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Title: Elucidating symbioses between *Drosophila suzukii* and fungal communities for improved insect and disease management in raspberry production

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Rationale:

In fruit crops, the interaction between frugivorous insects and fungi can alter pest and disease epidemiology, reducing marketable crop yields (Pfeiffer 2012; Biddinger et al. 2014) and in recent years, introduction of fruit pests have coincided with increases in fruit rot disease outbreaks (Pfeiffer 2012; Mustacich 2014). In addition, fungi can influence the immigration and persistence of insect pests; studies of the European grapevine moth suggest that mycophagic larvae feed on fungal hyphae (Mondy et al. 2004) and studies of Tephritid fruit fly-fruit rot interactions indicate that fly attraction is due to volatile host odors produced from rotten fruit (Machota et al. 2013).

However, insect-fungal symbioses are not always mutually beneficial; in some cases, fungal-related volatiles can act as repellents (Davis et al. 2013) resulting in insect preference for healthy over diseased berries (Tasin et al 2011), and some fungi have toxic effects (Trienens et al. 2010). Despite abundant evidence for insect-fungal interactions, the epidemiology of insect-fungal synergisms, such as the impacts of insects on disease development and the role of fungi in driving pest outbreaks, are poorly understood aspects of fruit crop production nationally.

Spotted wing drosophila (*Drosophila suzukii*) is a major driver of raspberry and blackberry fruit losses nationally. *Drosophila suzukii* is particularly problematic because females use their saw-like ovipositor to lay eggs in ripe or ripening fruit, instead of in wounded or overripe fruit like other drosophilids (Walsh et al. 2011). Red raspberries (*Rubus idaeus* L.) are particularly attractive host for *D. suzukii* (Bellamy et al. 2013, Burrack et al. 2013), and an estimate from a California economic analysis suggests that revenues could be decreased by 37% if *D. suzukii* is not managed in raspberry production (Goodhue et al. 2011).

Internal feeding by larvae damages fruit and decreases shelf life; in the field, egg laying is thought to facilitate infection by fungal fruit rots (Walsh et al. 2011) and emerging adults may be able to vector pathogens. *Botrytis cinerea* causes grey mold, one of the most economically significant diseases of fall raspberries nation-wide; although, studies of *D. suzukii-B. cinerea* interactions are lacking in raspberries (or any other host), European grapevine moth (*Lobesia botrana*) larvae vector *B. cinerea* spores and can facilitate colonization of developing fruit through oviposition wounds (Fermaud and LeMenn 1992).

In addition to *B. cinerea*, several other minor fungal pathogens also cause fruit rot, including *Cladosporium* species, *Colletotrichum* species, and diverse yeasts. Studies in raspberry indicate

that yeasts associated with fruit rots may also be spread by and attractive to *Drosophila* spp. (Chandler et al. 2014, Chandler et al. 2011). For instance, in preliminary two-choice flight tunnel experiments exposing 40 mated female *D. suzukii* to healthy grapes (*Vitis vinifera*, unknown red varietal) and grapes infected with sour bunch rot (caused by species of *Saccharomyces*, *Candida*, and *Hanseniospora*), *D. suzukii* females were strongly attracted to the grapes infected with sour bunch rot relative to the healthy grapes (Bellamy et al., unpublished data).

In addition to studies of plant pathogenic yeasts, analysis of *D. suzukii* microbial ecology, have revealed diverse yeast species residing on the surface of freshly laid *D. suzukii* eggs (Bellamy, unpublished) and studies in other crops indicate that other *Drosophila* spp. both vector (Reuter et al. 2007, Coluccio et al. 2008) and feed upon yeasts (Phaff et al. 1956, Anagnostous et al. 2010). In fact, baker's yeast plays a more important role in *Drosophila melanogaster* attraction to fruit than the fruit does (Becher et al. 2012). Research suggests that *D. suzukii* larvae and adults also feed upon yeasts, in particular *Hanseniaspora uvarum*, and it very likely that they spread these yeasts throughout their habitat (Hamby et al. 2012). *D. suzukii* females are also attracted to fungal volatiles emitted from yeasts (Schiedler et al. 2015).

