverage (Chapter 3), may have indirect effects on fungal disease epidemiology.

Within this framework, my overarching dissertation objectives were to advance our knowledge about *D. suzukii*'s interactions with raspberries and fungal microbes and to leverage that knowledge towards more sustainable pest management. Each chapter approaches this question from a different perspective. However, the potential downstream pest management applications that may emerge from each study overlap with one another, emphasizing the importance of developing an integrated insect and pathogen management program for caneberries.

Interactions Between Larval *Drosophila suzukii* and Yeast

Naturally occurring yeasts are widespread within fruiting systems and play an important role in *D. suzukii*'s life history. Like many species of *Drosophila, D. suzukii* closely associates with yeast microbes, and multiple studies have demonstrated that these associations benefit both adult and larval fitness (Bellutti et al., 2017; Bing et al., 2017; Mori et al., 2016; Spitaler et al., 2020). Complementing this growing body of literature, my work (Chapter 1) similarly demonstrates that yeast provide critical nutrients for larval development and that individual species of yeast differentially impact larval fitness and feeding behavior. In my assays, larvae exhibited a strong, consistent feeding preference for one yeast species, *Hanseniaspora uvarum*, a behavior that matches previous tests of adult yeast preferences (Scheidler et al., 2015) and previous surveys of yeast associations in field-collected adults and larvae (Hamby et al., 2012, Lewis et al., 2019). Collectively, these studies indicate a tight association between *D. suzukii* and *Hanseniaspora uvarum*,

suggesting that it may be an important species of yeast to target for downstream, pest management applications.

Each of *D. suzukii*'s interactions with yeast represent weak points in its life cycle that may potentially be exploited for more sustainable pest management (Hamby & Becher, 2016). For example, yeasts could be incorporated into insecticidal baits (Knight et al., 2015; Noble et al., 2019) or used to deliver insecticidal RNAi (Murphy et al., 2016). Recent research efforts have also explored using crop sanitizers to disrupt these naturally occurring interactions (Sial et al., 2019; van Timmeren et al., 2020). Crop sanitizers (e.g. hydrogen peroxide and/or peracetic acid) are used to kill microorganisms that have colonized the surface of crop plants, including yeasts and bacteria (Bachelli et al., 2013) as well as fruit rots such as *Botrytis* (Ayoub et al., 2018). They are most commonly applied post-harvest to manage microbial spoilage. However, it is possible that pre-harvest applications of sanitizer products to caneberries could remove yeasts from the surface of fruits, rendering the fruit less attractive to adult flies for oviposition (Sial et al., 2019; van Timmeren et al., 2020). Pre-harvest sanitizer applications may also positively benefit pathogen management. In addition to directly killing fungal propagules (Ayoub et al., 2018), reduced *D. suzukii* oviposition rates might minimize the number of wound-facilitated fungal infections that develop (Swett et al. 2019). Conversely, crop-sanitizer induced changes to the composition or density of the yeast community could positively increase fungal infections. Yeast, fungi, and bacteria engage in competitive interactions (Deveau et al., 2018; Janisiewicz et al., 2010), and reduced yeast density might increase a fungal pathogen's competitive ability. The outcome of these interactions are complex and will require further lab and field-based studies to fully understand.

***Drosophila suzukii* as a Potential Vector of Raspberry Fruit Rot Fungi**

Drosophila suzukii may have a strong, direct impact on fruit rot epidemiology. Feeding and oviposition wounds can facilitate new infections (Rombaut et al., 2017; Swett et al., 2019), and adult flies may increase disease incidence by transmitting fungal pathogens to healthy fruit (Chapter 2). Surveys of the adult *D. suzukii* fungal community demonstrate that flies acquire and carry viable fungal propagules of known primary fruit rot pathogens, most notably *Cladosporium cladosporioides, Cladosporium pseudocladosporioides*, and *Colletotrichum fiorinae*. Although these associations occurred at low rates, they were consistently observed across multiple field sites and sampling dates. Furthermore, laboratory studies demonstrate that adult flies are capable of transmitting fungal pathogens to new substrates. Together, this work suggests that it may be possible for *D. suzukii* to transmit fungi to fruit, although further work is needed to understand how these results will translate to more complex field conditions.

If *D. suzukii* is able to transmit plant pathogens, then management interventions that target the flies may act synergistically to also reduce fungal damage. Similar relationships have been seen in other systems. For example, *Drosophila* spp. can facilitate sour rot disease in grapes, and recent field studies have demonstrated that integrating insecticides that target *Drosophila* with antimicrobial agents that target the yeasts and bacteria responsible for grape sour rot significantly reduces disease incidence (Hall, Loeb, & Wilcox, 2018; M. E. Hall, Loeb, Cadle-Davidson, et al., 2018; Ioriatti et al., 2017). In onions, lowering the nitrogen fertilization rates corresponded with reduced thrip and bacterial bulb rot disease pressure in thrip resistant onion cultivars (Leach et al., 2020). Similarly, biological or cultural control tactics that reduce *D. suzukii* pressure, such as

exclusion netting (Leach et al., 2016; Schöneberg et al., 2021; Stockton et al., 2020), may also slow disease transmission rates and reduce insecticide and fungicide inputs needed within the system.

Optimizing Caneberry Spray Coverage for *Drosophila suzukii* Management

Improved spray coverage provides another opportunity to more effectively manage insects and pathogens. The dense vegetation found in mature caneberry plants can block pesticide dispersion, leading to stratified deposition patterns where the majority of the insecticide lands in the outer region of the plant canopy. This impedes our ability to control both insects and fungal pathogens. *D. suzukii* adults are more active in the interior and lower plant canopy (Diepenbrock & Burrack, 2017; Rice et al., 2017), and surveys of *Cladosporium* fruit rot have similarly found higher disease incidence within the interior plant canopy (Swett et al. 2019). Taking steps to optimize interior canopy spray coverage will therefore improve both insecticide and fungicide efficacy. Indeed, higher spray coverage levels positively correlate with increased adult *D. suzukii* mortality (Chapter 3) and improved control for fungal pathogens (Barber et al., 2003; Brink et al., 2006).

There are a number of potential approaches that can be taken to optimize spray coverage, including adjustments to the carrier water volume (Chapter 3), crop-specific sprayer calibration (Airblast 101), or the addition of adjuvants to the pesticide tank mix. Trellising or pruning may also increase pesticide spray penetration within the interior plant canopy (Vanee et al., 2000). In addition to directly increasing spray coverage and pesticide efficacy, canopy manipulation may indirectly impact insect and pathogen pressure. Reducing the canopy density can alter the microclimate, for example increasing sunlight penetration and temperature while decreasing the relative humidity. This may create

conditions less suitable for both *D. suzukii* (Schöneberg et al., 2020) and fruit rot fungi (Austin et al., 2011; Kraus et al., 2018).

Conclusions

The introduction of *Drosophila suzukii* to the United States has significantly disrupted IPM within caneberry production systems. Due to the combination of a low tolerance for larval infestation and a lack of effective management options, growers currently rely on calendar-based applications of insecticides to achieve consistent control. Recent advances in cultural and biological control tactics will increase the suite of management tactics available to control *D. suzukii*, allowing for more ecologically and economically sustainable management programs. As these programs are developed, it will be critical to continue accounting for tripartite interactions between *D. suzukii,* fungal microbes, and caneberry plants. Indeed, *D. suzukii* closely interacts with yeast and fungal microbes throughout its life history; in addition to providing new targets for sustainable pest management (Hamby & Becher, 2016), these interaction provide critical insights into the ecology of *D. suzukii* that need to be considered in the development of a sustainable IPM program (Peterson et al., 2018) Additionally, management interventions that target *D. suzukii* may indirectly impact fruit rot fungi. Understanding and accounting for these interactions will facilitate the development of an IPM program that targets the entire caneberry pest complex and helps growers produce crops in an economically and ecologically sustainable manner.

