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Biological, Behavioral and Preventative Management of the Invasive Insect Pest, Spotted Wing Drosophila (Drosophila suzukii Matsumura), in Maine Lowbush Blueberry (Vaccinium angustifolium Aiton)

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BIOLOGICAL, BEHAVIORAL, AND PREVENTATIVE MANAGEMENT OF THE INVASIVE INSECT PEST, SPOTTED WING DROSOPHILA (*DROSPHILA SUZUKII* MATSUMURA), IN MAINE LOWBUSH BLUEBERRY (*VACCINIUM ANGUSTIFOLIUM* AITON)

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THESIS ACCEPTANCE STATEMENT

On behalf of the Graduate Committee for Gabriel Al-Najjar I affirm that this manuscript is the final and accepted thesis project. Signatures of all committee members are on file with the Graduate School at the University of Maine, 42 Stodder Hall, Orono, Maine.

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BIOLOGICAL, BEHAVIORAL, AND PREVENTATIVE MANAGEMENT OF THE INVASIVE INSECT PEST, SPOTTED WING DROSOPHILA (*DROSPHILA SUZUKII* MATSUMURA), IN MAINE LOWBUSH BLUEBERRY (*VACCINIUM ANGUSTIFOLIUM* AITON)

By Gabriel Al-Najjar

Thesis Advisors: Dr. Francis A. Drummond and Dr. Eleanor Groden

An Abstract of the Thesis Presented
in Partial Fulfillment of the Requirements for the
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This research was conducted in order to identify the potential for utilization of various management techniques against the invasive *Drosophila suzukii* Matsumura, commonly referred to as the spotted wing drosophila (SWD), using Maine lowbush blueberry (*Vaccinium angustifolium* Aiton) as a model crop system. These included evaluations of three prospective approaches often considered when developing agricultural pest management programs for novel insect pests: 1) biological control through the intended release of natural enemies, in this case entomopathogenic fungi; 2) behavioral management through mass trap deployment in order to capture and kill adult SWD, and; 3) prevention through the deployment of insect exclusion netting during the pre-harvest fruit ripening period. The first assessment was accomplished through

complementary laboratory and field experiments. Mass-inoculation laboratory assays with four species of fungi resulted in significant mortality of SWD flies over five days post-exposure ($P < 0.0001$). While both *Beauveria bassiana* (strain GHA) and *Metarhizium anisopliae* (strain F-52) were among the most lethal isolates, only *B. bassiana* mycoses were shown to exert a significant dose-mortality response over a three day period following initial contact with conidia ($P < 0.0001$); based on the data obtained, the derived LC_{50} value corresponded to a pathogen surface density of approximately 16,000 conidia mm^{-2}. Although no detectable mortality effect was found during the *M. anisopliae* assay ($P = 0.64$), the frequency of sporulating fly cadavers increased substantially at elevated conidia doses of either fungal pathogen ($P < 0.0001$). A sub-lethal assessment of *B. bassiana* mycosis on reproductive development in immature *D. suzukii* females also generated support for decreased oocyte maturation rates in individual flies ($P = 0.02$). Coupled with the observable germination of conidia through SEM imaging, these results provide strong evidence for positive infection under laboratory conditions. Despite these promising results, however, the subsequent field evaluation of a commercially available *B. bassiana* (strain GHA) containing myco-insecticide yielded no additional evidence that could justify these entomopathogens as being feasible biocontrol agents in SWD management. Spraying blueberry enclosures prior to the introduction of 2,000 adult SWD failed to reduce the quantity of larvae inhabiting fruit samples, with 59 ± 63 (SD) vs 28 ± 19 obtained in sprayed vs unsprayed plots, respectively.

Objectives two and three entailed field experimentation only with lowbush blueberry. Mass trapping with volatile semiochemicals was evaluated at different trap concentrations. Varying the spatial arrangement of traps within study grids significantly influenced the quantity of SWD larvae infesting sampled blueberry fruits ($P = 0.0003$). The trap design and bait tested here were most effective when deployed at the lowest density (0.9 m trap spacing). Fruit samples collected from crops provided this treatment contained mean larval infestations of 1.5 ± 1.8 (SD). For comparison, the deployment of traps with 1.8 and 2.7 m of trap spacing resulted in larval sampling averages of 8.8 ± 11.1 (SD) and 17.3 ± 13.7, respectively. However, there was no detectable treatment effect of trap spacing on the mean number of adults captured in traps ($P = 0.40$). The results of this field investigation, in conjunction with those of other studies, might justify additional research on trap cropping in order to reduce the overall degree of chemical inputs required to adequately suppress fruit infestation.

The final objective produced results consistent with those of analogous investigations, which have shown insect-netting to be an effective preventative agent for physical exclusion of SWD flies from contacting viable host fruits prior to harvest intervals. Studies conducted in the lowbush blueberry agroecosystem during summer of 2014 and 2015 provide further support for this conclusion; net-protected fruits contained an average of 0.2 ± 0.2 (SD) larvae, in comparison to uncovered control fruits in which an average of 5.2 ± 3.9 larvae were sampled ($P < 0.0001$). In order to confidently implement novel

management techniques for suppressing SWD infestations, the observations gathered in this assessment cannot justify the immediate utilization of any technique as a replacement for insecticidal treatments. Even the positive results obtained from insect-netting experiments were constrained by limitations of spatial practicality with respect to application in large scale fruit growing operations. Therefore, additional experimentation will be necessary before identifying any of these techniques as viable approaches to incorporate with developing integrated pest management programs.

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THESIS INTRODUCTION

Spotted Wing Drosophila Geographic Distribution and Biology

Drosophila suzukii Matsumura (Diptera: Drosophilidae), commonly referred to as the spotted wing drosophila (SWD), is a polyphagous insect species native to Southeast Asia. Its current spatial geographic distribution extends far beyond Asia, since SWD has become an invasive agricultural pest in North America and Europe. This insect was first documented outside its native range on the Hawaiian Islands in 1980, and its subsequent expansion to the mainland of North American evidently did not occur until 2008 in California (Lee et al. 2011a). Since initial detection, this species has rapidly traversed North America to regions on the East Coast and then spread into Mexico and Canada. Its spread is partially due to the seasonal introduction of infested fruit, which exacerbates the annual persistence of this pest and allowed establishment of perennial SWD populations in climatically conducive geographic regions supporting wild and/or cultivated plant hosts (Cini et al. 2012, Asplen et al. 2015). The reproductive and developmental plasticity of this Dipteran is rather extraordinary; SWD have been observed exploiting floral resources in the absence of more preferred fruit hosts (Walsh et al. 2011).

SWD Phylogeny, Morphology and Phenology

Genetic sequencing and Bayesian analyses of mitochondrial cytochrome oxidase enzymes support the hypothesis of phylogenetic divergence of the *suzukii* and *melanogaster* subgroups within the *Drosophila* genus (Lewis et al.

2005); the latter subgroup includes the common vinegar fly, *Drosophila melanogaster* Meigen. Unlike most *Drosophila spp.* that oviposit in overripe or physically damaged fruit, SWD females utilize their sclerotized and serrated ovipositors to penetrate ripe or ripening berries and stone fruits with varying degrees of preference between not only plant species, but even among clones within a given cultivar (Burrack et al. 2013). Spotted wing drosophila oviposition stings leave fruit integuments with scars and tears, further threatening fruit integrity through increased vulnerability to microbial invasion by bacteria or yeasts, and secondary infestation of other insect pests with frugivorous juvenile stages (Louise et al. 1996, Asplen et al. 2015).

Pronounced sexually dimorphic traits make SWD adults morphologically distinguishable from most *Drosophila* species. In regions of Asia, accurate identification of SWD may be hampered by the presence of *Drosophila subpulchrella*, a closely related species within the *D. suzukii* species subgroup (Hauser 2011). During the first 48 h of adult maturation, males of each species develop a distinct, darkly pigmented spot distally on each wing (Asplen et al. 2015). While less obvious in relation to wing spots, fully mature males also possess two dark bands or combs on the first and second tarsal segments of each foreleg. In *D. suzukii* males, combs are oriented in a single row on each appendage; this contrasts with *D. subpulchrella* that has two distinct rows of combs on each tarsal segment (Hauser 2011). Females lack either of these attributes and must be identified by their prominent ovipositor. While the

potential introduction of species like *D. subpulchrella* to foreign continents must not be overlooked, these close relatives of *D. suzukii* have not been reported in North America or Europe (Takamori et al. 2006). Thus, in relevant geographic regions the capture of *Drosophila spp.* bearing the aforementioned physical traits is highly indicative of SWD presence.

Under favorable climatic conditions, adult female SWD live up to four weeks and are capable of producing well over 350 eggs, with peak laboratory oviposition rates exceeding 25 eggs day^{-1} at 25ºC (Gerdeman et al. 2013, Kinjo et al. 2014). Some individual females are capable of sustaining a degree of reproductive activity at temperatures exceeding 30°C, but no oviposition occurs below 10°C (Walsh et al. 2011, Lee et al. 2011a). Gravid female flies have also been described revisiting an individual fruit and laying multiple eggs in separate oviposition bouts. To my knowledge, no published studies have been conducted on the lower thermal thresholds for survival of juvenile SWD. Cold tolerance research has primarily focused on identifying alterations in pre-overwinter adult reproductive activity and survival in order to better define the pest's physiological cues for diapause (Tochen et al. 2014, Jakobs et al. 2015, Ryan et al. 2016).

The Oregon State University *D. suzukii* degree-day phenology model suggests that a single generation requires 494 degree days at base temperature 10 ºC. However, Wiman et al. (2014) suggests that degree days most effectively predict growth of insect populations characterized by at most a few generations with high synchrony. They state that a high degree of generational overlap has

been recognized in SWD populations, advocating for stage-specific models as an alternative population monitoring tool. Kinjo et al. (2014) have provided evidence that a high proportion of eggs hatch into larvae within 48 h of oviposition at 25ºC. They observed that constant temperatures ranging from 25 - 31°C promote larval development, with 100% mortality occurring consistently at 33°C. Under favorable climatic conditions, SWD larvae voraciously feed on internal host tissues as they develop through three instars, potentially completing larval development in as little as five days. It is widely understood that a high proportion of pupae metamorphose within fruit hosts, as opposed to externally on or in soils (Walsh et al. 2011). However, unpublished data by Ballman and Drummond (2014) suggest that a significantly greater proportion of pupae were found in soil samples as opposed to Maine lowbush blueberry fruits. Given the current and ongoing development of effective management guidelines that work to constrain the reproductive capacity of invading SWD populations, further investigation is necessary to shed light on the species' crop-specific population dynamics.

Under favorable conditions, SWD adults emerge from the puparium in as few as four days (Gerdeman et al., 2013). At about 25ºC, average development time from egg to adult has been shown to be gender-independent, taking an average of 22 days (Lee et al. 2011a, Tochen et al. 2014). The duration of time until reproductive maturity (pre-oviposition development) varies in different drosophilid taxa. It is understood that females of most species are not

reproductively active immediately after eclosion (Markow and O'Grady 2008). This is consistent with laboratory dissections of newly emerged SWD females (Alnajjar 2016), in which fully developed oocytes were not observed until approximately two days into adulthood at 25ºC ± 1 ºC. Kinjo et al. (2014) have further shown that temperatures above 25°C result in significant declines in individual growth and development rates, as well as decreased oviposition rates of females.

In conjunction with a reduction in survival rates, adult male gamete sterility is believed to occur at temperatures exceeding 30°C (Kanzawa 1934, Walsh 2011). However, no studies have specifically focused on the reproductive phenology of *D. suzukii* males. In many *Drosophila spp.*, spermatogenesis occurs during juvenile development and males eclose with viable gametes. The duration and extent of gamete elongation varies drastically across species; *D. melanogaster* sperm reach 1.9 mm in length and require 48 h of adult development, in contrast *Drosophila bifurca* sperm that ultimately extend 55 mm in length over the course of three weeks (Markow and O'Grady 2008). Currently SWD male gametogenesis has not been examined, and our knowledge concerning the pest's reproductive biology is therefore incomplete. Nonetheless, research continues to progress and facilitate a better understanding of the species' population growth mechanisms and rapid geographic expansion.

A Novel Agricultural Pest

When SWD was first detected in 2008, the California strawberry industry accounted for roughly $US 338 million in revenue, and the state raspberry production was estimated at $US 80 million (Goodhue et al. 2011). Regional raspberry and blackberry losses due to the 2008 SWD infestation reached about $US 42.9 million, or 20% of the total crop output in three California counties (Bolda et al. 2010). This generalist insect pest has also caused a significant reduction in highbush blueberry yields in Oregon and Washington. In 2008, these three states (California, Oregon and Washington) collectively accounted for over 25% of highbush blueberry production in the United States, and experienced an estimated revenue loss of approximately 40% due to SWD (Bolda et al. 2010). In combining the exhaustive list of host fruits (*see* Lee et al. 2011a) with the recent and advancing invasion in the United States, a country in which berry and stone fruit crops contributed roughly $US 4.5 billion to the economy in 2007 (Bruck 2011), the establishment of this insect undoubtedly poses a considerable threat to small fruit production. This is especially true with the occurrence of SWD in agricultural systems where geographic climatic patterns may result in greater population densities during or prior to the harvest of susceptible fruit.