Although some fungi are very attractive to adult *D. suzukii*, many others may be repellent. Observations by Hamby indicate that *D. suzukii* larvae do not perform well in fruit with advanced disease symptoms (covered in filamentous fungi or highly desiccated). However, whether larvae can complete a generation in diseased fruit prior to the onset of these advance systems is unknown. It is important to understand whether diseased fruit may contribute to *D. suzukii* pest pressure, because management can be targeted based on this information.

Beyond yeasts interactions, there are very few studies to elucidate the interactions *D. suzukii* has with fungal communities associated with raspberries. Questions in this sphere are various—what if any interactions does *D. suzukii* have with fruit rot fungi, including the highly important grey mold pathogen? Can fungal rotted berries be effectively utilized as a food source (e.g. mycophagy) for this insect? To address these and other questions, we proposed to broadly characterize the fungi associated with *D. suzukii*, in raspberry fields. To do this, we took a three pronged approach, to (1) characterized fungal communities associated with *D. suzukii* larvae in the raspberry fields (gut communities) using culture-based analysis, (2) conducted a survey to determine whether incidence and severity of specific fruit rot pathogens and / or secondary

yeasts correlate with *D. suzukii* population densities, and (3) evaluated how insects influence disease development when infested with pathogenic fungi in the laboratory.

Objectives:

1. To survey the fungi *D. suzukii* larvae feed upon (and may vector) and the impact *D. suzukii* infestation has upon fruit microbes.
2. To determine if fruit diseases incidence increases with *D. suzukii* adult and larval populations.
3. Determine whether *D. suzukii* can vector plant pathogenic microbes between fruit.

Objective 1. Survey the fungi *D. suzukii* larvae feed upon (and may vector) and the impact *D. suzukii* infestation has upon fruit microbes.

Methods. Two central Maryland diversified pick-your-own farms' fall (primocane fruiting) raspberry fields were used for this project. At site one three rows of 'Caroline' were used and at site two three rows of 'Jaclyn' were used. These sites were the source of all raspberries and *D. suzukii* used for this project.

In early August, at least five raspberry clusters composed of hard green raspberries (at least 5 fruit per cluster) were bagged per row at each site (3 rows per site) using nylon mesh 1 gallon paint strainer bags (Mater Craft) securely closed with wire to exclude *D. suzukii*. Bags were redeployed on fresh green raspberry clusters periodically during the season to ensure that un-infested ripe fruit were available when needed. These fruit were confirmed un-infested using larval floatation methods (described in Objective 2) at harvest. At two points during the harvest season (8/17/15 and 9/2/15) market ripe un-infested fruit were sterile collected from within the bag using whirl-pak filter bags. Un-bagged fruit (potentially infested) that was of similar ripeness were also collected using sterile methods for comparison. Fruit were selected that did not visually appear infected with *Botrytis*. Fruit were held in a cooler until they were returned to the laboratory for analysis on the same day. Fruit were macerated (3 replicate bags containing 3-5 individual berries collected from each replicate row for each site were used) within the whirl-pak bags without opening them and then duplicate sterile serial dilutions (1/10, 1/100, 1/1000, 1/10000) were performed on each bag of the juice. These replicate dilute juice suspensions were plated on Rose Bengal chloramphenicol agar (RBCA; prepared according to the manufacturer's

instructions; Oxoiod, United Kingdom; semi selective for yeast fungi) and *Botrytis*-selective media to quantify the fungal communities of the fruit juice. Colony forming units per mL fruit juice was calculated by counting the number of fungal colonies that grew on the plates for one dilution (between 20-500 colonies on the plate) per sample. Separate collections of similar fruit from the same clusters chosen for fungal analysis were used to evaluate ripeness and ensure it was similar by evaluating total soluble solids in the form of degrees BRIX using a refractometer (Atago PAL-1 from 0.0%- 53.0% BRIX). *Botrytis* incidence was infrequent (occurred in only 5 replicates) and was therefore not analyzed. Colony forming units data was analyzed for yeasts separately using a generalized linear model using the Fit Model platform of JMP ® Pro 11.0.0 (SAS Institute, Cary, NC) with fixed effects date of collection, site of collection, status (infested or un-infested), and all two-way interaction effects. Data were log transformed to meet the assumption of normality of residual errors assed using a Shapro-Wilk test. Data passed a Levene's test for homoscedasticity.