Appendix A: Supplemental Materials for Chapter One.

Supplemental Methods

Assay Arena Construction and Setup

Assay arenas were constructed using large (100 × 15 mm) petri dishes filled with ~ 20 mL of 2.74% water agar (deionized water and granulated agar; Fischer BioReagents, Fairlawn, NJ, USA). Once the agar solidified, two 7 mm cores were removed from either end of the petri dish using the large end of a sterile 1000 uL pipette tip (Mettler Toledo Rainin Instrument, LLC, Oakland, CA, United States). The holes were filled with freshly autoclaved potato dextrose agar (PDA; Difco™; Sparks, MD, USA) and allowed to cool. Yeast options were plated on each PDA core and allowed to incubate at room temperature 48 hours prior to starting the assay. To visualize larval feeding preferences, 6 uL of autoclaved neon pink or neon blue food coloring (McCormick and Company, Inc.) were pipetted onto each core five minutes prior to starting each assay.

All preference assays were conducted using second instar *Drosophila suzukii* larvae that were approximately 48 hours old. To standardize the age of the larvae, female *D. suzukii* adults were first placed on a grape juice agar plate for 48-72 hours, during which time they laid eggs within the media. Eggs were then collected using fine-tipped forceps, transferred to a small petri dish (60 × 15 mm) along with a small chunk of *Drosophila* diet, and incubated at 22°C for approximately 48 hours, at which point larvae were collected for assays.

To extract the larvae from the food, the diet was gently crushed and squirted with water, and larvae were removed using fine tipped paint brushes. All larvae were visually inspected under the microscope to confirm that they had reached the second instar by

examining their mouth parts and the posterior/anterior spiracles. Briefly, first instar larva have very small mouthparts that appear to be small black dots, and the second instar larvae have larger and clearer mouth hooks. Second instar larvae also have a clubbed anterior spiracle, while third instar larvae have a fanned anterior spiracle and dark orange rings on their posterior spiracle (Shingleton Lab, Michigan State University, Discriminating Among *Drosophila* Instars).

We then transferred approximately 40 second instar larvae into a small petri dish containing a single moistened sheet of filter paper, and held them without food for one hour to increase the likelihood of a choice being made. After the starvation period, 40 larvae were transferred into the assay arena (each larva was visually inspected to ensure that it had not been damaged during the one hour starvation period).

Yeast Feeding Confirmation

To confirm that our visual metric (color) of larval *D. suzukii* preference reflected actual larval feeding, we evaluated the gut microbial community in confirmation assays that were conducted separately from larval yeast preference assays. Confirmation assays were only conducted using *H. uvarum, P. kluyveri,* and *S. cerevisiae*. Each combination of yeast comparisons was replicated twice for these confirmation assays. Between replicates, we switched the color of food coloring (red or blue) used to stain each yeast species.

Larval preference assays were set up exactly as previously described for all yeast treatment combinations, and larvae were allowed one hour to feed. At the end of the hour feeding period, one larva typical of a red, blue, and purple specimen was removed from the assay arena, and the culturable gut microbial community was assessed (Hamby et al. 2012). Briefly, each larva was surface sterilized using 70% ethanol, placed in the center of a Rose

Bengal chloramphenicol agar plate (RBCA) and allowed to crawl over the surface for 30-60 minutes. Plates were prepared using Rose Bengal chloramphenicol agar base (Oxoid, United Kingdom) according to the manufacturer's instructions and amended with 0.1 g/L chloramphenicol (Sigma-Aldrich, St. Louis, MO, USA). While on the media, each larva deposited fecal pools containing fungi that survived digestion. We isolated and purified two colonies of each yeast morphological type. One strain was then randomly selected to be sequence identified to the species or genus level using the methods described in Lewis et al. 2018. As an additional control, we also isolated the culturable gut microbial community from two larvae that were starved for the one hour period but did not feed on any yeast.

Overall, these confirmation assays indicate that larval *D. suzukii* fed on yeasts in the larval feeding preference assays. Our yeast feeding assessments lined up fairly well with the larval gut microbial community, particularly in comparisons between *H. uvarum* and *S. cerevisiae* (Table S7). In two replicate assays involving *Pichia kluyveri*, we did isolate *P. kluyveri* from larvae scored as having fed exclusively on the alternative yeast (denoted in bold on Table S7). It is possible that this discrepancy reflects imperfect surface sterilization of the larvae in ethanol; larvae may have picked up yeast spores while moving through the arena. Alternatively, the food coloring that we used may not have stained *P. kluyveri* as well as the other yeast species; consequentially, larval preference for *P. kluyveri* may have been underestimated in these assays. We also found that the majority of larvae within this study had an additional yeast contaminant within their digestive tract, *Pichia occidentalis*. Given that we used non-sterile larvae in the preference assays, it seems likely that this yeast was present in our colony at the time of the experiments.

Nutritional Analysis

All nutritional analyses were performed by Medallion Labs (General Mills D.B.A. Medallion Labs, Minneapolis, MN, Analysis Conducted 28 November 2018 and 9 January 2019).

Protein (Dumas) Test

To determine total protein content a minimum of 20 grams of diet was combusted at a high temperature in the presence of pure O_2. Any non-nitrogenous combustion products (water vapor, oxygen, and CO_2) were removed with thermoelectic coolers and chemical sorbents, leaving only N_2 and NO_x. The latter product was reduced to N_2 by passage through a column packed with hot copper. The quantity of remaining N_2 was then determined and multiplied by a predetermined conversion factor (6.25), providing an estimate of total protein content as a percent of the original sample weight. This test had a detection limit of 0.125% nitrogen or 0.781% protein.

Fat by Gravimetric Test: Lipids were extracted from a minimum of 10 grams of fly diet via hydrolisis and partitionined into a mixed ether. The extract was dried and weighed, providing a measure of lipid residue as a percent of the total original sample weight. This test had a detection limit of 0.1% lipid.

Ash Test: Total ash content was determined by combusting a minimum of 10 grams of fly diet at a high temperature. Once the remaining residue cooled, it was weighed and reported as a percentage of the original sample weight. This test provided a measure of all non-combustible minerals within the fly diet, including salts, carbonates, and silicates. The detection limit for this test was 0.012%.

Moisture Test: To determine the total moisture content, a minimum of 10 grams of fly diet were heated in an oven for a specified period of time. The remaining fly diet was then weighed, and the percent weight loss calculated as an estimate of moisture. This test had a detection limit of 0.012%.

Carbohydrates and Calories by Calculation: The total carbohydrate content (g/100g diet) and total calories were estimated using results from the ash, fat, moisture, and protein analysis. Total calories and carbohydrate were calculated using the following equations respectively : $Carbohydrates = 100 - (\%Ash) - (\%\ Total\ Fat) - (\%\ Moisture) - (\%\ Protein)$ and $Calories = (4 \times Carbohydrates) + (9 \times Total\ Fat) + (4 \times Protein)$.

Supplemental Tables

Table S1. Recipe used to prepare experimental diets for the larval development studies. Diets were prepared following the standard yeast-cornmeal-molasses diet used to maintain our laboratory stocks. Diets were prepared using no yeast (Control) or with a standardized quantity of frozen and heat-killed *H. uvarum* (HU), *P. kluyveri* (PK), *I. terricola* (IT), or *S. cerevisiae* (SC). To compare the nutritional value of these experimental diets with a standard *Drosophila* stock recipe (used to rear our laboratory colony and purchased from a University of Maryland facility for the colony), we conducted proximate nutritional analysis on all diets used in the development study as well as for the laboratory standard diet that was prepared using freeze-dried *S. cerevisiae*.