SWD Documented Destruction

Lowbush (wild) blueberry (*Vaccinium angustifolium* Aiton) is a native and economically valued shrub of Maine. With over 24,300 hectares managed and harvest yields varying between 30 and 45 million kg. per year (Drummond et al. 2013), its cultivation contributes significantly to Maine's economy. In 2007, for

example, wild blueberry farmers generated roughly $250 million in farm gate revenue (Yarborough 2013). This agroecosystem has traditionally required management of only native insect pests, including the blueberry gall midge (*Dasineura oxycoccana* (Johnson)), blueberry spanworm (*Itame argillacearia* (Packard)), blueberry flea beetle (*Altica sylvia* Malloch), red striped fireworm (*Aroga trialbamaculella* Cham), strawberry rootworm (*Paria fragariae* Wilcox), blueberry sawfly (*Neopareophora litura* Klug) and blueberry maggot fly (*Rhagoletis mendax* Curran) (Drummond and Collins 1999). While invading SWD populations may result in devastating yield loss in the absence of sufficient control procedures, a degree of management efficacy has currently been achieved by implementing an integrated pest management (IPM) approach through which action is only taken to protect crops when necessary (Yarborough 2013, Drummond and Yarborough 2013). Notwithstanding, sustained reliance on a single treatment approach, however potent initially, can result in decreased efficacy over time. For this reason, it is recommended that crop protection involve rotational or concomitant application of multiple tactics throughout each growing season (Chaudhary 2008).

SWD Management in Maine Lowbush Blueberry

In October 2011, only three years after its introduction to the West coast of North America, *D. suzukii* was detected in Maine (Drummond and Yarborough 2013). Its population growth capacity and documented devastation during this time caused farmers to adopt a temporal shift in the harvest period for Maine

lowbush blueberry. The perceived potential yield loss incurred by SWD damage exceeds the presumed yield loss of conducting earlier harvests when a lower proportion of fruit may be in peak ripeness (Drummond and Yarborough 2013). Given the limited management options for this novel pest, earlier harvesting has become the recommended option for both organic and conventional lowbush blueberry growers. Unfortunately, the impacts of this management protocol on yields have yet to be quantified. Hence, while conducting earlier harvests has provided a temporary solution for mitigating the quantity of SWD infested fruits in lowbush blueberries, the economic feasibility of this approach has been questioned due to the harvest of considerable amounts of fruit before ripening. The sustainability of this tactic may also be short lived due to the potential of future climate change affecting SWD development and survival in the region. It is possible that temperature shifts favoring warmer winters will entail an increase in overwinter survival of SWD adults, and a shift toward earlier population increases in the summers that follow (Drummond 2016).

Currently, no quantitative assessments on revenue lost from SWD infestations in lowbush blueberry have been conducted. A portion of indirect economic costs in any given crop system are attributed to distribution restrictions of fruit grown in invaded regions, as well as the necessary pest prevention and monitoring protocols required to mitigate infestations (Lee et al. 2011a). The current IPM approach entails insecticidal applications upon the first detection of adult males captured in traps emitting volatile semiochemicals (Drummond and

Yarborough 2013). Bruck et al. (2011), and Collins and Drummond (2016a, 2016b) conducted insecticide efficacy experiments and found that, of the chemicals tested pyrethroid, organophosphate and spinosyn class-insecticides induced the highest mortality rates in adult SWD. Unfortunately, spray applications are unlikely to kill SWD juvenile stages, which are often protected from exposure within the fruits for a majority of their development. Insecticide tactics may not always be necessary, however, if monitoring and an early harvest approach are implemented appropriately (Drummond 2016).

It is important to consider that genetic resistance to insecticides has been well documented in insects (Brattsten et al. 1986, Campos et al. 2014) and suggests that persistent selection due to insecticides imposed on SWD populations may give rise to more physiologically tolerant or resistant genotypes. Fortunately, spatial overlap of SWD populations utilizing naturally occurring plant hosts with pestiferous agricultural populations is thought to sustain a degree of mixing and genetic flow, preventing homogeneity in the allelic frequencies conferring genotypic resistance. Therefore, the likelihood of insecticidal resistance arising in pestiferous SWD populations presumably increases with the degree of isolation from adjacent wild populations (Asplen et al. 2015).

It is also necessary to acknowledge that the three most lethal chemical classes to SWD adults also display high acute toxicity toward ecologically and economically valuable bee species (IPC 2011, Bunch et al. 2014, NPIC 2014). Although guidelines are available for minimizing toxin exposure to foraging wild

and commercial bees during lowbush blueberry bloom (Drummond and Stubbs 2003, Yarborough et al. 2015), recent bee diversity surveys in the Maine lowbush blueberry agroecosystem suggest that the co-occurrence of herbaceous plant species can provide additional floral resources for pollinator visitation after crop fruit set has begun (Bushman and Drummond 2015). Hester et al. (2001) have also provided evidence that runoff and drift increase the incidence of contact with wild and domestic bee populations. Runoff and drift can also redistribute pesticides into watersheds and surrounding landscapes, further facilitating direct contact of toxins with non-target organisms (Klöppel and Kördel 1997, Beketov et al. 2013). Laboratory assays conducted by Iwasa et al. (2004) further suggest possible acute synergistic physiological toxicity in bees. They observed drastic reductions in honey bee (*Apis meliffera*) LD_{50} values for acetamiprid, a neonicotinoid insecticide, after direct topical exposure to the fungicide propiconazole, a P450 enzyme inhibitor. While additional human toxicity analyses report minimal to no acute hazards from insecticides utilized in SWD management (IPC 2011, Bunch et al. 2014, NPIC 2014), the gap of research addressing chronic health complications should not be understated. Given that SWD is expected to persist as an agricultural pest and continue expanding its geographic distribution, the concerns outlined here necessitate the development of non-chemical management alternatives that are more compatible with the goals of human health and environmental conservation.

Non-Chemical Management Options for SWD

The utilization of physiologically attractive volatile chemicals has been proposed as a foundation for a mass "trap and kill" technique by Kanzawa (1934). While contemporary utilization of such chemicals for *D. suzukii* management is restricted to monitoring purposes, their implementation in behavioral control has not been extensively investigated. Currently, field studies have shown synergized attraction of SWD adults to acetic acid and ethanol mixtures, as opposed to either volatile alone (Landolt et al. 2011, 2012, Burrack et al. 2015). However, more assessments will be necessary to determine the capacity of these volatiles to sufficiently prevent infestation of fruit. Another alternative control to insecticides involves the installation of fine mesh netting as a physical barrier to *D. suzukii*. Data obtained thus far support the notion that protective crop netting effectively prevents highbush blueberry infestation by SWD (Cormier et al. 2015). Research has also been conducted in lowbush blueberry on mass trapping and exclusion netting (Yarborough et al. 2015, 2016). Both of these control tactics are described by Drummond and Yarborough (2013), but are characterized as not well tested and are not strongly recommended for SWD management in this crop system. The efficacy and practicality of implementing these protective strategies in other crop systems will therefore require further analysis.

Potential Biocontrol Agents of SWD

Biological control is frequently considered when developing IPM programs for invasive pests. This approach entails the release of naturally occurring

enemies to suppress pest populations once detected at the relevant abundance threshold. In northeastern Spain, Gabarra et al. (2014) have identified two hymenopteran parasitoids, *Pachycrepoideus vindemmiae* (Rondani) and *Trichopria* cf. *drosophilae* Perkins, consistently associated with SWD pupae surveyed from experimental plots of strawberry and raspberry. Their laboratory assessment demonstrated that both parasitoid taxa are able to utilize SWD as a reproductive host, indicating the potential for inoculative release. The investigators also describe one insect soil predator of SWD larvae and pupae, *Labidura riparia* Pallas, which, along with both parasitoid taxa, are able to reduce the pest's population growth in controlled environments. The author importantly notes that the potential agroecosystem impacts on trophic levels resulting from separate or concomitant release of insectivorous insects will require further research.

In Maine, a two-year sampling period (2014 and 2015) for parasitoids of SWD larvae and pupae has not resulted in positive detection (Ballman and Drummond 2014). However, predators have been found to exert mortality on SWD in lowbush blueberry fields, but the predatory species have not yet been identified, although members of the Carabidae and Gryllidae are suspect (Ballman and Drummond 2014). With the rapid geographic expansion of SWD, crop-specific pest management assessments have recently received more attention. Currently, the 20th century development and application of

entomopathogenic fungi is being examined as another possible biological control agent for this devastating agricultural pest.

Entomopathogenic Fungi; Applications in Agriculture

At the turn of the century an overwhelming majority of commercially developed myco-insecticide products contained one of three fungal species from the taxonomic division Ascomycota: *Beauveria bassiana* (Bals.) Vuill.*, Isaria fumosorosea* Wize (formerly *Paecilomyces fumosoroseus*), and *Metarhizium anisopliae var. anisopliae* (Metschn.) (Butt et al. 2001). Collectively, these fungi are implemented in a wide range of IPM programs and cause mycoses in a variety of species in the orders Coleoptera, Hemiptera, Blattodea, Orthoptera, Thysanoptera, Lepidoptera and Diptera. Microscopic conidia are asexually produced reproductive agents that require an arthropod host to fulfill completion of one life cycle. These naturally occurring entomopathogens have only very rarely been documented in cases concerning human health (Henke et al. 2002). In addition, their utilization in agriculture is believed to be innocuous toward non-target insect populations (Goettel et al. 2000, Shah and Pell 2003).

Pathogenicity Toward Insect Hosts

Fungal pathogenicity begins with physical localization of conidia on the insect cuticle. Favorable temperatures and a high relative humidity are the primary environmental stimuli for infection. Generally speaking, optimum growth temperatures for entomopathogenic fungi range from 20°C - 30°C. Under these climatic conditions, insect-pathogen enzymatic recognition signals physiologically

stimulate spore germination and hyphal growth on the insect integument (Tanada and Kaya 1993). Proteases are a key constituent of the pathogen's enzymatic arsenal and contribute significantly to the penetration of cuticular layers, with teneral insect integuments providing less protection against integument digestion (Shah and Pell 2003). Internally conjoined structures of the insect integument such as spiracles and sensory receptors may present additional, indirect routes of invasion for virulent conidia (Tanada and Kaya 1993). After successful host infiltration, often via appressorial formation, the fungus produces blastospores that propagate asexually and disperse throughout the insect's hemolymph, resulting in eventual host death via nutritional deficiency, toxin accumulation, or destruction of internal tissues (Shah and Pell 2003, Tanada and Kaya 1993). Under appropriate climatic conditions, the fungus enters a saprophytic reproductive stage in which hyphae and sporophores extend out of the insect cadaver and project into the atmosphere. Transmission of next generation conidia to subsequent hosts then becomes possible through physical contact with cadavers or aerial dispersal of conidia.

Perhaps equally paramount to effective mycoinsecticidal insect pest management; reduced consumption, growth and reproductive rates have been documented in insects exposed to sub lethal doses of entomopathogenic fungal inoculum. Gindin et al. (2006) observed non-lethal manifestations of mycosis in adult red palm weevils (*Rhynchophorus ferrugineus*), which displayed reductions in egg hatch and oviposition rates after direct topical exposure to *M. anisopliae*

conidia. Another study conducted by Moorthi et al. (2015) showed that inoculating early instar Oriental leafworm moth larvae (*Spodoptera litura*) to sub-lethal concentrations of *I. fumosorosea* or *B. bassiana* conidia significantly and persistently decreased consumption and development rates throughout subsequent life stages. The results of Moorthi et al. (2015) also suggest a dose-dependent reduction in oviposition rates of adult *S. litura* exposed to *I. fumosorosea* conidia as early instar larvae. Given the propensity of mycoses to cause both acute and chronic syndromes in a wide taxonomic spectrum of insect hosts, exploring these potential consequences of entomopathogenic fungal infection in adult SWD will aid in addressing the urgent needs of developing pest management programs.

Research Objectives

This project explored several alternative tactics to insecticidal SWD management. The first approach involved the use of entomopathogenic fungi as biocontrol agents of SWD in Maine lowbush blueberry. A preliminary *D. suzukii* adult lethality screening was accomplished through direct mass conidia inoculations of six entomopathogenic fungal strains within the species *B. bassiana, I. fumosorosea, M. anisopliae, and M. robertsii.* I then investigated the dose-dependent mortality response of SWD adults to infection by *B. bassiana* strain GHA and *M. anisopliae* strain F-52. I also explored the effect of sub-lethal *B. bassiana* exposure on ovarian maturation in teneral SWD females, and concluded with a subsequent SWD - mycoinsecticide efficacy test conducted with

controlled field experiments in Maine lowbush blueberry. Two other alternative control tactics were also investigated, mass trapping and exclusion netting. A field experiment was conducted to determine the effect of varying trap density on SWD adult recruitment/capture and larval infestation of lowbush blueberry fruits. A third field experiment was conducted to evaluate exclusion netting as a means of keeping SWD female adults from contacting and ovipositing in lowbush blueberry fruits.