Infested fruit were returned to the laboratory from each site as available from early August through early October. *Drosophila* larvae (2^{nd} to 3^{rd} instar) were removed from the fruit using Featherweight forceps (BioQuip, Rancho Dominguez, CA). Forceps were dipped into 95% ethanol and flamed twice in succession and allowed to cool for sterilization. The larva was surface sterilized by submergence in sterile autoclaved distilled water, followed by 70% ethanol, and then a final rinse in sterile autoclaved distilled water and placed in the center of a RBCA plate for 20-45 minutes depending on larval activity. Once a larva began to cross its own trail, it was removed using sterile forceps and placed in standard *Drosophila* medium. Only larvae that successfully eclosed as adult *D. suzukii* were used for this study. For yeast fungi, two colonies of each morphological type were isolated and identified for each RBCA plate. Colonies were serially plated on potato dextrose agar to ensure a pure culture, and then DNA sequencing methods were used to identify the strains. Briefly, the D1/D2 domain of the large (26S rRNA) subunit was amplified using the NL1 and NL4 primers (O'Donnell 1992) and submitted to Genewiz for Sanger sequencing. The rRNA sequences were compared against the NCBI database using the nucleotide Basic Local Alignment Search Tool (BLASTn) for taxonomic identification of the yeast isolates (http://blast.ncbi.nlm.nih.gov). A 98% match or higher to a published yeast species was used to assign species name, anything less was identified solely to genus level. Yeast cultures are cryopreserved in the Hamby lab. Filamentous fungi were pure

cultured with a single hyphal tip and identified to genus based on spore morphology. Species identification of all recovered fungal isolates is currently underway, using DNA sequence analysis.

Results. Fruit ripeness was similar for the fruit that were presumed infested (un-bagged) and confirmed un-infested (bagged), therefore we expect the bagging process did not impact the fruit quality. Significantly more yeast colony forming units occurred in the fruit juice at site 1 and in the infested (un-bagged) fruit, and a significant interaction was seen between site of collection and the infestation status of the fruit (Table 1).

Table 1. Comparison between infested (un-bagged) and un-infested (bagged) fruit (N = 3) ripeness and microbial communities (colony forming units (CFU) per mL fruit juice).

	Brix sugar content ± SE		Yeast CFU ± SE[a]		Botrytis CFU	
	Infested	Un-infested	Infested	Un-infested	Infested	Un-infested
Site 1						
8/17/15	10.7 ± 0.2	10.5 ± 0.3	$6.6 \times 10^6 \pm 3.9 \times 10^6$	$6.1 \times 10^4 \pm 1.4 \times 10^4$	0.0 ± 0.0	833.3 ± 833.3
9/2/15	10.9 ± 0.1	10.0 ± 0.3	$3.0 \times 10^5 \pm 9.0 \times 10^4$	$1.1 \times 10^5 \pm 4.6 \times 10^4$	1741.7 ± 1103.1	8.3 ± 8.3
Site 2						
8/17/15	9.1 ± 0.3	9.0 ± 0.6	$2.2 \times 10^4 \pm 3.2 \times 10^4$	$9.0 \times 10^4 \pm 3.8 \times 10^4$	0.0 ± 0.0	0.0 ± 0.0
9/2/15	10.3 ± 0.4	10.4 ± 0.4	$5.5 \times 10^4 \pm 7.6 \times 10^3$	$1.6 \times 10^5 \pm 4.4 \times 10^4$	16.7 ± 16.7	8.3 ± 8.3

[a]Overall Model $F_{6,41} = 11.37$, $p < 0.0001$; Model Effects: Date $F_{1,41} = 0.007$, $p = 0.9325$; Site $F_{1,41} = 20.16$, $p < 0.0001$; Status $F_{1,41} = 6.11$, $p = 0.0177$; Site*Date $F_{1,41} = 8.50$, $p = 0.0057$; Status*Site $F_{1,41} = 29.53$, $p = <0.0001$; Status*Date $F_{1,41} = 3.93$, $p = 0.0543$