Ingredient	Control	HU, SC, PK, or IT	Freeze-Dried SC*
Water	82.5 mL	82.5 mL	82.5 mL
Molasses	4.5 mL	4.5 mL	4.5 mL
Agar	0.468 g	0.468 g	0.468 g
Yeast	NA	5.4 g	5.4 g
Cornmeal	6.84 g	6.84 g	6.84 g
Tegosept	1.4 mL	1.4 mL	1.4 mL
Propionic acid	0.233 g	0.233 g	0.233 g

***** Diets were only prepared using freeze-dried *Saccharomyces cerevisiae* for use in proximate nutritional analysis

Table S2. Larval feeding in control yeast preference assays. Data is presented as the mean percentage ± standard error of larval *D. suzukii* that chose to feed on either the red or blue yeast in control yeast preference assays. HU = *Hanseniaspora uvarum*, IT = *Issatchenkia terricola*, PK = *Pichia kluyveri*, SC = *Saccharomyces cerevisiae*, WP = *Wickerhamomyces pijperi*.

Comparison	% Red Larvae	% Blue Larvae	% Non-Responders	*T*	df	*P* (t<0.05)
HU-HU	45.9 ± 4.2	43.1 ± 3.4	11.0 ± 2.3	0.644	5	0.5477
PK-PK	43.4 ± 4.0	43.7 ± 3.3	12.9 ± 3.9	-0.071	5	0.946
SC-SC	34.8 ± 5.3	36.6 ± 4.5	28.6 ± 9.5	-0.791	5	0.465
IT-IT	26.2 ± 2.1	29.2 ± 2.8	44.6 ± 3.3	-0.795	5	0.457
WP-WP	39.5 ± 5.2	37.7 ± 3.1	22.8 ± 6.9	0.474	5	0.656

Table S3. Molecular identification to confirm larval yeast feeding in laboratory preference assays. Yeast strains isolated from larvae that were scored as either red, blue, or purple in binary yeast preference assays. All yeast strains were isolated and sequence identified to species. Data for each strain identified is presented, including the percent homology, the number of base pairs in the sequence, and the GenBank match accession number. The percentage of total colonies belonging to each morphospecies was visually estimated. Strains in bold represent identifications that contradict our visual assessment of yeast feeding.

	Rep	Red Yeast	Blue Yeast	Specimen color	% Homology	Bp	GenBank Match Accession #	% of Colonies	Genus	Species
HU vs PK	1	HU	PK	Red	100	547	KT922472.1	100%	*Hanseniaspora*	*uvarum*
		HU	PK	Blue	100	526	U76348.1	100%	*Pichia*	*occidentalis*
		HU	PK	Purple	99.8	550	U75727.1	99%	*Pichia*	*kluyveri*
		HU	PK	Purple	100	551	DQ104733.1	1%	*Pichia*	*kluyveri*
	2	PK	HU	Red	100	537	AB847520.1	100%	*Pichia*	*occidentalis*
		PK	HU	Blue	100	548	KT922893.1	80%	*Hanseniaspora*	*uvarum*
		PK	**HU**	**Blue**	**99.8**	**538**	**U75727.1**	**10%**	***Pichia***	***kluyveri***
		PK	HU	Blue	100	542	KT9230371.1	10%	*Hanseniaspora*	*uvarum*
		PK	HU	Purple	99.8	532	DQ104733.1	70%	*Pichia*	*kluyveri*
		PK	HU	Purple	99.8	536	U75727.1	30%	*Pichia*	*kluyveri*
HU vs SC	1	HU	SC	Red	100	553	KT922893.1	99%	*Hanseniaspora*	*uvarum*
		HU	SC	Red	100	504	KT923037.1	1%	*Hanseniaspora*	*uvarum*
		HU	SC	Blue	100	541	HM165257.1	98%	*Saccharomyces*	*cerevisiae*
		HU	SC	Blue	100	535	U76348.1	2%	*Pichia*	*occidentalis*
		HU	SC	Purple	99.3	560	HM165257.1	95%	*Saccharomyces*	*cerevisiae*
		HU	SC	Purple	100	534	U76348.1	5%	*Pichia*	*occidentalis*
	2	SC	HU	Purple	100	552	GP011558.1	99%	*Saccharomyces*	*cerevisiae*
		SC	HU	Purple	100	549	CP011558.1	1%	*Saccharomyces*	*cerevisiae*
		SC	HU	Blue	99.8	549	KT923037.1	99%	*Hanseniaspora*	*uvarum*
		SC	HU	Blue	100	550	KT923037.1	1%	*Hanseniaspora*	*uvarum*
		SC	HU	Red	100	553	HM165257.1	100%	*Saccharomyces*	*cerevisiae*

Table S4. Proximate nutrient analysis of experimental diets used in *D. suzukii* development studies. Diets were analyzed for total caloric content (presented as the calories per 100 grams) and the relative percentage of ash, moisture, carbohydrates, fats, and protein using standard proximate methods. All nutritional analysis were performed by Medallion Labs Inc. and repeated twice using diet prepared on two separate dates (Rep 1 and Rep 2). In some samples, the percentage of total fat and protein fell below the minimum detectable threshold and is indicated by the notations <0.5 and <0.781 respectively.

Treatment	Calories (per 100g)		Percent Ash		Percent Moisture		Percent Carb		Percent Fat		Percent Protein	
	Rep 1	Rep 2	Rep 1	Rep 2	Rep 1	Rep 2	Rep 1	Rep 2	Rep 1	Rep 2	Rep 1	Rep 2
CON	38	37	0.222	0.218	90.271	90.486	9.5	9.3	< 0.5	<0.5	< 0.781	<.781
HU	44	42	0.276	0.332	88.781	89.094	10.9	9.4	< 0.5	<0.5	< 0.781	1.16
IT	41	40	0.207	0.281	89.486	89.786	9.2	9.9	< 0.5	<0.5	1.12	<.781
PK	41	38	0.272	0.267	89.548	90.324	9.2	9.4	< 0.5	<0.5	1.01	<.781
SC	41	39	0.245	0.318	89.48	89.886	8.6	9.8	< 0.5	<0.5	1.69	<.781
DRY	52	53	0.385	0.462	86.653	86.191	10.4	11.1	< 0.5	<0.5	2.57	2.27

Table S5. Larval *D. suzukii* feeding preferences for all comparisons involving *Pichia kluyveri*. Data is presented as the mean percentage of larvae ± standard error that responded to each yeast (N=12 replicate binary choice assays). HU = *Hanseniaspora uvarum*, IT = *Issatchenkia terricola*, PK = *Pichia kluyveri*, SC = *Saccharomyces cerevisiae*, WP = *Wickerhamomyces pijperi*.

Comparison	% PK	% Other	*T*	df	*P*(T<0.05)
PK - IT	34.8 ± 2.0	35.2 ± 3.1	0.114	11	0.912
PK - HU	27.1 ± 2.2	59.7 ± 2.5	7.468	11	< 0.001
PK - SC	33.7 ± 2.5	42.2 ± 2.6	-2.057	11	0.032
PK - WP	35.7 ± 2.4	37.2 ± 3.0	-0.516	11	0.616

Table S6. Larval *D. suzukii* feeding preferences for all comparisons involving *Issatchenkia terricola*. Data is presented as the mean percentage of larvae ± standard error that responded to each yeast (N=12 replicate binary choice assays). HU = *Hanseniaspora uvarum*, IT = *Issatchenkia terricola*, PK = *Pichia kluyveri*, SC = *Saccharomyces cerevisiae*, WP = *Wickerhamomyces pijperi*.

Comparison	% IT	% Other	T	df	*P*(T<0.05)
IT - PK	35.2 ± 3.1	34.8 ± 2.0	0.114	11	0.912
IT - HU	23.1 ± 2.1	61.4 ± 2.6	8.601	11	< 0.001
IT - SC	31.5 ± 4.8	24.8 ± 3.7	-1.211	11	0.251
IT - WP	41.6 ± 3.8	32.7 ± 3.1	1.775	11	0.104

Table S7. Larval *D. suzukii* feeding preferences for all comparisons involving *Wickerhamomyces pijperi*. Data is presented as the mean percentage of larvae ± standard error that responded to each yeast (N=12 replicate binary choice assays). HU = *Hanseniaspora uvarum*, IT = *Issatchenkia terricola*, PK = *Pichia kluyveri*, SC = *Saccharomyces cerevisiae*, WP = *Wickerhamomyces pijperi*.