CHAPTER I

Laboratory and Field Susceptibility of *Drosophila suzukii* Matsumura (Diptera: Drosophilidae) to Entomopathogenic Fungal Mycoses

Abstract

Spotted wing drosophila (*Drosophila suzukii* Matsumura) is an introduced generalist insect pest of berry and stone fruits in North America and Europe. Concerns over the environmentally obtrusive utilization of insecticides in agricultural pest management have facilitated a desire to shift away from chemical control where feasible. We tested the susceptibility of adult *D. suzukii* to infection by four species of entomopathogenic fungi. The species and strains that we evaluated were *Beauveria bassiana* (Bals.) Vuill., strains GHA and HF-23; *Isaria fumosorosea* Wize strains FE-9901 and Apopka 97; *Metarhizium anisopliae* var *anisopliae* (Metschn.) strain F-52; and *Metarhizium robertsii* (Clavicipitaceae) strain DW-346. All fungal mycoses throughout five days resulted in significantly greater mortality rates in comparison to non-treated flies ($P < 0.0001$). In a follow-up lethal concentration bioassay of the two most virulent isolates, increasing pathogen dosages from 0-16,000 *B. bassiana* (strain GHA) conidia mm^{-2} not only induced greater mortality rates in SWD flies ($P < 0.0001$) but also positively influenced the proportion of sporulating *D. suzukii* cadavers ($P < 0.0001$). While fly inoculations of 0-4,000 *M. anisopliae* conidia did not result in any measurable mortality response ($P = 0.693$), a greater frequency of cadavers

sporulated at higher pathogen concentrations ($P < 0.0001$). Scanning electron micrographs of both entomopathogens show germinating *B. bassiana* and *M. anisopliae* conidia on *D. suzukii* integument. Evidence is also provided for sub-lethal manifestations of *Beauveria bassiana* laboratory mycosis in teneral virgin females; oocyte maturation rates were significantly curtailed through one week of adulthood development ($X^2_{(1, n=13)} = 5.34$, $P = 0.02$.). Despite these promising laboratory results, however, an average of 59 ± 63 (SD) larvae were found infesting fruit samples 235 degree-days after the introduction of SWD flies to enclosures following a myco-insecticide application. This was greater than the 28 ± 19 (SD) mean larval counts from fruits not protected with the biocontrol agent. Zero larvae inhabited fruits collected from SWD exclusion control cages. Taken together, the results presented here show that entomopathogenic fungi are lethal towards *D. suzukii* in the laboratory with virulent capacity to induce chronic syndromes in the sub-lethal stages of mycosis. Yet the inability of the myco-insecticide to provide effective fruit protection against SWD necessitates further investigation for effective and economical application of entomopathogenic fungal strains in *D. suzukii* biocontrol programs.

Introduction

Drosophila suzukii Matsumura (Diptera: Drosophilidae), commonly referred to as the spotted wing drosophila (SWD), is a polyphagous insect pest species native to Southeast Asia. Currently, the species' distribution extends beyond Asia, and SWD has recently been described as an invasive agricultural pest in North America and Europe. Its establishment and persistence in these non-native continents is believed to be exacerbated seasonally by unintentional introduction of infested fruits and the potential establishment of perennial SWD populations in climatically conducive geographic regions that support wild and/or cultivated host plants (Asplen et al. 2015, Cini et al. 2012).

Unlike most *Drosophila spp.* that oviposit in overripe or physically damaged fruit, SWD females utilize a sclerotized and serrated ovipositor to directly penetrate ripe or ripening berries and stone fruits (Isaacs et al. 2012). The resulting oviposition stings leave fruit skins with scars and tears, further threatening fruit integrity through increased vulnerability to microbial invasion by bacteria or yeasts, and secondary infestation of other insect pests with frugivorous juvenile stages (Louise et al. 1996, Asplen et al. 2015). Under favorable climatic conditions, SWD population growth can proceed exponentially. Female flies live up to four weeks, during which individuals are capable of producing well over 350 eggs with peak laboratory oviposition rates exceeding 25 eggs day^{-1} at 25°C (Walsh et al. 2011, Gerdeman et al. 2013, Kinjo et al. 2014).

Lowbush blueberry (*Vaccinium angustifolium* Aiton) is a native shrub and agricultural crop in Maine. With over 24,300 hectares managed and harvest yields varying between 30 and 45 million kg. per year (Yarborough 2013), its cultivation contributes significantly to state's economy. In October 2011, only three years after its introduction to the West coast of North America, *D. suzukii* was reported in lowbush blueberry fruits (Drummond and Yarborough 2013). Given the limited management options for this novel pest, early harvesting has become the recommended option for both organic and conventional lowbush blueberry growers. However, it has been proposed that temperature shifts favoring warmer winters will induce an increase in overwinter survival of SWD adults, and a shift toward earlier population increases in the summers that follow (Separovic et al. 2013, Drummond 2016).

Current and Prospective SWD Management

The current integrated pest management (IPM) recommendation for this widespread insect pest entails precautionary insecticidal treatment immediately after adult male detection in baited traps emitting volatile semiochemicals (Drummond and Yarborough 2013). Unfortunately, spray applications have limited effect on SWD juvenile stages, which occur within host fruits for a majority of their development. Insecticide resistance has been observed and documented in numerous insect pests (Brattsten et al. 1986, Campos et al. 2014). While it has been suggested that persistent insecticidal selection pressures imposed on growing SWD populations may result in the development

of tolerance or resistance, the pest's phenology generally dictates that peaks in population abundance often occur through the post-harvest interval of many vulnerable crops in northern geographic regions (Haviland and Beers 2012). In addition, the utilization of naturally occurring plants as hosts by wild SWD populations could effectively dilute resistance genes in regions with spatial overlap between wild and agricultural SWD populations. It is worth noting, however, that many insecticide classes lethal to *D. suzukii* adults also display toxicity toward native and domestic bee species (Johnson et al. 2010, Bunch et al. 2014).

Biological control is frequently considered when developing IPM programs for pestiferous species (Chaudhary 2008). This approach entails the deliberate exploitation of predators or pathogens through conservation of naturally occurring antagonists, or via classical, inoculative or inundative release. The adoption of a given approach requires investigating the potential development of problematic trophic interactions manifested between control agents, the target pest species, and overlapping populations of non-target insects inhabiting the landscape (Hajek 2004).

At the turn of the century, entomopathogenic fungi were successfully utilized for management of various widespread and destructive insect pests in North American agriculture including the Colorado potato beetle, *Leptinotarsa decemlineata* (Say), and the European corn borer, *Ostrinia nubilalis* (Hubner) (Butt et al. 2001). At that time, an overwhelming majority of commercially

developed myco-insecticide products contained one of three fungal species from the taxonomic division Ascomycota: *Beauveria bassiana* (Bals.) Vuill.*, Isaria fumosorosea* Wize (formerly *Paecilomyces fumosoroseus*), and *Metarhizium anisopliae var. anisopliae* (Metschn.) (Butt et al. 2001). Investigations of the resulting mycoses have shown induction of both lethal and sub-lethal host syndromes in species of the orders Coleoptera, Hemiptera, Blattodea, Orthoptera, Thysanoptera, Lepidoptera and Diptera (Butt et al. 2001, Shah and Pell 2003).

Research Objectives

The goal of my research was to explore the potential for utilization of entomopathogenic fungi as biocontrol agents of SWD. A preliminary *D. suzukii* adult lethality screening was accomplished through direct mass conidia inoculations of six entomopathogenic fungal strains within the species *Beauveria bassiana, Isaria fumosorosea, Metarhizium anisopliae,* and *Meterhizium robertsii* (Clavicipitaceae). We then investigated the dose-dependent mortality response of SWD adults to infection by *B. bassiana* strain GHA and *M. anisopliae* strain F-52. The effect on ovarian maturation of sub-lethal *B. bassiana* exposure to teneral SWD females was also evaluated. In addition, a follow up myco-insecticide efficacy trial was conducted in controlled field cage experiments in Maine lowbush blueberry.

Methods

Isolation & Viability Estimations of Entomopathogenic Fungi

Beauveria bassiana strains GHA and HF-23, and *I. fumosorosea* strains FE-9901 and Apopka 97 were isolated from the myco-insecticides Botanigard®, Balance®, Nofly® and Preferal®; respectively. A small sample of each formulated material was suspended in 300µL aqueous 0.01% Tween® solution. *Beauveria bassiana* dilutions were cultured on a dodine growth medium consisting of 30 g/L wheat germ. Hodgson Mill® brand wheat germ was autoclaved in 1L dH_2O for 20 min and then strained through four layers of cheesecloth. Deionized H_2O was then added as necessary, to bring the volume up to one L. The following ingredients were added to 1 L of the liquid by stirring: 20g/L agar, 0.25g/L Chloramphenicol, 0.8 mL 0.1% Benomyl/dH_2O solution, 2 mL 0.5% Crystal Violet/dH_2O solution, and 0.3 g/L Dodine (65%). The mixture was again autoclaved for 20 min. One liter of the mixture was distributed evenly among about 40 sterile 100 mm x 15 mm petri dishes. Suspensions of *I. fumosorosea* were plated on Sabouraud dextrose agar (SDA) with added antibiotics streptomycin and penicillin each at 100 mg mL^{-1} media mixture. After approximately ten days of incubation at 25 ± 1°C, spores of individual *I. fumosorosea* and *B. bassiana* colony forming units (CFU) were collected and vortexed in 0.01% Tween® and transferred to cultures containing SDA or SDA with yeast (SDAy), respectively. Conidia of *M. robertsii* (Clavicipitaceae) were provided by the USDA ARS Collection of Entomopathogenic Fungi and cultured on ¼ potency SDA. *Galleria melonella* (Linnaeus, 1758) were inoculated with *M. anisopliae* conidia from laboratory cultures. Conidia obtained from sporulating *G.*

melonella cadavers were suspended in 0.01% Tween® and plated on ¼ strength SDA. All cultured fungi were incubated in a scotophase growth chamber at 25 ± 1°C for 10-14 days and stored in a non-illuminated laboratory refrigerator at 4 ± 1°C. Viability tests were conducted prior to each bioassay. This entailed suspension of conidia samples obtained from cultured fungi in 300µL 0.01%Tween®, plating on the fungus' respective growth medium, and incubation in a scotophase growth chamber at 25 ± 1°C for 20h. A sampling grid was then superimposed on the exterior surface of each plate. Random grid sections were magnified at 40x under a phase contrast microscope in order to quantify the relative proportion of germinating vs non-germinated conidia. A minimum of 200 conidia were sampled from any given culture.

Acquisition of SWD for Experimentation

All *D. suzukii* utilized in laboratory experiments were taken from reared colonies originating from captured adults in Maine lowbush blueberry fields in Washington Co., Maine. The laboratory colonies were infused annually with captured wild flies to maintain genetic diversity similar to field populations. Each *D. suzukii* culture was provided with Carolina Formula 4-24® instant *Drosophila* media and maintained in the laboratory under growth chamber conditions set to 25 ± 1°C and a 12 h L/D cycle.

Qualitative High Dose Fungal Inoculation of Flies

An initial study was designed to determine which fungal isolates would be the most promising for further assessments for biological control of spotted wing

drosophila. Viability tests for cultures of *B. bassiana* strain GHA (GHA) and HF-23 (HF-23), *I. fumosorosea* strains FE-9901 (FE) and Apopka 97 (AP), and *M. anisopliae* strain F-52 and *M. robertsii* strain DW-346 yielded roughly 95%, 93%, 92%, 95%, 90% and 84% spore germination, respectively. Four hundred and twenty adult SWD (1:1 sex ratio) were CO_2 immobilized and distributed equally among 21 culture vials. A culture sampling loop was utilized for delicate swabbing of cultured conidia until uniform spore coverage was achieved on the loop's tip. Conidia were then placed in sterile 1mL centrifuge vials. Three blank vials were included as a control treatment and each treatment was replicated three times. One set of twenty flies was introduced to each aggregate of conidia or the control and vortexed at low intensity for approximately 30 seconds. These treated flies were then transferred back to culture vials and placed in a growth chamber for five days (the period at which control mortality reached 20%). Mortality among fungal species and strains were assessed with nominal logistic regression and a subsequent series of binomial pairwise contrasts with a significance level of $\alpha = 0.05$. (JMP®, Version 12.0.1 SAS Institute Inc., Cary, NC, 1989-2007).

Conidia Concentration Mortality Assays

Dose-mortality assays of *B. bassiana* strain GHA (95% viability) and *M. anisopliae* strain F-52 (92% viability) conidia were conducted on 0-3 day old adult flies. The experiment was initiated by removing all existing adults from thriving colonies of SWD. After three days of colony development in a growth

chamber, three hundred 0-3 day old adults (1:1 sex ratio) were collected, immobilized with CO_2 and divided evenly into 30 culture tubes. Great care was taken to examine immobilized flies by grasping the wings so gender identification could be made with a brief observation of the genitalia.

One layer of filter paper was placed in each of thirty, 4.5 cm petri dishes. Suspensions of 1.2 x 10^5 - 10^9 GHA conidia mL^{-1} and 2.1 x 10^4 - 10^8 F-52 conidia mL^{-1} 0.01% Tween® were prepared within 90 minutes of the application. To allow for mortality comparisons between the two fungal pathogens, an additional suspension of 1.9 x 10^8 *B. bassiana* conidia mL^{-1} 0.01% Tween® was included as a treatment during the *M. anisopliae* assay. Prior to spraying, filter paper surfaces were slightly misted with dH_2O and covered with one layer of 0.22 μm GV millipore filter paper. Control groups were exposed to 0.01% Tween® only, and all treatments were replicated five times. Conidia suspensions were homogenized using a vortex mixer before being applied to the pre-moistened Millipore filters using a Burkard® computer controlled sprayer with a click setting of 6 and psi of 10. Five replicate dishes with Millipore filters plus a dish of water agar were treated with each concentration of each fungus (approximately 0.25μL applied per treatment dish). Control groups were exposed to 0.01% Tween® only. Water agar plates were observed under 40x with a phase contrast microscope to determine the exact density of conidia per mm^2 deposited on the filters.

After spraying, water droplets were allowed to evaporate for roughly 15 minutes to prevent drowning of immobilized SWD. Ten male and ten female adults were then released onto each treated surface. Dishes were rubber banded, placed in plastic bags with a moist paper towel, and incubated in the dark at 25 ± 1ºC for 24 h. Flies were then immobilized with CO_2, transferred to a culture vial and placed back into the growth chamber with a 12 h L/D cycle.

Starting 24 h after initial conidia contact, dead flies were collected daily for six days. Cadavers removed from the culture tubes were surface sterilized in 10% benzalkonium chloride, followed by two rinses in dH_2O, and allowed to dry by blotting on filter paper. Cadavers were then placed in individual wells of 48 well microtiter plates. Plates were held in plastic bags with a moistened paper towel to encourage sporulation of infected individuals. An extra set of eight adults was exposed to both fungal pathogens at the corresponding dosage of 1 x 10^8 conidia mL^{-1} application. Approximately 24 h after inoculation, these specimens were preserved in 70% EtOH for qualitative scanning electron microscopy examination of spore germination on the fly integument.