Botrytis occurred at both sites in both infested and un-infested fruit, but overall infection was low in the fruit used for this study. A total of 26 larvae were successfully evaluated for fungal feeding, and 60 unique yeast isolates were identified. *Drosophila* larvae fed upon both yeasts and hyphal fungi, with yeasts occurring much more frequently. As seen in California, *H. uvarum* was the yeast most commonly fed upon by larval *D. suzukii* (Table 2). *Cladosporium* was the dominant hyphal fungus recovered from larval frass, with 24-64% of larvae across the two sites feeding upon it. An average of 2.33-4.18 propagules (spores or hyphal fragments, measured as colony forming units) per larva occurred in these in plate walks (Table 3). *Botrytis*, *Penicillium* and two unknown hyphal species were also recovered from larval frass, at lower frequencies

(one larvae at one or both sites); where present, frass density ranged between 1 and 2 propagules per plate. To the authors' knowledge, this is the first study to describe *Cladosporium* as a component of the microbial community in SWD larval frass. It is not clear why *Cladosporium* was so common in larval frass, compared to *Botrytis*, which was more common in the field; it is possible that the highly melanized *Cladosporium* spores are more tolerant of antagonistic conditions in the gut. Alternatively, SWD larvae may exhibit a preference for feeding upon *Cladosporium* relative to *Botrytis*.

Table 2. Fungal species fed upon by larval *D. suzukii* (collected from August to October).

	Site 1 n = 14	Site 2 n = 12	Total Isolations
Aureobasidium pullulans	0	1	1
Candida azyma	0	1	1
Candida oleophila	1	0	1
Candida railensis	1	0	1
Candida sp.	1	0	1
Candida zemplinina	0	1	1
Cryptococcus magnus	1	0	1
Hanseniaspora opuntiae	6	1	7
Hanseniaspora thailandica	1	0	1
Hanseniaspora uvarum	12	12	24
Issatchenkia terricola	4	1	5
Metschnikowia fruticola	0	1	1
Pichia kluyveri	0	3	3
Pseudozyma hubeiensis	1	0	1
Pseudozyma sp.	2	0	2
Saturnispora diversa	0	5	5
Wickerhamomyces pijperi	0	4	4

Table 3. Incidence and intensity of SWD larvae infestation by hyphal fungi at two sites

	Site 1 (n=14)		Site 2 (n=12)	
Genus[a]	Percent of larvae infested	CFUs per larvae[b]	Percent of larvae infested	CFUs per larvae[b]
Cladosporium	64%	4.18	25%	2.33
Botrytis	7%	1.00	0	nd
Penicillium	0	nd	8%	1.00
Unknown #1	7%	1.00	8%	2.00
Unknown #2	7%	1.00	0	nd

[a]Identified to genus based on morphological traits

[b]Average number of colony forming units (CFUs) on those plates where the fungus was observed (conditional intensity of infestation); no data (nd) in cases where no larvae were infested.

Objective 2. To determine if fruit diseases incidence increases with *D. suzukii* adult and larval populations.

Methods: At the same sites described above, fruit disease and *D. suzukii* populations were evaluated. *D. suzukii* adults were monitored every week between July and October 2015 with three Trécé Pherocon® SWD (1 per row at both of the abovementioned sites) traps using apple cider vinegar as a drowning solution. As soon as commercially ripe fruit were available, 10 raspberries (as close to marketable ripeness as possible) were collected per row where adult traps are deployed. Fruit were immediately returned to the lab and larval infestation was evaluated. Berries were crushed and soaked in a sugar-water solution of 950 ml of water mixed with ¼ cup of white cane sugar for approximately 10 minutes to allow the larvae to float. This mixture will was gently agitated and poured through mesh window screen into a series of three sieves with the coarsest at the top and the finest at the bottom (2.36 mm mesh, 500 µm mesh, 300 µm mesh) 76.2 mm diameter and 50.8 mm height sieves (W.S. Tyler Industrial Group, Mentor, OH). Total *Drosophila* larvae per raspberry sample were counted by inspecting both sides of the sieves under a dissecting microscope.