Comparison	% WP	% Other	*T*	df	*P*(T<0.05)
WP - IT	32.7 ± 3.1	41.6 ± 3.8	1.775	11	0.104
WP - PK	37.2 ± 3.0	35.7 ± 2.4	-0.516	11	0.616
WP - HU	30.1 ± 3.9	50.9 ± 3.8	3.042	11	0.011
WP - SC	45.9 ± 4.3	28.6 ± 3.5	-2.286	11	0.043

Supplemental Figures

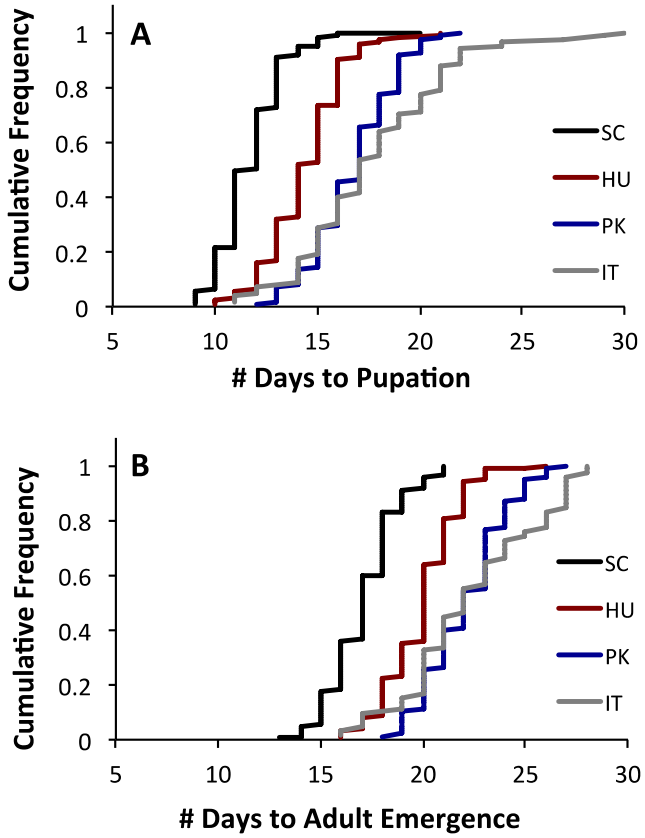

Figure S1. Cumulative frequency curves for the (A) larval (1st instar larvae to pupa) development time and (B) total (1st instar to adult) development time.

Appendix B: Supplemental Materials Chapter Two

Supplemental Tables

Table S1. Complete list of fungal genera isolated from field-collected *Drosophila suzukii* in 2018, 2019, and 2020 showing the number of flies with each fungal genus present or absent in the interior and exterior regions.

	2018		2019		2020	
Genus	**Exterior**	**Interior**	**Exterior**	**Interior**	**Exterior**	**Interior**
Cladosporium	8	0	5	1	20	4
Penicillium	6	1	3	3	4	4
Mucor	4	4	0	1	6	4
Aspergillus	8	4	2	0	2	2
Fusarium	5	0	1	0	6	4
Gilbertella	0	0	2	1	5	3
Colletotrichum	1	0	1	2	3	1
Geotrichum	1	0	0	0	1	6
Botrytis	3	0	0	0	2	1
Alternaria	1	1	0	0	2	0
Bjerkandera	2	2	0	0	0	0
Curvularia	1	1	0	0	2	0
Gonatobotryum	0	0	1	1	1	1
Sarocladium	1	0	1	0	1	1
Stereum	1	1	0	0	2	0
Epicoccum	0	0	1	1	1	0
Pilidium	0	1	0	0	1	1
Talaromyces	0	1	0	1	0	1
Trametes	2	1	0	0	0	0
Irpex	1	1	0	0	0	0
Cercospora	0	0	0	0	1	0
Ceriporia	1	0	0	0	0	0
Chaetomella	1	0	0	0	0	0
Clonostachys	0	0	0	0	1	0
Coniella	0	0	0	0	0	1
Gibellulopsis	0	0	0	0	1	0
Isaria	0	0	0	0	1	0
Lecanicillium	0	0	0	0	1	0
Monochaetia	0	0	0	0	1	0
Myrothecium	0	0	0	0	1	0
Pestalotiopsis	0	0	0	0	0	1
Pithomyces	0	0	0	0	1	0
Pseudopithomyces	0	0	1	0	0	0
Schizophyllum	1	0	0	0	0	0
Seimatosporium	0	0	0	0	1	0
Trichoderma	1	0	0	0	0	0
Tyromyces	0	0	0	0	1	0

Supplemental Figures

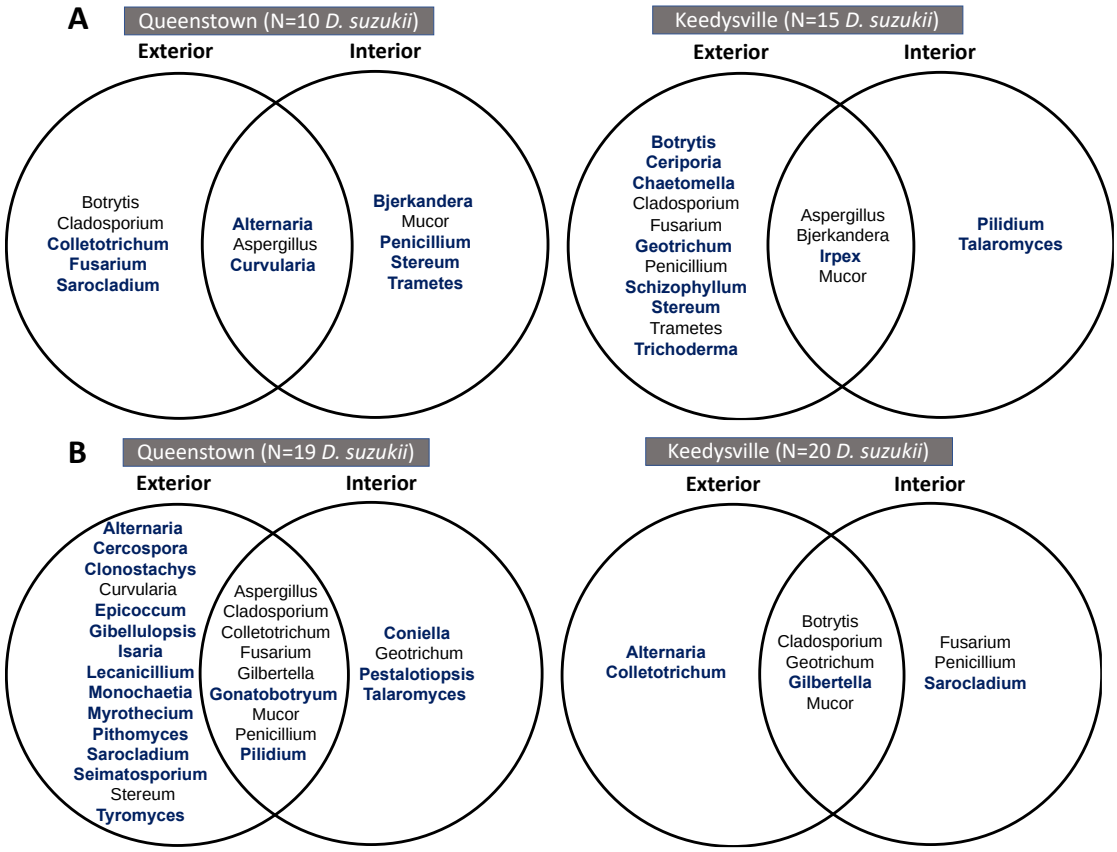

Figure S1. Venn Diagram showing distribution of genera that accumulated externally, internally, or in both regions at the Queenstown and Keedysville field sites in (A) 2018 and (B) 2020 (data pooled across sampling dates). Bolded / blue text denotes genera that were only isolated from one fly.