The dose-mortality response of flies to these entomopathogenic fungi was quantified with nominal logistic regression (JMP®, Version 12.0.1 SAS Institute Inc., Cary, NC, 1989-2007). Designating a control mortality cutoff of 20%, both logit models were constructed with three days of mortality and sporulation data. Unfortunately, the intended gender-specific response assessment was not possible due to inconsistently greater ratios of males observed in some

replicates. This was attributed to the visual identification of individuals as being male based on the presence of wing spots. Given that newly emerged SWD males require additional post-eclosion maturation time for the pigmentation of wing spots, their absence during fly collections could have led to the disproportionate collection of individuals that were incorrectly identified as female.

SWD Oocyte Maturation During *B. bassiana* Mycosis

An experiment was conducted to assess sublethal dose effects of *B. bassiana* on SWD fecundity. Fungal culture viability was estimated to be approximately 95% using the methods previously outlined. Thriving SWD cultures were immobilized with CO_2 and all live adults were removed. Immature SWD in the cultures were then allowed to continue development for 20 h in a growth chamber. Newly emerged flies were anaesthetized and examined to ascertain gender by applying slight pressure to the mediolateral abdominal region of flies in order to force protrusion of copulatory organs. A suspension of 1×10^8 conidia mL^{-1} 0.01% Tween® and a 0.01% Tween® only control were applied to eighteen 4.5cm petri dishes with Millipore filters on top of moistened filter papers using the protocols described above for dose-response bioassays.

After spraying, a small amount *Drosophila* media was placed on the periphery of each dish such that there was minimal hindrance of conidia-treated surfaces from contacting fly appendages. Flies were then introduced to sprayed Millipore filters, and each dish was sealed with Parafilm® for moisture retention.

Sexual maturation was allowed to progress under growth chamber conditions at 25 ± 1ºC with a 12h L/D cycle. An OMEGA® OM-90 series temperature/humidity data logger was placed directly in the growth chamber and in a sprayed and sealed petri dish without flies to monitor relative humidity during the incubation period. Prior to dissections, any mortality was noted but cadavers were allowed to remain in petri dishes. Three replicated SWD dissections were conducted on each of 5, 6 and 7 days of post-eclosion adult maturation. To accomplish this, live flies were euthanized in 70% ethanol and ovaries were carefully extracted in order to count the number of fully developed oocytes held by each female, with treatment samples divided up by replicate and dissection day. Overall, six total dishes (three of each treatment) were processed at any given dissection time. At this time, cadavers were removed from dishes, surface sterilized, placed in individual wells of 48 well microtiter plates with a moist paper towel, and monitored for sporulation. An additional set of eight adult SWD was exposed to the experimental *B. bassiana* conidia dosage. About twenty hours after inoculation, these individuals were euthanized in 70% EtOH so infection could be qualitatively confirmed in individuals via scanning electron microscopy. A generalized linear model was utilized under the assumption of exponentially distributed response data with firth bias adjusted estimates (JMP®, Version 12.0.1 SAS Institute Inc., Cary, NC, 1989-2007). Time (fly post-eclosion age in days) and treatment (exposure dose of conidia) were included in the model as independent variables.

Scanning Electron Microscopy

We were interested in documenting fly integument penetration by germinating *B. bassiana* and *M. anisopliae* conidia. Based on preliminary specimen preparation trials of uninfected flies, the utilization of aldehyde tissue fixation techniques (*see* Pekrul and Grula 1979) was deemed unnecessary for SEM imaging of the SWD fly integument. Rather, specimens held in 70% ethanol directly underwent a sequential series of ethanol dehydrations in 70%, 80%, 85%, 90%, 95% and 100% ethanol, entailing complete submersion in each dilution for three separate seven-minute cycles. Specimens were held in 100% ethanol after final dehydration, and desiccated with a Tousimis Samdri® PVT-3 Critical – Point Dryer. Each fly was then grounded to the base of a mounting stub with silver conduction paint and coated with a 35nm layer of Gold/Palladium (Au/Pd) in a Cressington® 108 Auto/SE Sputter Coater. Specimens were examined and images were obtained under an AMRay®-1820 scanning electron microscope.

SWD - Mycoinsecticide Efficacy Assessment

A field-cage study was conducted in the summer of 2015 to assess the biological control potential of *B. bassiana* conidia applied on lowbush blueberry. Nine nylon mesh cages obtained from Young's Canvas Shop® were erected over lowbush blueberry plants on 30-Jul 2015 at Blueberry Hill Farm in Jonesboro, Maine. One set of three, 6 m x 2.5 m cages were tan mesh with coverage area of approximately 15 m^2 with 121 holes per cm^2, and the remaining cages

consisted of black mesh with dimensions 6 m x 2.7 m enclosing approximately 16 m^2 of crop with about 81 holes per cm^2. Three statistical blocks (replicates) with each of three treatments consisted of one set of tan cages, and two sets of black cages. One red Solo® cup baited with sugar water and yeast was deployed on a metallic 76 cm plant support post in each cage for one week in order to detect the presence of any adults prior to initiating the experiment. No SWD were detected prematurely, before the initiation of the experiment, in any of the nine cages.

On 12-Aug, the day prior to SWD release, flies from laboratory-reared colonies were aggregated in culture vials containing *Drosophila* media. Estimations of SWD abundance in each culture vial were qualitatively obtained via visual comparison with colonies of the following known fly densities: 50, 100, 150 and 200 flies. Colonies were distributed evenly among six sets totaling 2,000 flies each, and held in an air conditioned room until transport to the Jonesboro, ME field site (University of Maine Blueberry Hill Experiment Farm) on the following day.

Beauveria bassiana Spray Applications and Conidia Sampling

Preceding SWD introduction on 13-Aug, the formulated *B. bassiana* (strain GHA) product Mycotrol® was applied at the recommended application rate of 2.3 L ha^{-1} ($2.5x10^{13}$ conidia ha^{-1}) to one randomly selected release cage in each of the three blocks of cages using a CO_2 powered R & D Sprayers® backpack sprayer fitted with a hollow-cone nozzle. In order to assess the effect of cage

shading on conidia longevity on foliage and fruit, a non-caged 43 m^2 crop section of field was divided into three sampling plots and sprayed with Mycotrol® at the same recommended rate and application date as the enclosure cage applications. At 0 (ca. ten minutes), 24, 48 and 96 h after application, six blueberry leaves were collected from the *B. bassiana* treated plants in each of the treated cages and non-caged plots. In each cage and plot, two leaves were sampled from each of three areas: high, medium and low stem positions. Eleven mm diameter disks were cut from each leaf using a copper cork-borer, and the six disks cut from the same cage or plot were pooled in one sterile centrifuge vial and stored in a chilled cooler containing ice packs.

Immediately upon arrival at the laboratory, each of the pooled leaf disks sampled were homogenized in 30 mL 0.01% Tween® for 1.0 min. Conidia suspensions from these samples were serially diluted to 0.1x the stock concentration, and three 0.5 mL aliquots were each cultured on individual petri dishes containing a Dodine® wheat germ growth medium. These cultures were then incubated in the dark at 25 ± 1ºC until growth progressed sufficiently for conidia and conidiophore observations via phase contrast microscopy. Plates containing morphological analogues of *B. bassiana* were divided into equal quadrants numbered 1-4. A random number generator was utilized to randomly designate a quadrant from each plate, from which the number of suspected *B. bassiana* CFU's was counted and multiplied by four. This number represents the approximate relative abundance of *B. bassiana* conidia occupying leaf disks

sampled from cage-shaded and non-shaded study areas throughout the experiment. The main effects and interaction of time and shading were analyzed by ANOVA (RBD) on log-transformed 0.1x dilution conidia counts. An inverse-prediction was then utilized to estimate the half-life expectancy of conidia based on the sampling data. (JMP®, Version 12.0.1 SAS Institute Inc., Cary, NC, 1989-2007).

Spotted Wing Drosophila Release

Immediately following the initial leaf sampling bout at 0 h, 2,000 flies were introduced into each of six of the nine cages. The treatment cages for the experiment were as follows: 1) Mycotrol spray application and 2,000 flies released (S), 2) no Mycotrol® application and 2,000 flies released (NS), and 3) an exclusion control cage with no Mycotrol® application and no flies released (C). Each vial was opened by hand and allowed to remain undisturbed overnight. Flies still localized on media were forcibly tapped onto a petri dish containing no physiologically attractive volatiles. After one hour, flies occupying the petri dish were euthanized in 70% ethanol, removed from the study area and subtracted from the release total.

Sampling Cages for SWD Adults and Larvae

The Oregon State University (2015) *D. suzukii* degree-day phenology model was utilized to project population growth rates so fruit sampling bouts could be conducted when a high proportion of offspring from introduced flies would have developed to the third larval instar. On 25-Aug, approximately 235

degree-days after SWD release, study plot fruits were sampled in order determine the impact of treatment on maggot infestations. Five total samples (ca. 473mL each) of blueberries occupying high, medium and low stem positions were collected from various randomly selected clones. An additional sample of ground berries was obtained to compensate for displacement of fruits from stems due to internal structural damages that result from feeding of SWD larvae. All samples were held in a laboratory refrigerator and processed within one week of the collection date. Fruits were gently crushed and mixed with 10% saline solution to facilitate dissociation of insect larvae from the fruit pulp. The control treatment cage fruit was assessed to determine whether wild SWD and Blueberry flies (*Rhagoletis mendax* (Curran)) not released might have entered into the sealed cages and confounded the results. Samples were strained into a black tray after thirty minutes and SWD larval counts were conducted. Weighted averages for stem and ground larval abundances were transformed into ordinal ranks with 0 = 0 larvae, 1 = 1-10 larvae, 2 = 11-100 larvae, 3 = over 100 larvae. Ranked data were then analyzed via ordinal logistic regression (JMP®, Version 12.0.1 SAS Institute Inc., Cary, NC, 1989-2007).

To quantify adult abundance within study enclosures, one red Solo® cup baited with sugar water and yeast was deployed on a metallic 76 cm plant support post in each of the nine cages. Traps were allowed to capture flies undisturbed for six days, after which the contents were filtered and examined under a dissecting microscope for both male and female adult SWD. The total

number of captured SWD was divided by the predicted quantity of SWD introduced to release cages in order to assess the capture efficacy of individual traps. An ANOVA (RCB) was then conducted along with a subsequent Tukey post-hoc test (JMP®, Version 12.0.1 SAS Institute Inc., Cary, NC, 1989-2007).

Results

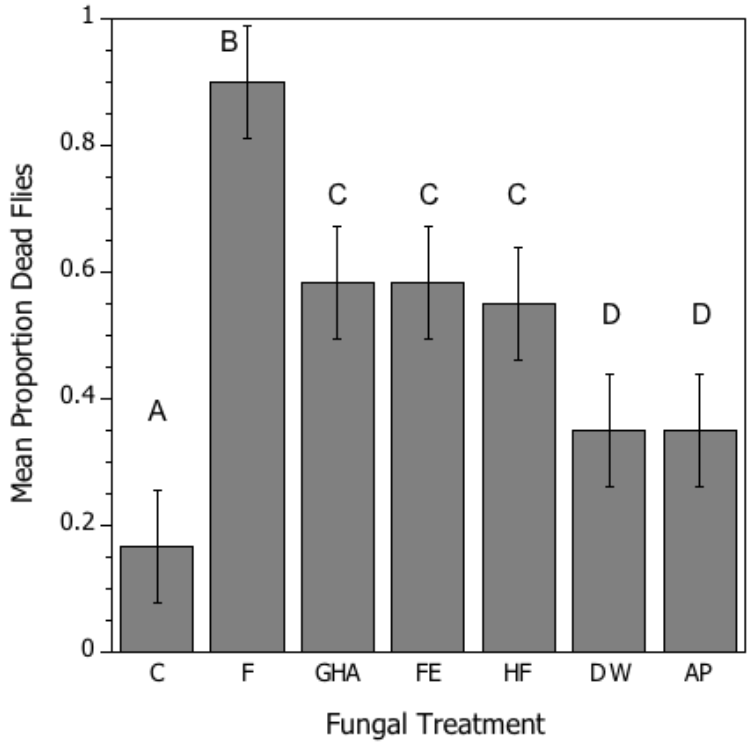
Qualitative High Dose Inoculation of Flies

Single dose inoculation of adult SWD with any fungal pathogen resulted in an overall significant difference in mortality of treated vs control groups of flies at five days post exposure ($X^2_{(6,\ n=20)} = 88.19$, $P < 0.0001$). Fly mortality and pairwise contrast results are shown in figure 2.1. In the case of all fungi, exposures resulted in higher rates of mortality than the control. Mortality as a result of exposure to *M. anisopliae* F-52 was greater than all other fungal species and strains. The group of fungal pathogens resulting in the next highest level of mortality were; *B. bassiana* (GHA), *B. bassiana* (HF-23), and *I. fumosorosea* (FE-9901). The fungi *I. fumosorosea* (Apopka 97) and *M. robertsii* (Clavicipitaceae) (DW-346), although resulting in mortality rates significantly greater than the control, were the poorest performing fungi of all strains tested.

Conidia Concentration Mortality Assays

Fly mortality exceeded 20% in one control replicate of each pathogen assay. Each dataset was analyzed with and without the outliers, with logit model outputs indicating no notable difference in *B. bassiana* mortality, and a nonsensical inverse *M. anisopliae* dose effect. Under exclusion of these outliers,

Figure 2.1 Average proportion of dead SWD adults five days after mass conidia inoculation. Treatments tested include a control (Tween only, C), *M. anisoplae* strain F-52 (F), *B. bassiana* strains GHA (GHA); HF-23 (HF), *I. fumosorosea* strains FE9901 (FE); Apopka 97 (AP), and *M. robertsii* strain DW-346 (DW). Error bars were constructed using 1 standard error of the mean. Each column represents the average mortality measurement of three replications. Pairwise contrast results are shown as letters above each column. Two columns displaying dissimilar letters denotes a significant difference in fly mortality among those treatments.