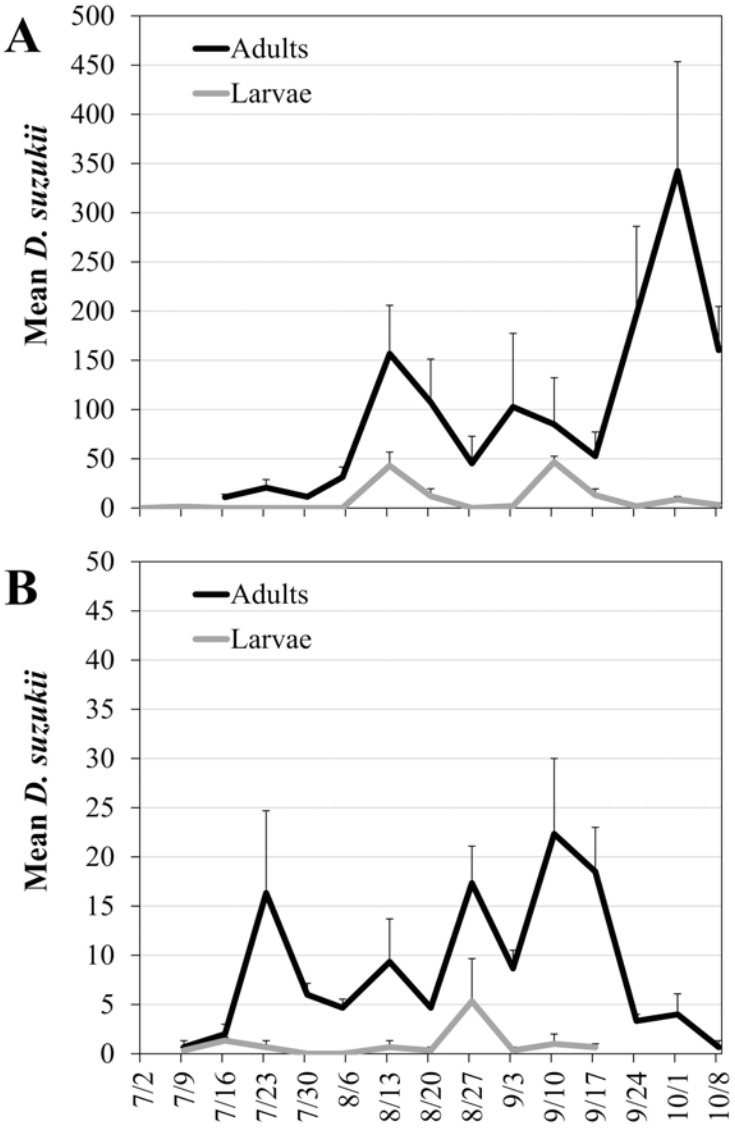

Figure 1. Mean plus SE (N = 3) adults and larval *D. suzukii*. A. Site 1 B. Site 2

Incidence and severity of all fruit rot diseases and *D. suzukii* infestations were quantified twice (8/13/15 and 8/28/15) during the fall raspberry harvest for bagged and un-bagged fruit. Bagged fruit rot disease incidence was measured visually as the fruit was collected and fruit were confirmed uninfested by *D. suzukii* by returning the fruit to the lab and using larval flotation methods (described above) on a subsample of 3 fruit per row on each sample date. Because bagged fruit contained a finite number of market ripe fruit at that time point, sample size varied by replicate row for total fruit collected. Forty un-bagged berries per row were randomly selected, and rated for presence of *D. suzukii* larvae, *Botrytis* and *Cladosporium* fruit rots *in situ* on both dates.