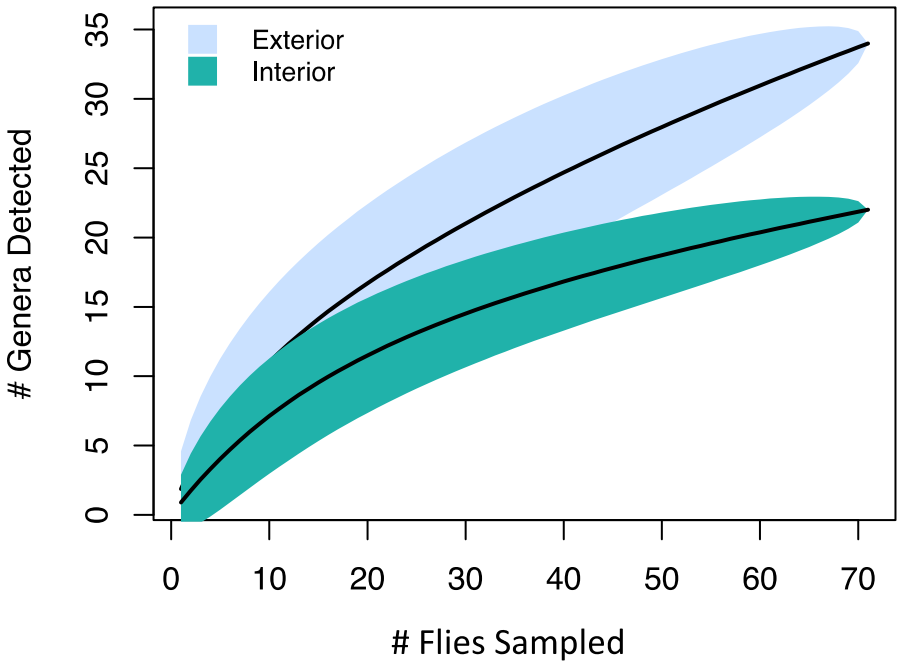

Figure S2. Species accumulation curve showing the number of fungal genera detected when a given number of field-collected adult *Drosophila suzukii* were sampled, split between internal and external fungi. Data were pooled across all years and sampling sites. This figure was developed using the vegan package (Oksanen et al. 2020) in R and plotted with the R Base package.

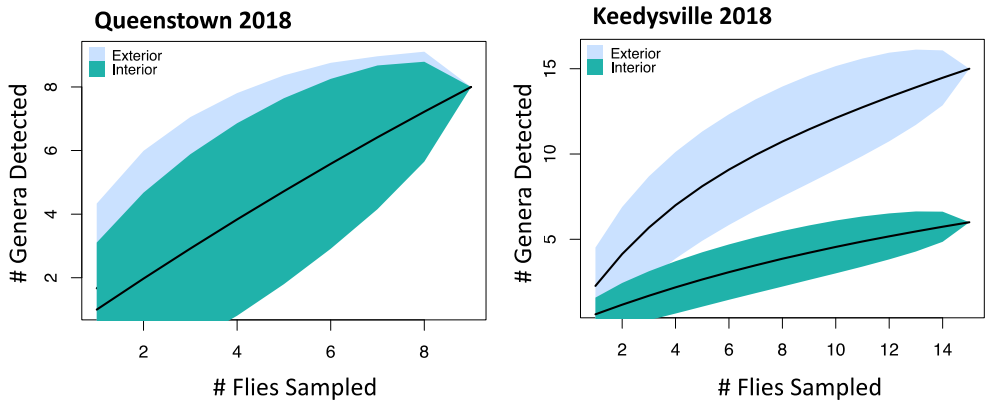

Figure S3. Species accumulation curve for *D. suzukii*'s fungal associations in 2018 at Queenstown and Keedysville. Each graph shows the number of fungal genera detected when a given number of field-collected adult *Drosophila suzukii* were sampled, split between internal and external fungi. The x- and y-axis on each graph are scaled differently due to varying overall genus richness and sample numbers across each site.

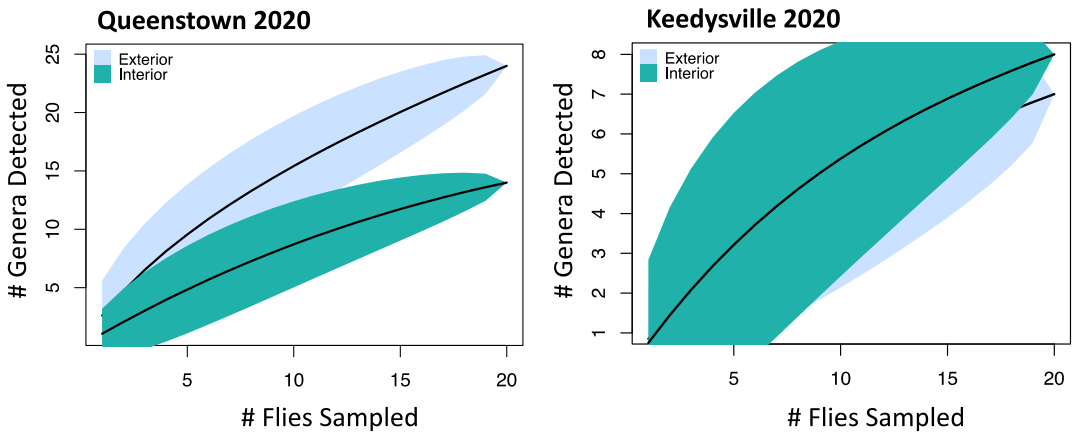

Figure S4. Species accumulation curve for *D. suzukii's* fungal associations in 2020 at Queenstown and Keedysville. Each graph shows the number of fungal genera detected when a given number of field-collected adult *Drosophila suzukii* were sampled, split between internal and external fungi. The y-axis on each graph are scaled differently due to varying overall genus richness across each site.

Appendix C: Supplemental Materials for Chapter 3

Supplemental Methods

Laboratory Bioassay Spray Coverage Treatments

Raspberries were treated with either zeta-cypermethrin or water (untreated control), and each fruit was sprayed at either a high and a low spray coverage rate. To create variation in spray-coverage rates, we used a metronome to adjust the speed at which a Preval® paint canister moved along a track in front of the fruit. The low coverage treatment was generated by moving the sprayer quickly, at a rate of 140 beats per minute with a 2.5 cm length of track covered each beat. The high coverage treatment was generated by moving the sprayer slowly at 20 beats per minute. Each raspberry was sprayed twice, with the berry rotated 180° between sprays to ensure that all surfaces were covered. During both passes of the sprayer, we quantified the percent spray coverage that the raspberry received on each side using spray cards. This approach approximated the spray card coverage assessments that were used in the field, allowing us to better understand the relationship between spray card coverage values and efficacy.

Laboratory treatment spray coverage levels were estimated by deploying paper spray cards behind each berry used in the assay. The estimated percent spray coverage received by each individual berry varied. However, summarizing the data across the high and low concentration bioassays, moving the sprayer slowly (high coverage treatment) generated an average percent coverage of 84.6 ± 1.5% (N=80 berries) while moving the sprayer quickly (low coverage treatment) resulted in an average percent coverage of 21.8 ± 1.4% (N=80 berries).