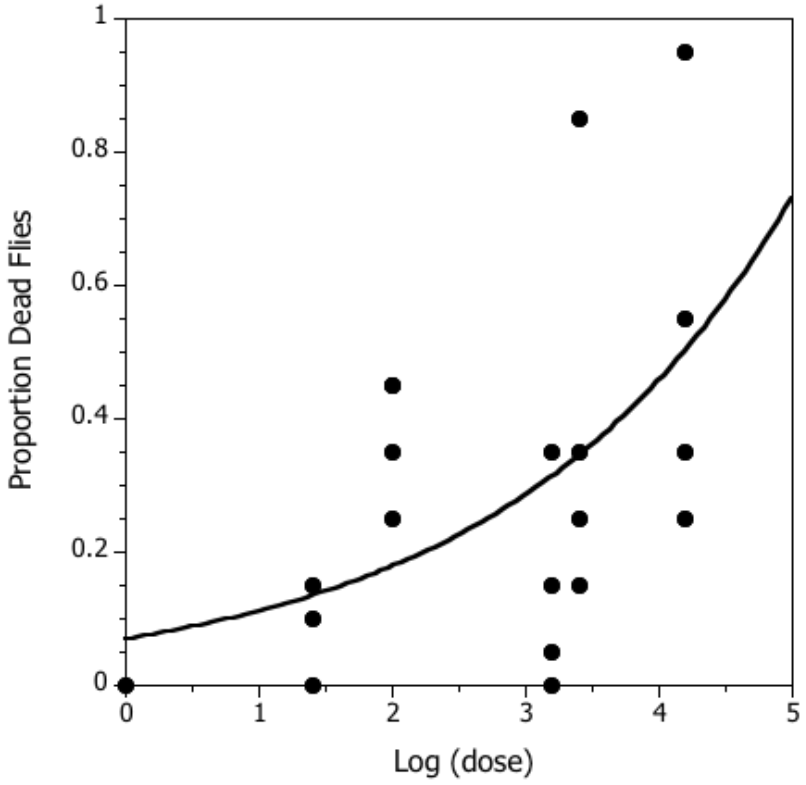
evidence for a positive relationship between *B. bassiana* conidia exposure concentration and adult mortality was provided by logistic regression ($P <$ 0.0001, Table 2.1). Based on the mortality data obtained (Fig. 2.2), the pathogen's predicted log-transformed LD 10, 25, 50, 75, 90 and 99 values were as follows: 1.1, 2.7, 4.2, 5.8, 7.4, and 10.8 conidia mm^{-2} respectively. It should

Table 2.1 Fly mortality and sporulation logit results after exposures to varying concentrations of *B. bassiana* (strain GHA) or *M. anisopliae* (strain F-52) on conidia-treated surfaces. Critical dose values for mortality and sporulation are provided for the threshold at which 50% of the population or cadavers would have died (LD_{50}) three days after conidia exposure, or where 50% of fly cadavers will have sporulated (SD_{50}) three weeks after death.

Fungal Isolate & Response Variable	n	Slope ± SE	LD_{50} or SD_{50}	95% CI	X^2
GHA Mortality	480	0.70 ± 0.10	4.2	0.52 – 0.91	50.06
F-52 Mortality[a]	480	0.05 ± 0.12	N/A	N/A	0.15
GHA Sporulation	122	2.39 ± 0.45	3.4	1.62 - 3.39	28.71
F-52 Sporulation	61	1.92 ± 0.49	2.8	2.32 - 3.35	15.46

[a] logistic regression not significant

Figure 2.2 Proportional mortality of SWD flies three days after indirect topical exposure to varying *B. bassiana* strain GHA conidia surface doses (Conidia mm^{-2}). The Log (dose) impact on mortality is represented as an exponential relationship. The highest Log (dose) tested in the bioassay was 4.2, which corresponds with the predicted LD_{50} based on these data.



be noted that the greatest log-dose tested in this assay was 4.2 and constituted a surface conidia concentration of approximately 16,000 conidia mm^{-2}. A mortality increase was not observed in response to increasing levels of *M. anisopliae* conidia (P = 0.693, Table 2.1) with a significant difference in fly mortality noted between the two pathogens during this assay ($X^2_{(1, N = 160)}$ = 5.03, P = 0.025). Both fungi were able to utilize *D.suzukii* for growth, development

and asexual reproduction, as mycelia were observed on dead flies three weeks after surface sterilization (Fig. 2.3). Furthermore, in the case of both entomopathogens, cadaver sporulation frequencies increased in response to higher conidia dosages ($P < 0.0001$, Table 2.1, Fig. 2.4). No sporulation on cadavers of control replicates were observed during either assay.

Scanning Electron Microscopy

Scanning electron micrographs of inoculated flies show germination of both *B. bassiana* and *M. anisopliae* conidia on fly integuments (Fig. 2.5). Hyphae of both fungal strains are seen directly penetrating the cuticle and do not appear constrained to indirect invasion mechanisms through natural openings of the exoskeleton.

Oocyte Maturation During *B. bassiana* Mycosis

The histograms in figure 2.6 depict an exponential distribution of egg count data. We estimate that a surface dosage of 2,900 conidia mm^{-2} was administered to the treated flies. Integuments of euthanized specimens exposed to fungal treatments for qualitative SEM examination supported the assumption of positive infection by *B. bassiana* in individuals exposed to this conidia concentration. These data show that *B. bassiana* exposure and likely mycosis suppressed oocyte maturation rates in immature females ($X^2_{(1, n=13)} = 5.34$, $P = 0.02$, Fig. 2.7). At the sampling times of five and six days post exposure, flies displayed similar oocyte maturation rates. However, by day seven, egg accumulation appears to have been impacted by *B. bassiana* exposure with a

Figure 2.3 Entomopathogenic fungi sporulating on *D. suzukii* cadavers. Pictures were taken roughly three weeks after indirect topical exposure to *B. bassiana* (left) or *M. anisopliae* (right) conidia. (Bar = 0.75 mm for both images)

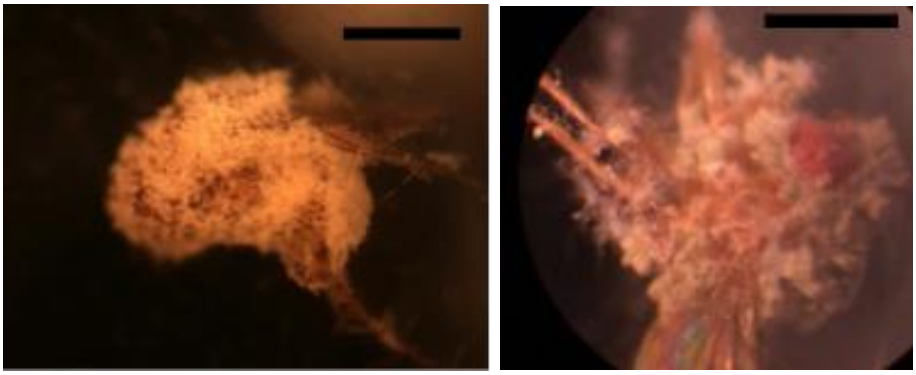
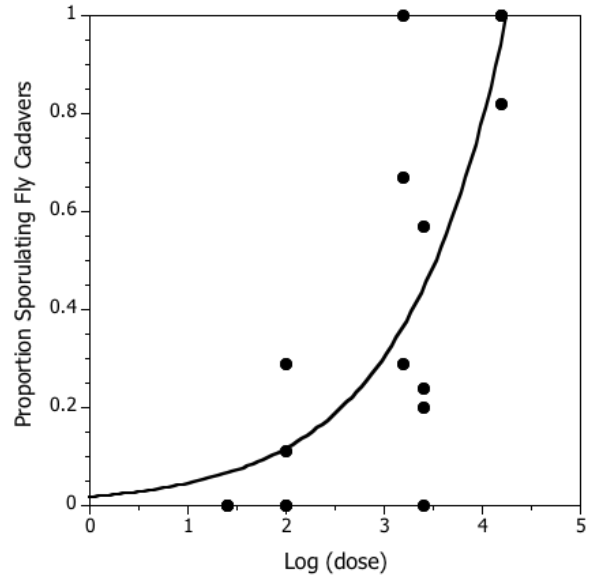


Figure 2.4 Proportion sporulating *D. suzukii* fly cadavers at 0-16,000 or 0-4,000 conidia mm^{-2} of *B. bassiana* (top) and *M. anisopliae* (bottom), respectively. The data presented here were gathered three weeks after mortality of inoculated flies. One replicate of each bioassay was excluded due to greater than 20% control mortality.



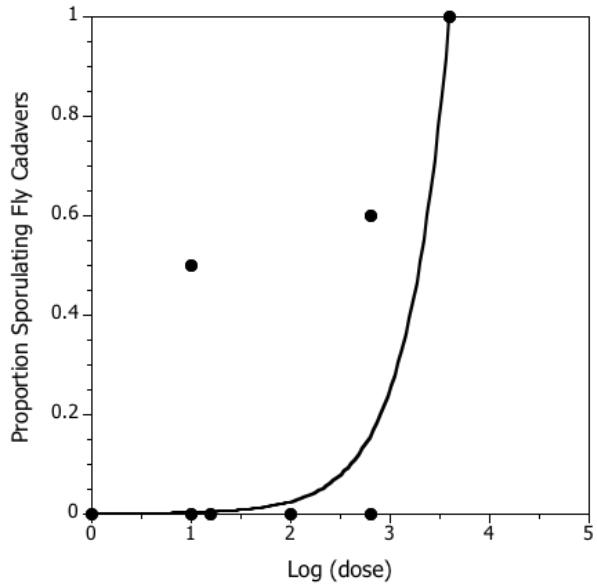


Figure 2.5 SEM micrographs of germinating conidia on *D. suzukii fly* integuments. (A and B) Germinating *B. bassiana* conidia (arrows) on the host femur and pronotum, respectively. Marked conidia of image B denote the formation of germ tubes. (C and D) Germinating *M. anisopliae* conidia on the host tibia and femur, respectively. Indirect hyphal penetration through a seta follicle (black arrow) can be seen in the upper portion of image C, with white arrows signifying appressorial (ap) formation and direct penetration observed in the remaining *M. anisopliae* conidia of micrographs C and D. Image E shows the host's terminally located tarsal segments after surface inoculation with the maximum *M. anisopliae* conidia dose tested. (Bar = 10 µm for A; bar = 20 µm for B and E; bar = 5 µm for the rest.)

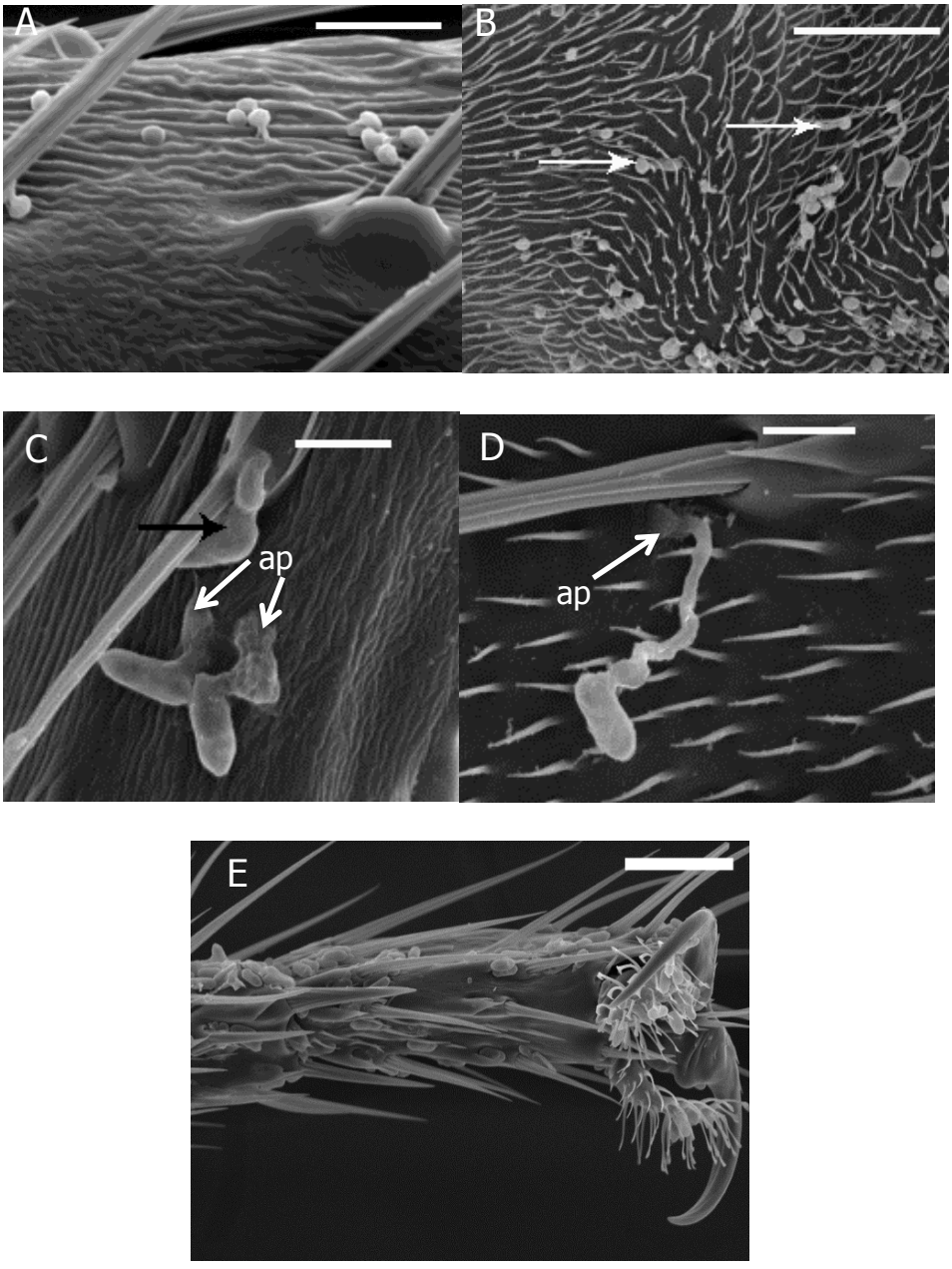
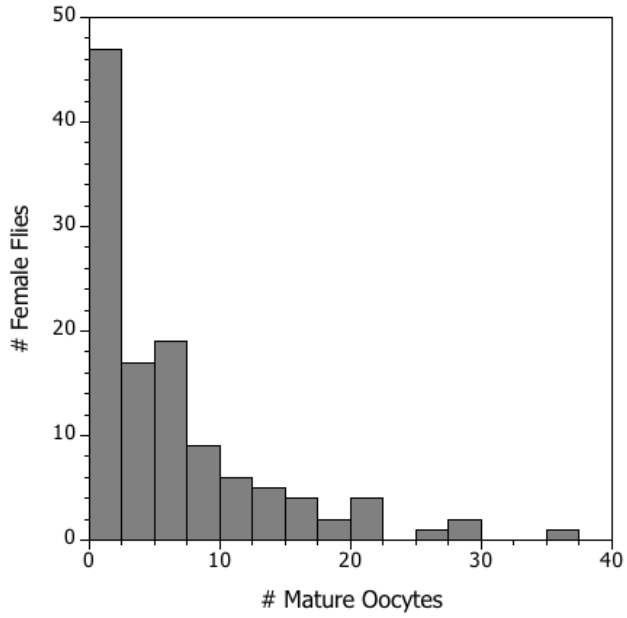