Results: Site 1 consistently had a higher (orders of magnitude) *D. suzukii* adult and larval population than Site 2, and the population gradually built over the season (Figure 1). There was a weak positive correlation between SWD and fruit rot infested berries for both *Botrytis* fruit rot and *Cladosporium* fruit rot across sites (Figure 2), when comparing un-bagged fruit that were simultaneously evaluated for infestation and rot. Comparing the two sites, *Botrytis* disease incidence was higher at the site with higher SWD populations (Site 1) than the site with lower populations (Site 2), though this may be an effect of variation in pest management practices between the sites (Figure 3). *Cladosporium* levels were similar at the two sites, with slightly higher incidence at Site 2. SWD larvae were present in an average of 60% of *Botrytis*-infected

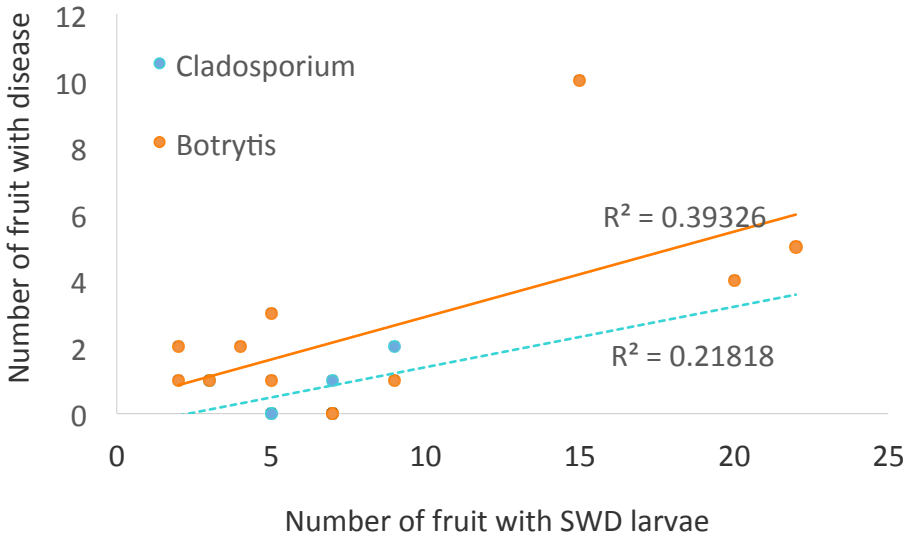

Figure 2. Correlation between fruit rot and SWD infestation levels by row, as evaluated by concurrent visual inspection of un-bagged fruit, for the two sites across two dates

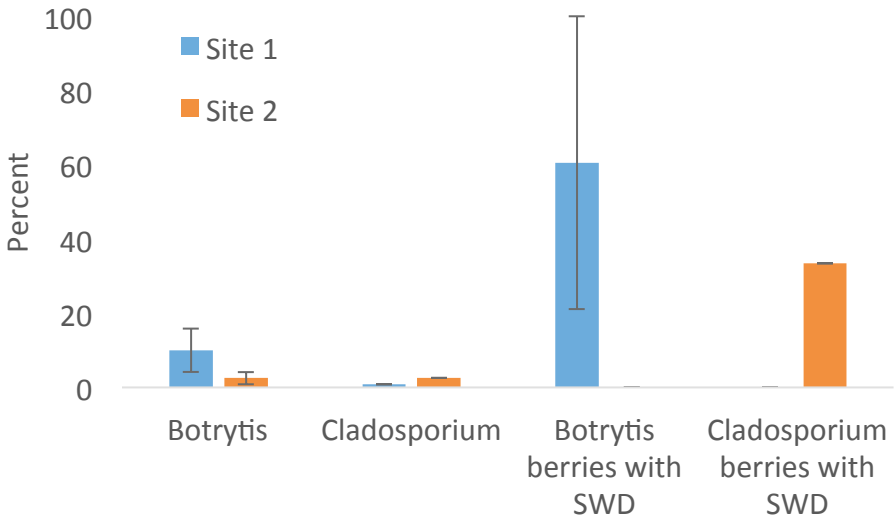

Figure 3. Comparison of *Botrytis* and *Cladosporium* fruit rot incidence and the percent of fruit rot infected berries with SWD larvae at Site 1 (high SWD populations) and Site 2 (low SWD poplations), as evaluated by visual inspection of un-bagged fruit across two time points.

berries at Site 1 (*Botrytis* was uncommon at Site 2). At Site 2, SWD were larvae were recovered from 40% of *Cladosporium* infested berries (*Cladosporium* was uncommon at Site 1). These

analyses demonstrate that SWD can co-occur with both fruit rot fungi, and indicate that SWD may facilitate fruit rot. Data over a second field season, with more repeat measures over time will clarify relationships between these pests in red raspberries.