Raspberries, chosen to be a similar size and randomly assigned to each treatment, were mounted 20.3 cm above the ground using a wooden dowel and positioned 61.0 cm away from the paint canister sprayer and spray track (Figure S1). To ensure even spray patterns between replicates, the sprayer was turned on 15.2 cm downstream of the raspberry. At this distance, pesticide residues from the initial start of the sprayer could not reach the raspberry, creating a buffer that allowed the sprayer to fully prime before treating the fruit. We did not quantify the amount of insecticide deposited on each berry. However, deposition would have varied between replicates due to differences in coverage and berry surface area.

Plot Descriptions for Carrier Water Volume Spray Coverage Trials

From 2016 – 2017, airblast sprayer trials were conducted using a mixed berry planting at the Western Maryland Research and Education Center in Keedysville, MD (Washington Co.). The planting consisted of four rows of plants, alternating between primocane red raspberries and floricane black raspberries (two rows each; Figure S7). Trials were conducted only using the primocane red raspberries (c.v. Caroline). To standardize for differences in stand vigor between and along the rows, carrier water volume treatments were applied to half of each row on alternating sides of the field (Figure S7), and spray cards were placed in representative sections of the row. Spray cards were deployed in the outer corner of each row along a 2.5 m section to minimize drift between treatments. During the first 2016 spray trial, two sets of replicate spray cards were deployed in each corner (four replicates total). For the remaining 2016 and 2017 trials, three sets of replicate spray cards were deployed in each corner (six replicates total). For all spray trials we analyzed the side of the card facing outward toward the row middle. Therefore, we

alternated which side of the row cards the spray cards were deployed on so that half of the replicates were positioned on opposite sides of the row and faced in opposite direction (Figure S7).

Backpack spray trials in 2017 were conducted using two mixed berry plantings maintained at Washington Co. and Queen Anne's Co. Each planting consisted of four consecutive rows each of primocane blackberries, floricane blackberries, primocane black raspberries, floricane black raspberries, primocane red raspberries, and floricane red raspberries. Only the primocane blackberries (c.v. Prime-Ark) and floricane red raspberries (c.v. Prelude) were used in the backpack spray trials. Because these plantings were on a slope, we assigned the two carrier water volume treatments to alternating rows of blackberries or raspberries, allowing us to account for potential confounding effects due to irrigation and stand vigor (Figure S8). Spray cards were deployed in three replicate plants per row, with spray cards distributed throughout the entire row and again deployed such that card direction alternated between each replicate as previously described (half of the replicates were positioned on each side of the row). At the Washington Co. site, the same primocane blackberry planting was also used for the 2018 spray trials with the airblast sprayer and two-sided row crop head; to minimize drift between carrier water volume treatments, we only sprayed through the nozzles on the right side of the sprayer.

In 2019, airblast sprayer trials were conducted at the Washington Co. field site using a twelve-row planting of primocane red raspberries (c.v. Caroline) that were approximately six months old. We again assigned carrier water volume treatments to alternating rows of plants, selecting rows that had vigorous plant growth, intact trellising, and were separated by at least three rows of buffer plants. We quantified spray coverage in

three replicate plants per row, and spray cards were spread throughout and deployed on alternate sides of the row between replicates.

Supplemental Tables

Table S1. Average percent coverage ± standard error observed for raspberries treated in 468 and 935 liters per hectare (L/ha) laboratory bioassays using a sprayer speed of 20 and 140 beats per minute (BPM), with either the water control (CON) or zeta-cypermethrin (ZC). Data were analyzed separately for each insecticide concentration using a mixed model ANOVA in the nlme package (Pinheiro et al. 2017); models included insecticide treatment, sprayer speed, and the treatment by speed interaction as fixed effects. For each concentration, values that do not share a letter are significantly different ($\alpha = 0.05$), and means separations were performed for a single significant fixed effect (bold).

Concentration	Insecticide	Speed (BPM)	Mean % Coverage ± SE	N
468 L/ha[a]	CON	20	83.78 ± 3.09 b	20
		140	16.11 ± 1.41 a	20
	ZC	20	87.78 ± 2.49 b	20
		140	22.18 ± 2.81 a	20
935 L/ha[b]	CON	20	80.94 ± 4.00 C	20
		140	27.44 ± 3.13 B	20
	ZC	20	85.81 ± 2.57 C	20
		140	21.63 ± 3.20 A	20

a. Trt $F_{1,73} = 6.82$, $P = 0.011$; **Speed $F_{1,73} = 1001.47$, $P < 0.001$**; Trt*Speed $F_{1,73} = 1.33$, $P = 0.252$

b. Trt $F_{1,73} = 0.03$, $P = 0.859$; Speed $F_{1,73} = 498.78$, $P < 0.001$; **Trt*Speed $F_{1,73} = 4.11$, $P = 0.046$**

Table S2. Summary of blackberry and raspberry carrier water volume spray trials that were conducted at the Washington Co. and Queen Anne's Co. field sites using different types of sprayer equipment.

Site	Sprayer	Crop	Trial Dates
Washington Co.	Airblast	Raspberries	31 August (2016) 21 September (2016) 22 August (2017)
	Airblast + Row Crop Head	Blackberries Raspberries	7 August (2018) 22 October (2019)
	Backpack	Blackberries Raspberries	22 August (2017) 22 August (2017)
Queen Anne's Co.	Backpack	Blackberries Raspberries	16 August (2017) 16 August (2017)

Table S3. Summary of sprayer settings for commercial spray coverage trials conducted from 2016 – 2017.

Year	Site	Carrier Water Volume (L/ha)	Sprayer	Speed (km/hour)	Pressure (PSI)
2016	One	524	Tifone Storm 1032 Airblast Sprayer	3.7	220
2017	One	524	Tifone Storm 1032 Airblast Sprayer	3.7	220
2017	Two	935	AgTec Airblast Sprayer	3.2	30

Table S4. Mean larval infestation density (larvae per starting female SWD) ± standard error (SE) in raspberries that were treated at either a 468 or 935 liters per hectare (L/ha) application rate and with either a high (~85% coverage, 20 BPM) or low (~22% coverage, 140 BPM) spray coverage rate. Data were analyzed separately for each application rate and insecticide treatment using a Kruskal-Wallis test. No significant differences in larval infestation due to spray coverage were found ($\alpha = 0.05$).

Application Rate	Insecticide	Spray Coverage	N	Mean # Larvae per Female ± SE
468 L/ha [a]	Water (control)	High	20	9.85 ± 1.65
		Low	20	8.20 ± 1.17
	Zeta-cypermethrin	High	20	0.14 ± 0.14
		Low	20	0.06 ± 0.03
935 L/ha [b]	Water (control)	High	20	1.70 ± 0.43
		Low	19 [c]	2.63 ± 1.16
	Zeta-cypermethrin	High	20	0.09 ± 0.05
		Low	20	0.15 ± 0.08

a. Water Coverage $\chi^2_1 = 0.51$, P = 0.473; Zeta-cypermethrin Coverage $\chi^2_1 = 0.92$, P = 0.336
b. Water Coverage $\chi^2_1 = 0.02$, P = 0.876; Zeta-cypermethrin Coverage $\chi^2_1 = 0.87$, P = 0.351
c. Larval infestation data was accidentally not recorded for one raspberry, so the berry was excluded from analysis