Figure 2.6 Histograms of egg maturation data in female SWD after sub-lethal *B. bassiana* exposure. Data were analyzed under the assumption of exponentially distributed data of both non-treated (top) and treated (bottom) flies. The total number of mature oocytes counted in each individual is represented here.



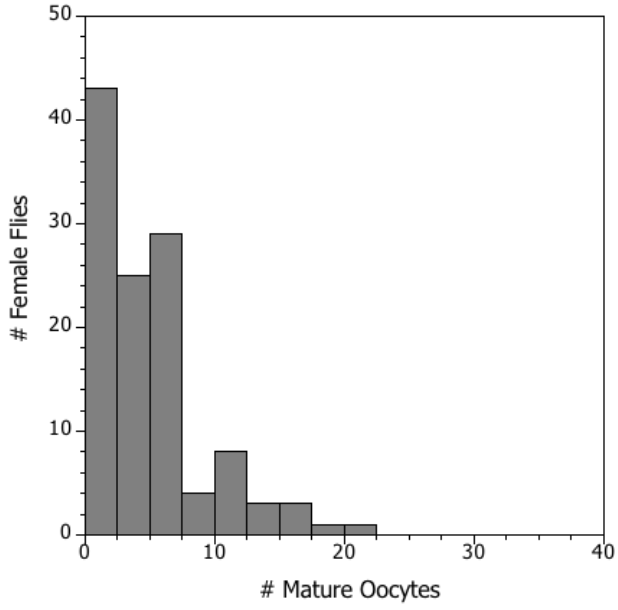
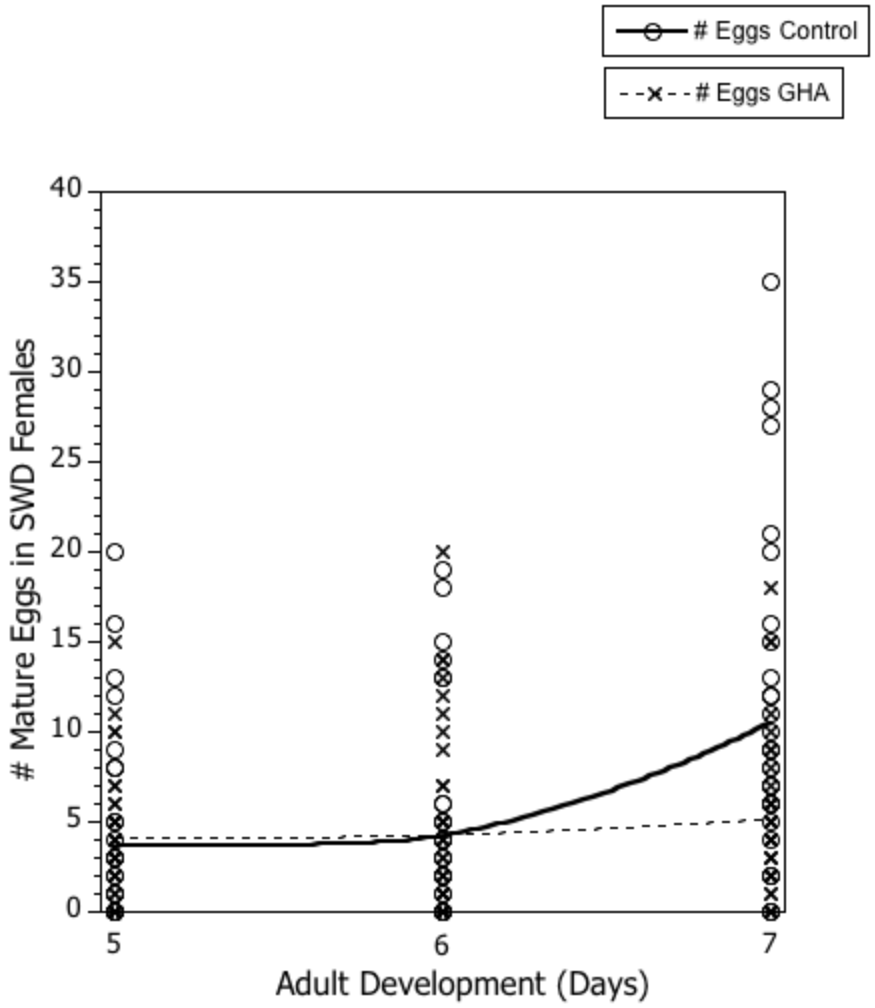


Figure 2.7 Oocyte maturation in teneral *D. suzukii* females exposed to control (O) or *B. bassiana* (X) inoculated surfaces. Treatment exposure was initiated no later than 21 h after pupal eclosion for any given individual. Dissections were conducted on sets of females after 5, 6 and 7 days of adulthood development.



significant difference in oocyte number observed between control vs treated flies.

Myco-insecticide Efficacy Against SWD in Lowbush Blueberry

The decay of *B. bassiana* conidia sampled on lowbush blueberry foliage was impacted by the model's parameters, cage-shading and post-application time ($F = 9.02$; df = 7, 16; $P = 0.0002$). However, reductions in conidia viability were most strongly correlated with time ($P < 0.0001$), and do not appear to have

been influenced by shading of cages ($P = 0.54$). The total counts at each collection interval were plotted over time and fitted with a logarithmic decay function (Figure 2.8), and the inverse prediction approximated a *B. bassiana* conidia half-life of about 3.4 h after initial application. Based on the measured quantities of conidia on the leaves sampled at 0 h (ca. 10 min) post-spray, the actual application rate tested in this study is estimated to be roughly 3,500 conidia per mm^2.

The ordinal logistic model suggests that a significant treatment effect exists with SWD larvae ($X^2_{(2, N = 9)} = 14.23$, $P = 0.0008$). Larval infestation in fruits were lower in the no release cages than cages where flies were released but there was no difference in larval infestation between the Mycotrol® treated release cages and the release cages that did not receive a spray application. Similarly, analysis of the adult abundance suggests a significant treatment effect ($F = 5.46$; df = 2, 6; $P = 0.045$), which again, is due to the lower quantity of adults observed in the no release cages compared with the release cages, with no significant difference in SWD adults between cages receiving the *B. bassiana* application in comparison to the untreated release cages. The mean SWD capture rate of individual traps was found to be 15.7 % ± 7.6 % (SD) of the release total. Lastly, including an estimate of successful release density as a covariate did not explain any additional variation in measurements of adult abundance among sprayed and unsprayed release cages ($F = 0.15$; df = 2, 3; $P = 0.86$).

Figure 2.8 Decay in *B. bassiana* conidia viability from Mycotrol® protected foliage over time, fitted by logarithmic regression. Sample collections were obtained 0 (ca. 10 min), 24, 48 and 96 h after the myco-insecticide application. Each data point at a given time interval represents the mean *B. bassiana* cfu abundance of three cultures prepared with leaf suspensions sampled from a single study plot. All averages are plotted together since a cage shading effect on conidia longevity was not found.

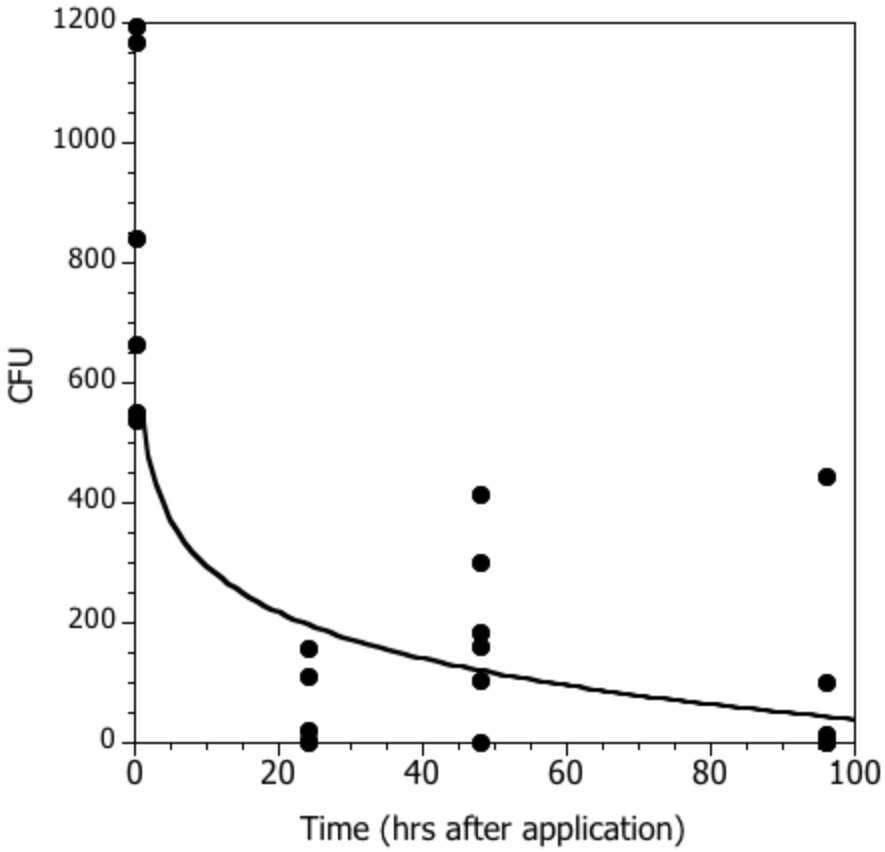
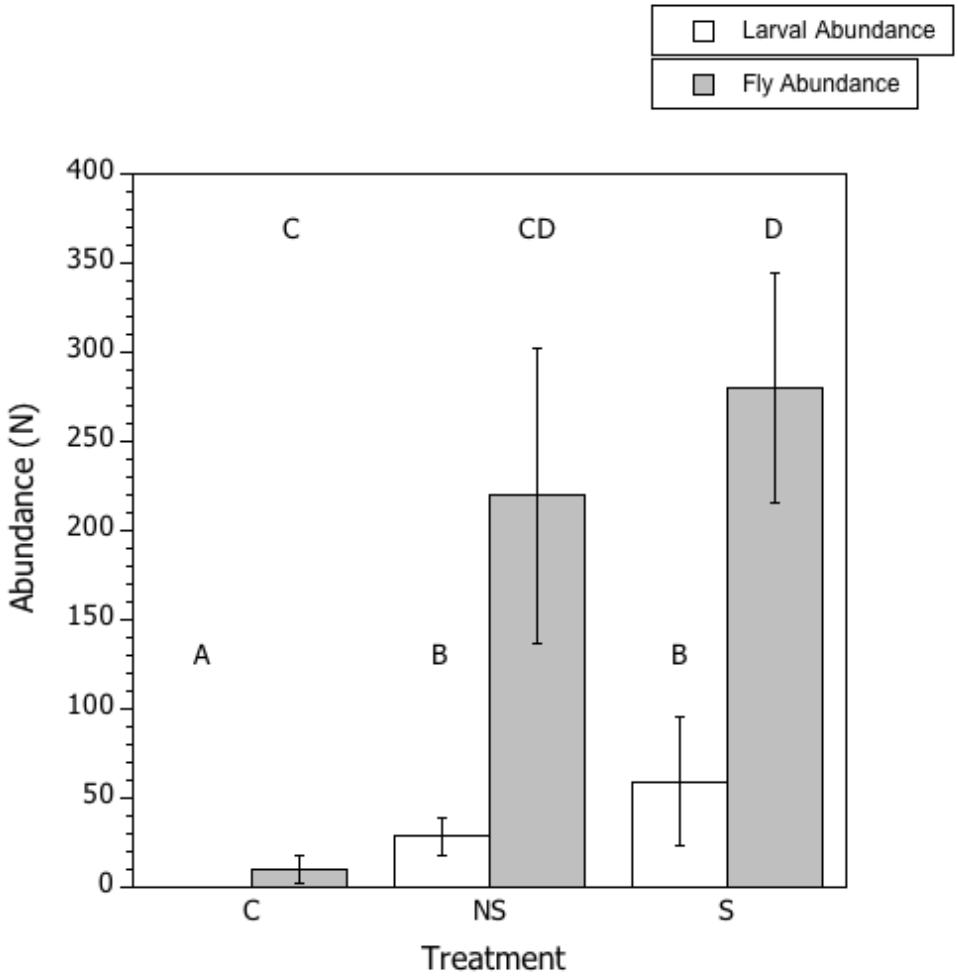


Figure 2.9. Average abundance (N) of individual SWD larvae and flies sampled in each of the three control cages (C), unsprayed release cages (NS), and *B. bassiana* protected release cages (S). Error bars were constructed using one standard error of the mean.