Objective 3. Determine whether *D. suzukii* can vector plant pathogenic microbes between fruit. **Methods:** Un-infested (bagged and confirmed un-infested as described in Objective 1) ripe raspberries exhibited no external fruit rot symptoms were collected from both field sites and used within one day of collection on 9/4/15 and 9/9/15 for laboratory experiments. Raspberry clusters were placed in floral water picks inside test tube racks and kept cool until use. Sterile Bugdorm cubical (299 cm) cages were covered with plastic sheets (over the mesh portion), lined with clean bench paper on the bottom, and sprayed with sterile autoclaved distilled water to increase internal humidity. Floral water picks were transferred to sterile test tube racks and introduced into the cages to ensure that each treatment received at least five ripe berries (more than five were often present). Treatments included a control where no flies and no spores were introduced to the fruit, *D. suzukii* only where laboratory reared *D. suzukii* (20 males, 20 females) were introduced, and *D. suzukii* (20 males, 20 females) with *Botrytis* spores. After a 24 hour treatment period where the clusters and flies were left at room temperature (~73°F (23°C)) on the bench top, the fruit clusters were sterilely removed from the cages and inspected to ensure no flies remained. They were then transferred to incubation bags and held at a 63°F (17°C) for 3-5 days before incidence of *Botrytis* and *Cladosporium*, as well as other post-harvest fruit rots were evaluated. Treatments were replicated two times for each site.

Results: Flies exposed to *Botrytis* spores increased the number of *Botrytis* infected fruit in our laboratory studies relative to fruit that were not exposed to flies carrying spores (Figure 4).

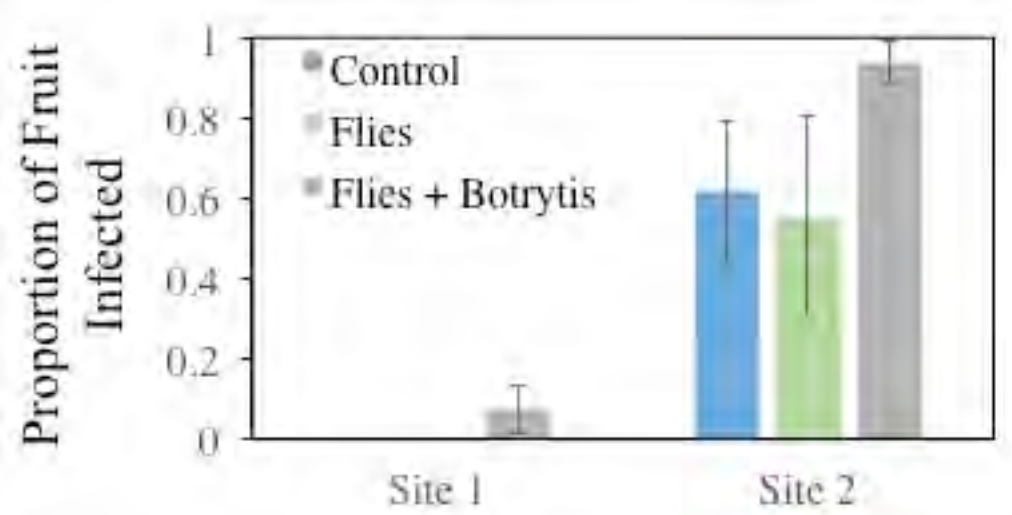

Figure 4. Mean ± SE (N = 2) proportion of fruit infected with *Botrytis* fruit rot for each laboratory treatment for each site.