Supplemental Figures

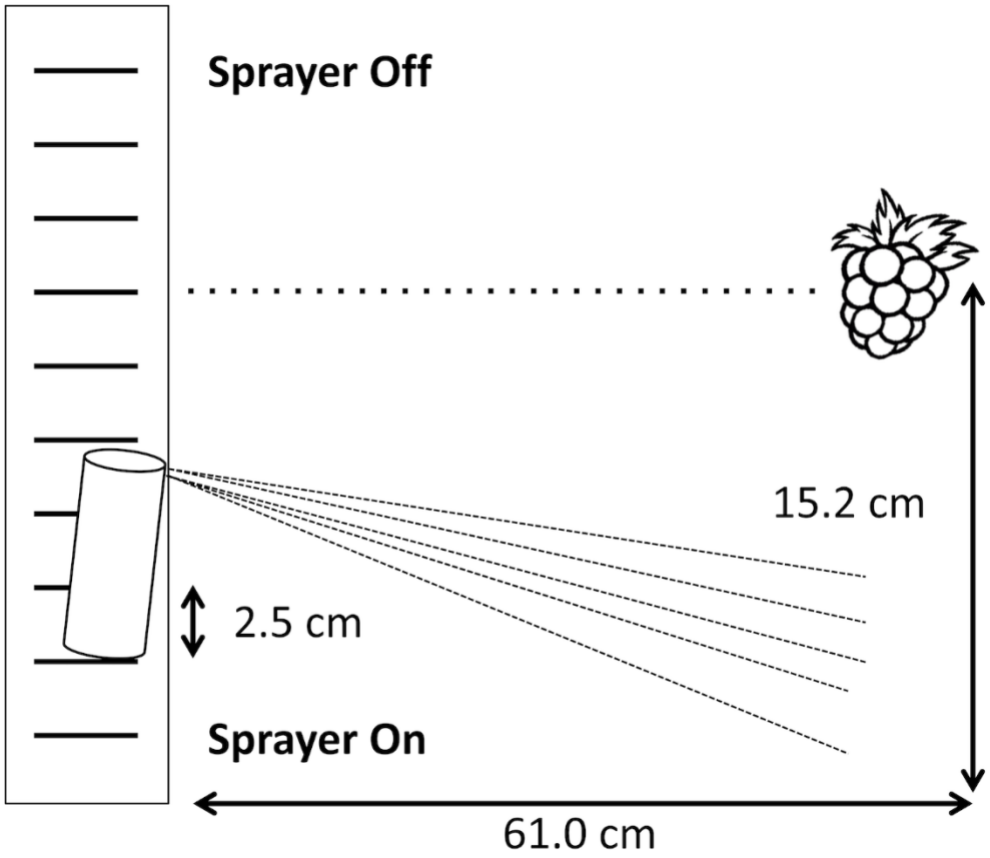

Figure S1. Visual summary of bioassay spray application. On each day that bioassays were conducted (five replicates per day), a fresh Preval® paint canister sprayer moved along a 22.7 centimeter (cm) track that was positioned 61.0 cm away from one raspberry (mounted 20.3 cm above ground using a wooden dowel). To ensure that the sprayer was fully primed prior to hitting the raspberry, spraying was initiated 15.2 cm downstream, moving along the full 22.7 cm track at a speed of 2.5 cm per beat.

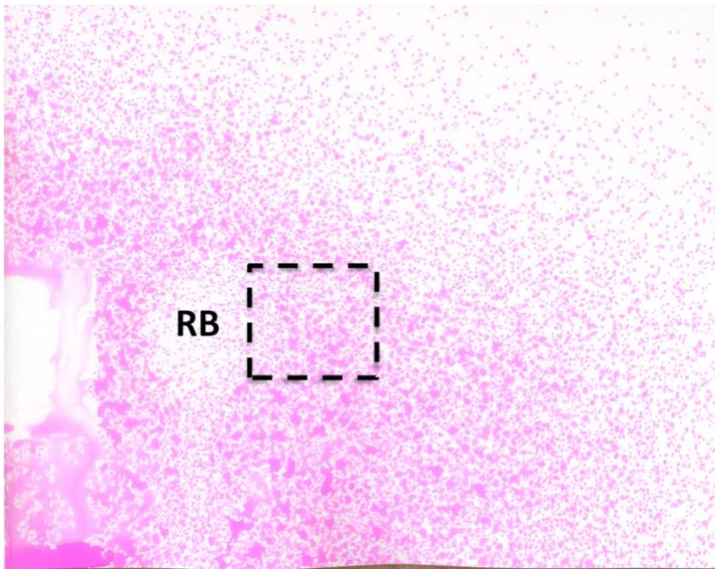

Figure S2. Procedure for spray coverage quantification in laboratory bioassays. Due to variation in spray coverage patterns along the vertical and horizontal gradients within each spray card, scanned images were cropped prior to analysis to capture an approximately 10 square centimeter area directly right of the raspberry (RB).

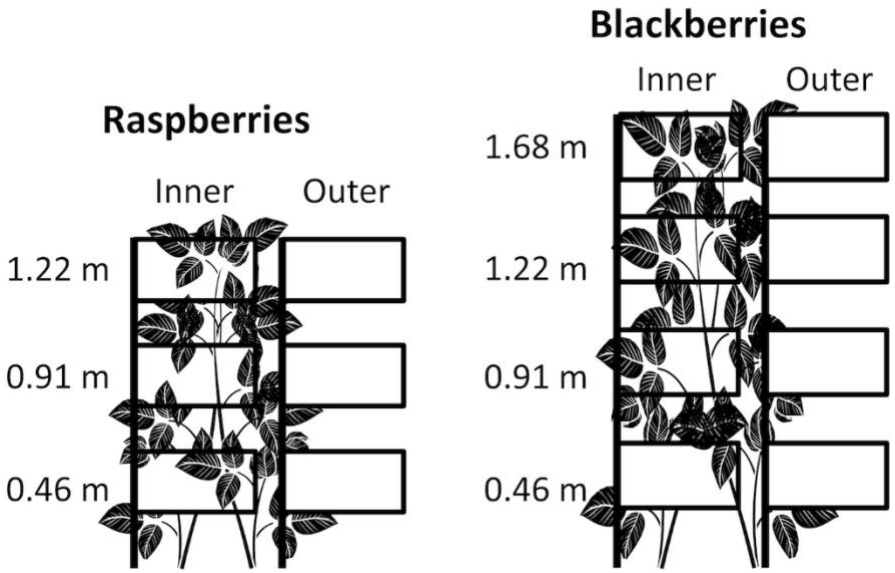

Figure S3. Schematic of spray card deployment for spray coverage trials in raspberries and blackberries. Spray cards were deployed on wooden dowels in the inner plant canopy (center of the plant, surrounded by foliage) and the outer plant canopy (along the plant margins). In raspberries, spray cards were positioned at three heights: 0.46 meters (m), 0.91 m, and 1.22 m above ground for a total of six cards per location. In blackberries, spray cards were deployed at four heights: 0.46 m, 0.91 m, 1.22 m, and 1.68 m above for a total of eight cards per plant. Each location/plant was treated as an experimental replicate and data were subset by height prior to analysis.

Figure S4. Spray cards deployed in the inner and outer canopy of primocane red raspberries (right) and blackberries (left). The side facing outwards toward the nearest row middle (in front of the dowel as viewed in this photo) was used for analysis.

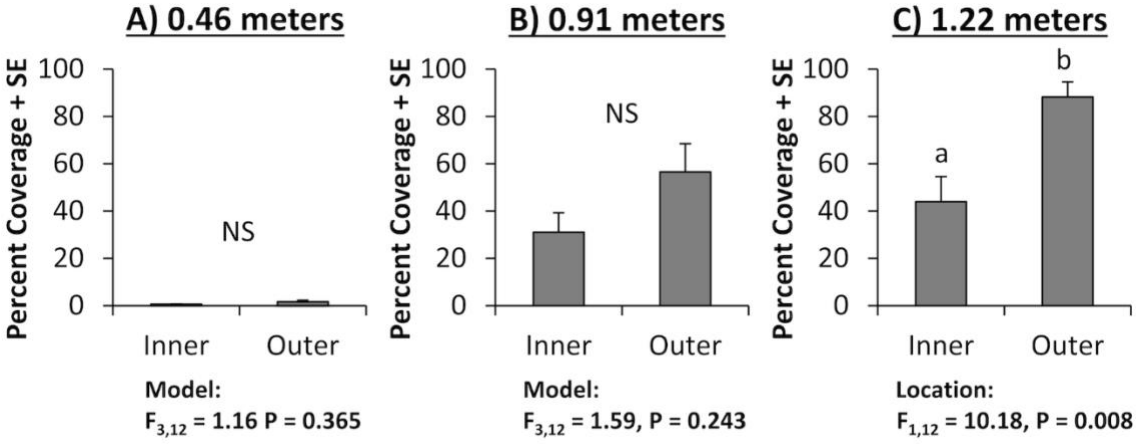

Figure S5. Mean percent spray coverage and standard error in raspberries sprayed at 468 and 935 liters per hectare (L/ha) using an airblast sprayer at Keedysville, MD on 31 August 2016. Data were subset and analyzed separately by spray card height: A) 0.46 meters above ground, B) 0.91 meters above ground, and C) 1.22 meters above ground. Each analysis included carrier water volume treatment, canopy location, and the location by treatment interaction as fixed effects. No significant (NS) effects due to carrier water volume treatment or the interaction effect were observed at any height. Within a graph, bars that do not share a letter have significant differences in spray coverage between the inner and outer canopy by Tukey's HSD (α=0.05).