Discussion

Laboratory Assessments

Collectively, the results of this investigation suggest that, while the entomopathogens tested are virulent toward *D. suzukii* flies following laboratory inoculation, their utilization as biocontrol agents in SWD management does not appear promising, at least after a single spray application. Laboratory induced mycoses resulted in both lethal and sub-lethal effects in SWD flies. After exposure to pathogen surface concentrations ranging from 0 - 16,000 *B. bassiana* conidia/mm^2, a positive dose-mortality response was observed. While no dose-response assessment was conducted for comparison, Cuthbertson et al. (2014b) also reported significant increases in adult *D. suzukii* mortality after direct topical contact with *B. bassiana* conidia.

It is important to note that all six fungal strains elevated *D. suzukii* mortality rates after a mass lethal conidia exposure. Interestingly, *Metarhizium anisopliae* strain F-52 induced the greatest mortality of the six strains tested, but failed to produce a significant fly mortality effect during the follow up dose-response assay. Despite this inconsistency, conidia of both pathogen assays were observed germinating on flies euthanized no more than 24 h after physical contact with the insect exoskeleton. Investigations on the contrasting invasion mechanics of direct and indirect host integument invasion by entomopathogenic fungi have been conducted previously on other insect taxa, including pest species of the orders Coleoptera and Lepidoptera (Pekrul and Grula 1979, Talaei-

hassanloui et al. 2007, Guerri-Agullo et al. 2009). The formation of appressoria at the distal end of a conidia germ tube is generally regarded as evidence for direct enzymatic degradation and hyphal invasion of the host cuticle, due in part to the high mitochondrial composition and metabolic activity of cells forming the structure. On *D. suzukii* flies, possible appressoria formation and integument penetration was observed (Fig. 2.5). Furthermore, the frequency of sporulation appeared to have increased with conidia dose in the case of both entomopathogenic fungi, with the formation of mycelium noted in fly cadavers (Fig. 2.3). Collectively, these results provide complimentary evidence for positive laboratory infection of both entomopathogenic fungi toward adult *D. suzukii*, even in the absence of a significant dose-mortality response to *M. anisopliae* induced mycosis. Given our understanding of the differing mechanisms by which activity of *B. bassiana* and *M. anisopliae* mycotoxins affect host longevity (Sowjanya Sree et al. 2008, Qadri et al. 2011), it is possible that over a longer time period than examined here, infection by *M. anisopliae* at similar concentrations would have substantially elevated the proportional mortality of SWD flies.

Given the apparent virulence of these biocontrol agents toward *D. suzukii* adults, their perceived potential for implementation in SWD management necessitated further examination of the physiological impacts of sub-lethal infections that trigger diversion and prioritization of energy and resources from development and reproduction into the host immune response. Insect fecundity

reductions have been documented in red palm weevils (*Rhynchophorus ferrugineus*) treated with sub lethal doses of *M. anisopliae* inoculum, manifesting in reduced egg hatch and oviposition rates over time (Ginden et al. 2006). Exposure of adult yellow fever mosquitoes (*Aedes aegypti*) to *B. bassiana* conidia significantly altered the reproductive output of individuals. On average, control flies laid a greater quantity of eggs throughout their lifetime in comparison to pathogen inoculated *A. aegypti* (Darbro et al. 2012). Interestingly, the opposite trend was observed in adult *A. aegypti* through the first 48 h post-exposure. During this time period, the mean oviposition rate of *B. bassiana* treated females exceeded that of individuals exposed to the control treatment. In response to indirect topical *B. bassiana* inoculation of SWD females at a conidia surface density (2,900 conidia mm^{-2}) corresponding approximately with the second highest pathogen dose tested in the preceding concentration bioassay (2,400 conidia mm^{-2}), virgin female oocyte development rates were similar in control vs diseased individuals until seven days of adult maturation. At day seven, the ovaries of pathogen inoculated females contained significantly less mature oocytes than those of control flies. However, given time constraints of this study, it cannot be concluded whether oocyte development becomes delayed or remains stagnant during the sub-lethal phase of fungal infection in *D. suzukii* females.

The capacity for various entomopathogenic fungi to significantly suppress insect population growth rates has been described previously in a number of

insect hosts, including SWD (Ginden et al. 2006, Cuthbertson et al. 2014b, Moorthi et al. 2015). In addition to reducing host fecundity, immature host growth and development rate declines have been described in insect pests during laboratory inoculations with entomopathogenic fungi. Direct topical exposure of Oriental leafworm moth larvae (*Spodoptera litura* (Fabricius)) to sub-lethal concentrations of *I. fumosorosea* or *B. bassiana* mycotoxins persistently suppressed development and consumption rates of individuals throughout subsequent life stages (Moorthi et al. 2015). While fungal pathogenicity in juvenile *D. suzukii* host systems were not examined in this study, similar pathogen effects could restrict the recruitment of reproductively vigorous adults to invading populations during early stage infestations.

Field Assessment

Beauveria bassiana is applied as an insecticide alternative for Dipteran pest management in animal agriculture. In a comparative study conducted by Kaufman et al. (2005), the degree of control achieved through applications of the product Balance® in poultry facilities exceeded that of traditionally employed pyrethrins for control of house fly populations (*Musca domestica* Linnaeus). Preliminary testing of the fungal isolate utilized by Balance®, *B. bassiana* strain HF-23, yielded a significant mortality response in adult SWD. In relation to mortality data and available literature of the other entomopathogenic fungi tested, *M. anisopliae* and *B. bassiana* strain GHA were selected for further biocontrol evaluation in this assessment. However, despite strong evidence for

laboratory infection of flies by *B. bassiana*, no results were obtained that justify the implementation of these fungal pathogens in *D. suzukii* pest management. Direct spraying of caged lowbush blueberry crops with the product Mycotrol® failed to reduce the relative abundance of larvae and flies sampled in study enclosures.

With the implemented application methodology of spraying the foliage, SWD adults needed to contact and pick up conidia on their legs, mouthparts or abdomen. In complimentary laboratory assessments, *D. suzukii* adults displayed acute and chronic vulnerability to mycoses in response to a surface inoculation approach thought to represent a practical pathogen-insect contact mechanism for wild adult *D. suzukii*. The SEM micrographs of *M. anisopliae* inoculated flies illustrate that a considerable quantity of sensilla and setae are found on the species' tarsal segments. These structures protrude from the exoskeleton and substantially influence the surface area found on this body region, seemingly resulting in a considerable concentration of conidia on this body region. Once picked up, conidia may then be physically transferred to other body regions through grooming or copulatory behaviors. Based on the lowbush blueberry foliage sampled immediately after spraying, the predicted coverage tested in the field study (3,500 conidia mm^{-2}) corresponded fairly closely in magnitude with the second highest dose tested during the *B. bassiana* laboratory assay (2,500 conidia mm^{-2}). This conidia concentration significantly increased mortality rates of flies under laboratory conditions utilizing an analogous, indirect surface

exposure protocol to that implemented in the field. Given that *Beauveria bassiana* conidia obtained from leaf samples were successfully cultured on a selective growth medium, and that there was no significant impact of spraying on the abundance of adult SWD captured in release cages, it is plausible to suspect that uncontrolled factors may have impeded the germination of virulent spores after their attachment to the insect cuticle.

Insufficient relative humidity (RH) has been shown to limit infection and control efficacy of *B. bassiana* toward arthropod hosts (Shipp et al. 2003). While no critical RH thresholds were derived from the experiment, diet inoculation with 1×10^8 *B. bassiana* strain HQ917687 conidia mL^{-1} supplied to *M. domestica* flies and larvae at varying laboratory RH levels generally resulted in greater mortality rates in more humid laboratory climates with temperatures ranging from 20 – 35°C (Mishra et al. 2013). Temperatures outside of this range were shown to negate the virulence of fungal conidia in both *M. domestica* life stages, regardless of climatic RH composition.

Throughout the duration of the August 2015 field study in Jonesboro, ME, precipitation events were extremely sparse; according to logged weather data taken at the field site, from initiation of the experiment until the collection of fruit samples a total rainfall of 8.6 mm is estimated to have fallen under daily temperature extremes ranging from 12.7 – 29.6°C. Moreover, from myco-insecticide applications up through the 96 h leaf sampling, a mere 0.25 mm of precipitation was detected at the field site. Given approximated conidia viability

half-life of about 3.4 h, the precipitation experienced over this time interval may have been inadequate for sustaining relative humidity conditions needed for successful germination. This would effectively prevent substantial rates of SWD infection and enabled flies to inflict the severe fruit infestations observed. Unfortunately, no SEM micrographs were obtained from fly integuments of this experiment to provide evidence in support of this hypothesis. For further consideration as biocontrol agents of SWD, future mycoinsecticidal efficacy experiments against *D. suzukii* should contemplate laboratory or greenhouse assessments in order to obtain information on the inoculation efficacy of entomopathogenic fungal conidia on fruits and leaves. It will also be worth exploring the necessity for multiple vs single myco-insecticide applications in an attempt to increase the probability of pathogen-host contact during a period of sufficient RH.

Future Assessments

One necessary consideration of insect pest biocontrol is the stage-specific physiological and phenological vulnerability of a pest species to a given management technique. In the context of lowbush blueberry infestations with *D. suzukii*, unpublished data by Ballman and Drummond (2014) show that the localization of pupating individuals may be highly dependent on the type of fruit host with a greater frequency of pupae metamorphosed in soil substrates as opposed to within lowbush blueberry fruits. These findings are contrary to the purported pupation sites proposed by Walsh et al. (2011), but appear to be more

conducive with the pupation strategies described by Asplen et al. (2015). Given the management goal of sustaining low densities of reproductively active adult SWD (Walsh et al. 2011), it is possible that the targeting of viable soil-dwelling pupae could aid in achieving this goal.

Pupal Susceptibility

Formulations containing entomopathogenic fungi may be incorporated into the irrigation systems for the localization of virulent conidia in the soil. Given the apparent pupation strategy of *D. suzukii* in the lowbush blueberry agroecosystem, this method of delivery could substantially impede the recruitment of reproductively active adults into an invading population. Although not tested here, pathogenicity toward juvenile SWD could retard the propagation of gametes and maturation of reproductive organs. We observed this in ovarian development rates of *B. bassiana* infected female SWD throughout first week adult maturation in virgin females. Coupled with the post-eclosion time requirements for completing reproductive development in both sexes of many *Drosophila spp.* (Markow and Grady 2008), expanding mycoinsecticidal exposures to include juvenile *D. suzukii* might enhance the degree of control obtained beyond that achieved through exclusive targeting of fly populations. Beris et al. (2012) have shown that laboratory induction of pupal mycoses in Mediterranean fruit fly (*Ceratitis capitate* (Wiedemann, 1824)) incurred a slight mortality response, and consequent reductions in adult longevity. Therefore, the capacity for entomopathogenic fungi to disrupt juvenile as well as adult

maturation, and curtail the average longevity of infected individuals, should be acknowledged during future evaluations on the potential application of myco-insecticides in crops susceptible to *D. suzukii* invasion.

Potential Concomitant Release

The association of Hymenopteran parasitoids with SWD pupal hosts has been described in some regions of Europe, as has predation by soil dwelling insects (Gabarra et al. 2014). Further laboratory evaluations have facilitated the consideration of these natural enemies in biological control for *D. suzukii*. Concomitant treatments are often recommended in pest management practices for sustained efficacy (Chaudhary 2008), and the cumulative effects of entomopathogenic fungal applications into agroecosystems harboring predators and parasitoids could further restrict pest population growth rates during early stage infestations. Despite evidence supporting the compatibility of predator and parasitoid release in conjunction with entomopathogenic fungi to combat pestiferous insect populations in the field (Labbé et al. 2009), specific targeting *D. suzukii* pupae will inevitably bring the pathogen in close proximity with beneficial insects, naturally occurring or otherwise. Evaluations might therefore consider looking at the potential conflicts, if any, of this myco-insecticide treatment protocol on the abundance of antagonistic parasitoids and predators as pertains to biocontrol efforts for novel pests such as SWD.

One alternative to irrigation or spray delivery involves the auto-dissemination of conidia in reservoirs deployed within no-kill traps emitting

attractive, volatile semiochemicals. Mycoses are then able to propagate throughout an invading insect population via horizontal transmission mechanisms during copulatory and/or aggregation behaviors. El-Sufty et al. (2011) designed a dry, mass inoculation device for red palm weevil adults. The investigation included an aggregation pheromone and found that trap visitors frequently experienced mortality increases in the laboratory. Deploying devices in date plantations not only resulted in a significant mortality response over a two year sampling period, but also appears to have manifested in successful and extensive dispersal of the fungus via horizontal contact with control populations. The demonstrated capacity for fungal pathogens to reproduce within a *D. suzukii* host, coupled with sustained efforts in the identification of species-specific symbioses of SWD with microbial yeasts (Hamby et al. 2012), could aid in the development of an analogous inoculation trap for utilization in *D. suzukii* management as a medium for selective infection.