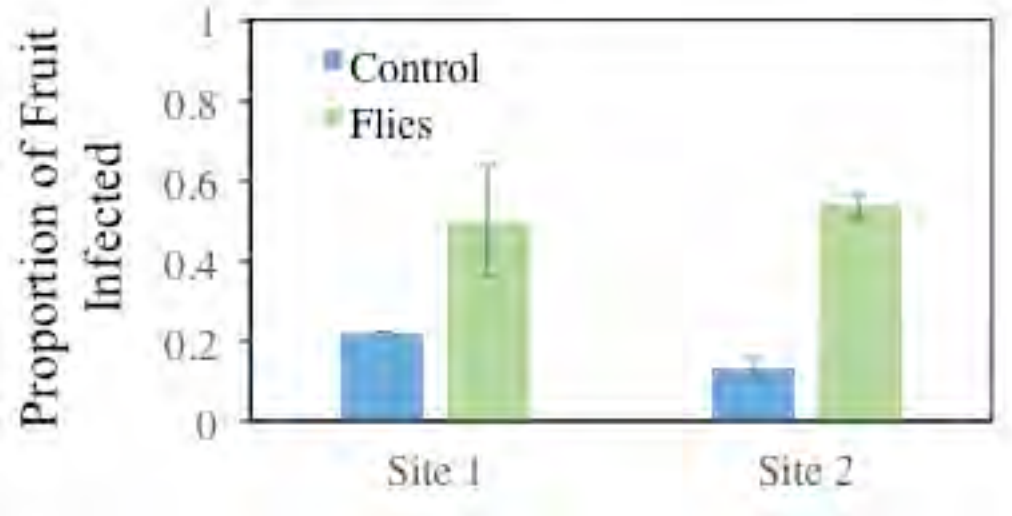

Figure 5. Mean ± SE (N =2) proportion of fruit infected with *Cladosporium* fruit rot for each laboratory treatment for each site.

However, we do not know if the number of spores the laboratory flies were carrying would be similar to the spore exposure of flies in the field, nor do we know if flies in the field would visit un-infected fruit within 24 hours of spore exposure. Therefore, this laboratory experiment is a worst-case scenario. Site 2 had a much higher background level of *Botrytis*, probably due to infections that occurred during flowering. Interestingly, the introduction of laboratory flies and subsequent wounding of the field collected fruit by flies increased the number of fruit that were

infected with *Cladosporium* fruit rot at both sites (Figure 5). Therefore, it is likely these fruit carried latent *Cladosporium* infections that better developed into rot after the flies wounded the fruit.

Discussion: Spotted wing drosophila, *D. suzukii*, and fruit rot pathogens occur together in Mid-Atlantic fall red raspberry fields, and are likely impacting one another. However, we are just scratching the surface of these potential impacts with the preliminary laboratory and field studies that were conducted with NABG-RF research funds in 2015. *D. suzukii* larvae are feeding upon yeasts and hyphal fungi in Mid-Atlantic fall red raspberries and are impacting the microbial communities of these fruit. We have confirmed that *D. suzukii* is associated with both *B. cinerea* and *Cladosporium* infected berries, and that wounds can increase incidence of *Cladosporium* under controlled laboatory conditions. *Cladosporium* is not typically observed as a pre-harvest pathogen--disease development of this wound-faciliated pathogen in the field may primarily be a result of feeding activity of *D. suzukii*. We also observed that if flies are exposed to *Botrytis* spores they may be able to transport them to healthy fruit and initiate *Botrytis* infections. Do these larvae later die when the fruit rot infection advances (the fungus covers the fruit entirely), or do they finish development before the infection progresses and emerge successfully? If these flies successfully emerge, will they then carry spores to other fruit that are not exhibiting disease symptoms? Does *D. suzukii* interact with all fruit rot pathogens similarly, or are there differences in fungal physiology and host interactions that influence this association? If *D. suzukii* are important to the development of fruit rot, this may mean that (1) the disease is less severe in early season raspberries that are less affected by the flies and (2) controlling *D. suzukii* could help to minimize pre-and post harvest losses from fruit rots. Second year data will help clarify these relationships.

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