Figure S6. Two-sided row crop head attachment used for 2018 and 2019 airblast sprayer trials detached (top) and mounted to the airblast sprayer (bottom).

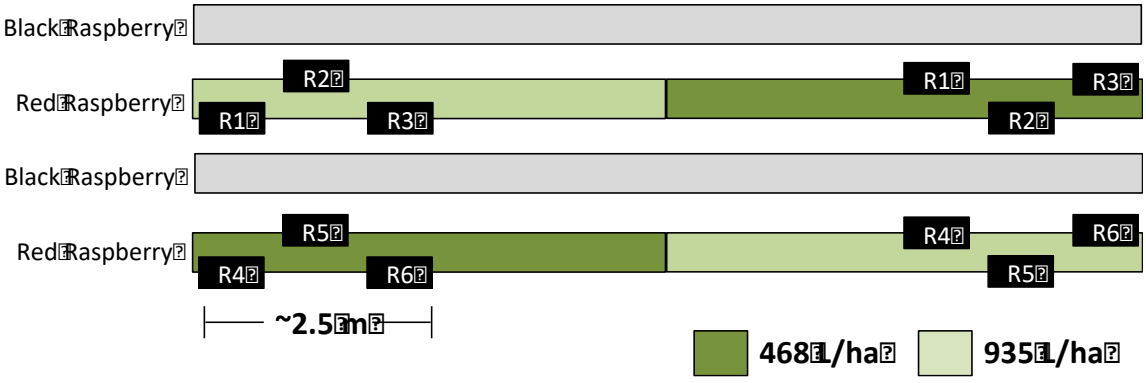

Figure S7. General plot map for 2016 and 2017 airblast sprayer trials that were conducted in primocane red raspberries (c.v. Caroline). Carrier water volume treatments were applied to opposite halves of two rows of primocane red raspberries (dark green = 468 liters per hectare; light green = 935 liters per hectare). Spray cards were deployed along an approximately 2.5 meter section of row in each corner, with three replicate sets of spray cards placed in each corner and spray cards alternating to face opposite directions of the row between replicates (individual replicates are denoted by the black boxes labeled R1 – R6).

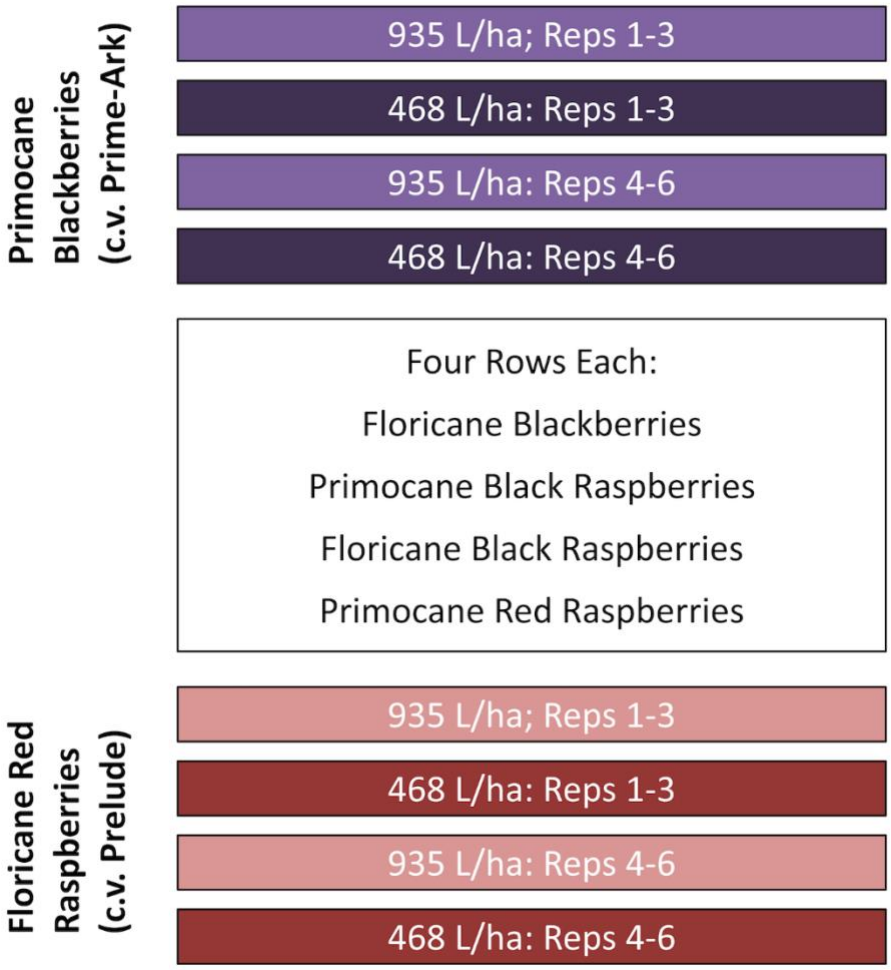

Figure S8. General plot map of the mixed fruit planting used for the 2017 backpack sprayer trials in primocane blackberries (c.v. Prime-Ark), the 2017 backpack sprayer trials in floricane red raspberries (c.v. Prelude), and the 2018 airblast sprayer with row crop head trials in primocane blackberries. The primocane blackberry and floricane red raspberry plantings were separated by 16 rows of plants (four rows each of floricane blackberries, primocane black raspberries, floricane black raspberries, and floricane red raspberries) that were not used for any study. In all spray trials, carrier water volume treatments alternated between rows to account for potential confounding effects in stand density due to uneven irrigation along the plot. Three replicate sets of spray cards were deployed in each row, with spray cards alternating to face opposite directions of the row between replicates.

Appendix D: Approval of Previously Published Work

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March 26, 2021

Dr. Steve Fetter
Associate Provost and Dean, The Graduate School
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College Park, MD 20742

Dear Dr. Fetter,

Margaret Lewis, UID 114533975, has the approval of her examining committee to include her previously published work in her dissertation. These works: Lewis, M.T., Hamby, K.A. 2020. Optimizing bramble spray coverage for *Drosophila suzukii* (Diptera: Drosophilidae) management on diversified fruit farms. J. Econ. Entomol. 113(6):2820-2831, https://doi.org/10.1093/jee/toaa237, and Lewis, M.T., Hamby, K.A. 2019. Differential impacts of yeasts on feeding behavior and development in larval *Drosophila suzukii* (Diptera: Drosophilidae). Sci. Rep. 9(1):13370, https://doi.org/10.1038/s41598-019-48863-1 have been cited appropriately throughout the dissertation and a preface discussing her contributions has been included and approved by the committee. Margaret conceived the manuscripts, conducted the work, and prepared the manuscripts; her committee has determined that she made a substantial contribution to these works.

Please do not hesitate to contact me if you have further questions.

Sincerely,

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