CHAPTER II

Behavioral and Preventative Management of *Drosophila suzukii* Matsumura (Diptera: Drosophilidae) in Maine Lowbush Blueberry (*Vaccinium angustifolium* Aiton) Through Mass Trap Deployment and Insect Exclusion-Netting

Abstract

The management of spotted wing drosophila (*Drosophila suzukii* Matsumura) in invaded agroecosystems continues to receive significant attention. While complementary monitoring and insecticidal application procedures can provide effective protection against this insect pest in some cropping systems, more sustainable alternatives are currently under development. In this investigation, we explored the efficacy of mass-trapping with volatile semiochemicals and the use of insect exclusion netting as independent management protocols for *D. suzukii* in Maine lowbush blueberry (*Vaccinium angustifolium* Aiton). We found the utilization of exclusion netting to be an effective preventative tactic against SWD invasion, with consistent and significant reductions in larval infestations observed during field trials conducted in 2014 and 2015. Total average larval composition of fruit samples collected from net-protected and unprotected lowbush blueberry plants were 0.2 ± 0.2 (SD) and 5.2 ± 3.9 larvae per fruit sample of 250 berries, respectively. Mass-trapping was not effective in mitigating the severity of SWD infestations; an average of 11.0 ±

17.3 (SD) larvae were counted in fruit samples of control plots (1.8 m trap spacing), in comparison to a nonsignificant trend in a lower density of 8.7 ± 6.4 larvae found inhabiting fruits sampled in medium density trapping grids (1.8 m trap spacing). Among trapping grids, varying the concentration of traps significantly influenced larval infestations of fruit samples ($F = 7.45$; df = 4, 31; $P = 0.0003$). During the experiment, placing 16 evenly distributed traps throughout a 9x9 m^2 crop area resulted in total average abundance of 1.5 ± 1.8 (SD) SWD larvae, as opposed to averages of 8.8 ± 11.1 (SD) and 17.3 ± 13.7 (SD) larvae with 49 and 121 traps per 9x9 m^2 study area, respectively. Differences in larval abundance between the latter trapping grid treatments was not significant. Interestingly, no density-dependent response between trap spacing and average SWD adult captured per trap was observed in the data, with 13.3 ± 10.4 (SD), 17.1 ± 21.1, 21.3 ± 15.3 flies estimated in low, medium and high density trapping grids, respectively. Apparently, the capture rates of individual traps did not correlate with deployment density, suggesting that more traps per unit area only work to concentrate adults in that area. This technique could therefore be considered as part of a trap cropping strategy for management of *D. suzukii*.

Introduction

Drosophila suzukii Matsumura, commonly referred to as the spotted wing drosophila (SWD), is a recently established invasive insect pest of North American and European small fruit cultivation. The occurrence of SWD fruit

infestations severe enough for rejection by potential consumers was not reported consistently prior to its invasion of these non-native regions. The overall degree of destruction incurred within recently colonized agroecosystems vastly exceeds the negligible infestations described in the species' native range (Kanzawa 1934, Goodhue et al. 2011). The damage potential of this exotic pest in crop systems of foreign regions drastically exceeds the criterion for quality fruit marketability. Revenue losses attributed to SWD contamination in North America have been estimated in various Western Pacific U.S. regions, and amounted to roughly $US 42.9 million in three California counties alone (Bolda et al. 2011).

SWD and Maine Lowbush Blueberry

Prior to the 2011 establishment of *D. suzukii* in Maine, over 24,300 ha of lowbush blueberry (*Vaccinium angustifolium* Aiton) farmland generated roughly $250 million of farm gate revenue in 2007 (Yarborough 2013). Currently, no estimations of monetary loss in lowbush blueberry have been developed to include the direct *D. suzukii* damage of internal fruit tissues, secondary microbial contamination, or the compounding costs of implementing monitoring and treatment protocols. A widely adopted response to male detection during adult monitoring surveys entails the targeting of adults through application of pyrethroid, organophosphate or spinosyn class insecticides to suppress population growth before significant crop damage is inflicted (Bruck 2011, Collins and Drummond 2016a, b). Unfortunately, many of these chemicals threaten the health of wild and domesticated pollinator species, insect natural enemies, and

persist in surrounding landscapes and watersheds after runoff or drift dispersal (Hester et al. 2001, Beketov et al. 2013). The development of environmentally and economically sustainable *D. suzukii* integrated pest management programs is currently an ongoing process in North American and European small fruit agriculture. Ideally, findings will be translatable across a wide spectrum of agroecosystems given the species' generalist phytophagy and high generational turnover that aid in its rapid spread and alarming crop injury potentials (Asplen et al. 2015).

Prospective Behavioral & Cultural SWD Management

The use of capture-and-kill traps emitting volatile semiochemicals constitutes an effective chemical pre-treatment monitoring tool of early stage *D. suzukii* invasions throughout the fruit ripening and harvest periods. Currently, exploiting the positive chemotactic response of SWD adults to ethanol molecules emitted by yeast-containing baits is a widely implemented bait attraction protocol (Walsh 2011, Yarborough et al. 2013, Burrack et al. 2015). The utilization of physiologically attractive compounds in mass capture and kill of *D. suzukii* was first proposed by Kanzawa (1934). Since that time, results produced by two recently conducted mass-trapping efficacy evaluations have yielded conflicting results (Wu et al. 2007, Hampton et al. 2014). Thus, the implementation of this approach in the absence of complimentary control measures is widely regarded as an impractical and ineffective option for reducing the abundance of SWD larvae in fruit.

By exploiting the phagostimulatory reflex of insects to desirable food (Cevik and Erden 2012), the infusion of boric acid with aqueous sucrose solutions has provided an effective oral exposure mechanism for toxin delivery in both urban and vector associated pests; insecticidal treatments in environments in and around homes have received scrutiny due to the high degree of direct spatial overlap between humans and target insects. Research has shown that boric acid ingestion by the German cockroach (*Blatella germanica* (Linnaeus)) and the Asian tiger mosquito, *Aedes albopictus* Skuse, can suppress population growth through a combination of chronic and acute toxicity. Naranjo et al. (2013) also described a reduction in the post-treatment oviposition rate of *A. albopictus*. According to Markow and Grady (2008), reproductive maturation of *D. melanogaster* females progresses more rapidly when nutritional resources are available for immature fly consumption. Given recent laboratory observations on SWD ovarian development (Al-Najjar 2016) which provides evidence for a 5-6 day reproductive immaturity in teneral female flies, the ingestion of boric acid by foraging SWD females during this critical developmental stage might physiologically disrupt or delay the progression of reproductive maturity. Hampton et al. (2014) found that a majority of flies in close proximity to trapping grids exclusively contact the external surface of traps and remain free to propagate. Considering this, the incorporation of a boric acid and sucrose solution on the exterior surface of baited traps could expose a proportion of these uncaptured *D. suzukii* to the toxin and ultimately enhance the control

efficacy of mass-trapping. In the absence of a gustatory rejection by *D. suzukii* upon the ingestion of toxins in a food source, this strategy might provide a mechanism for increasing the proportion of flies treated upon contact with a trap surface.

Another preventative approach involves the deployment of fine mesh netting at ripening and pre-harvest crop intervals as a physical hindrance to ovipositing *D. suzukii* contacting potential host fruits. This technique has been effectively implemented in IPM programs for a variety of insect pests (Lloyd et al. 2005, Dib et al. 2010, Sauphanor et al. 2012), and has received attention in the ongoing efforts to provide organic growers with chemical management alternatives for SWD in a variety of agricultural systems (Schattman 2015, Cormier et al. 2015). The works of Cormier et al. (2015) also addressed the significance of net-shading on plant photosynthetic activity by quantifying the chlorophyll and sugar composition of blueberry fruits upon concluding the study. They found no apparent reduction in fruit quality from berries sampled in protected vs unprotected crops. However, given the spatial limitations of the field and greenhouse evaluations conducted thus far, it is unknown whether exclusion netting can meet the criterion for economical practicality of SWD management in large-scale crop systems.

Research Objectives

Neither of the proposed management approaches has yet been demonstrated to provide a level of efficacy against *D. suzukii* infestations needed

in large-scale crop systems. We conducted two independent field studies with the intention of further exploring the SWD control efficacy of these techniques in Maine lowbush blueberry. The first study involved quantifying the severity of fruit infestations in response to boric acid applications on ethanol emitting traps deployed at varying densities within trapping grids. The second investigation examined the prevention efficacy of insect netting in lowbush blueberry fruits against SWD larval infestation.

Methods

Trap Composition

Each trap consisted of one red Solo® cup with roughly seven, standard 3.2 mm-diameter punched holes inserted about 2.5 cm below the cup's upper rim. Within experimental grids, individual traps were positioned on a green, metallic 76 cm plant support post and baited with approximately 5 cm of a mixture containing 15 mL dry yeast colonies, 60 mL white granulated sugar, and 0.35 L H_2O. Control traps were filled with about 5 cm of water only. Each red Solo® cup was provided a red foam photoabsorption cap to seal the trap and prevent the interference of light with fermentation reactions. The external surface of each mass-trapping experimental trap was then uniformly sprayed with a mixture of 1% borate L^{-1} 25% (*w/v*) sucrose/water solution. Traps intended for monitoring purposes during these assessments did not have boric acid solution administered to external surfaces.

Salt Extraction of Larvae from Blueberry Samples

Each fruit sample occupied a volume of about 160mL, and was gathered from random clones within the given study plot. All fruit samples were processed within one week of being gathered. To accomplish this, each sample was gently crushed in a plastic bag and suspended in 10% saline solution in order to induce disassociation of SWD larvae from fruit pulp. The remaining contents were then filtered through a fine mesh sieve and suspended in a black bottom metallic tray filled with water. A lamp was utilized to illuminate the contents of samples so the abundance of *D. suzukii* larvae inhabiting fruit samples could be determined (Drummond et al. 2016).

Trap Sampling for Adults

Sampled traps were gathered at different intervals depending on the experiment, and analyzed within one week of being collected. Contents of each were rinsed with H_2O and filtered through a sieve in the laboratory. The remaining trap contents were emptied into a white metal tray filled halfway with about 5 cm water. Male and female *D. suzukii* counts were then obtained from each trap sample.

2013 Preliminary Mass Trapping Field Study

On 25 July 2013, a single 7.6 m x 4.9 m study area was set up at the University of Maine Blueberry Hill Research Farm in Jonesboro, ME, for a preliminary investigation of the potential of mass trapping for suppression of SWD in pre-harvest fruits. Twelve traps were deployed in a 3 x 4 grid with approximately 1.8 m of spacing between traps. Traps were collected every seven

days, during which freshly baited traps were exchanged with sampled ones. Those containing flies were taken back to the laboratory where the abundance of male and female SWD was determined.

On the final collection date, 14 September, two samples of blueberries were gathered from random areas inside and outside the grid to determine female SWD oviposition activity. Externally sampled plant clones were located a minimum of 9.1 m from the peripheral border of the trapping grid. Each blueberry sample was processed in the laboratory by using the methods described previously.

2014 Replicated Mass Trapping Field Study

On 27 August 2014, prior to the detection of juvenile SWD in preliminary fruit monitoring bouts, twelve 9.1 m x 9.1 m study grids were established in lowbush blueberry. Experimental traps were deployed and spaced at 0.9 m (low density), 1.8 m (medium density) and 2.7 m (high density) in order to determine the impact of varying trap density on *D. suzukii* abundance. Control traps were spaced 1.8 m apart. All treatment grids were replicated three times, in replicate blocks in the same 16.2 ha field at the University of Maine Blueberry Hill Research Farm in Jonesboro, ME. Trapping grids within a block were positioned a minimum distance of 9 m from one another. For collection of adult abundance data, three randomly chosen monitoring traps were taken from each trapping grid. During weekly trap collections, all traps were cleaned and recharged with freshly prepared bait and boric acid solution. In addition, three blueberry

samples (160 mL each) of random clones located within each study area were collected weekly so that larval infestations could be quantified. Trap contents and blueberry samples were analyzed for *D. suzukii* by utilizing the processing methods described previously.

Since treatments were not replicated in the preliminary mass-trapping assessment, only the 2014 study results were statistically analyzed. Analyses of variance (ANOVA, RCB) were utilized to examine the impact of treatment and block on the observed variation in adult and larval SWD abundance within trapping grids. An initial test entailed two separately conducted ANOVAs to assess how varying trap density impacted *D. suzukii* adult and larval abundances within study plots. A third ANOVA examined the relative abundance of *D. suzukii* larvae found infesting fruit samples collected in control vs medium density experimental plots. Significant effects were subject to subsequent Tukey post-hoc tests. (JMP®, Version *12*.0.1 SAS Institute Inc., Cary, NC, 1989-2007).

Insect Exclusion Efficacy

Insect Netting (25 Mesh - 13' wide x 50' long) with mesh size meeting the predetermined dimensions for effective exclusion of SWD adults (Caprile et al. 2013) was tested at Blueberry Hill Farm in Jonesboro, ME during the summers 2014 and 2015. In 2014, one replication was initiated on 9 July and the other on 18 July; in 2015, two replications were set up on 29 July, with a third replication initiated on 30 July. All of these replicates were deployed prior to capture of any adult *D. suzukii* and fruit being susceptible to attack. Unprotected plots served as

control treatments for each replicate. In addition, ethanol baited monitoring traps were deployed in close proximity to study areas for determining the onset of invasions. Upon initial capture of adult *D. suzukii,* externally located blueberries were periodically sampled from the periphery of study plots in order to track fruit infestations. On 17 September, after fruit had been infested by colonizing *D. suzukii*, exclusion nets were taken down and five samples (160 mL each) were obtained from each study plot for determining the abundance of *D. suzukii* larvae per fruit sample. This was accomplished by utilizing the previously described salt extraction method for fruit samples.

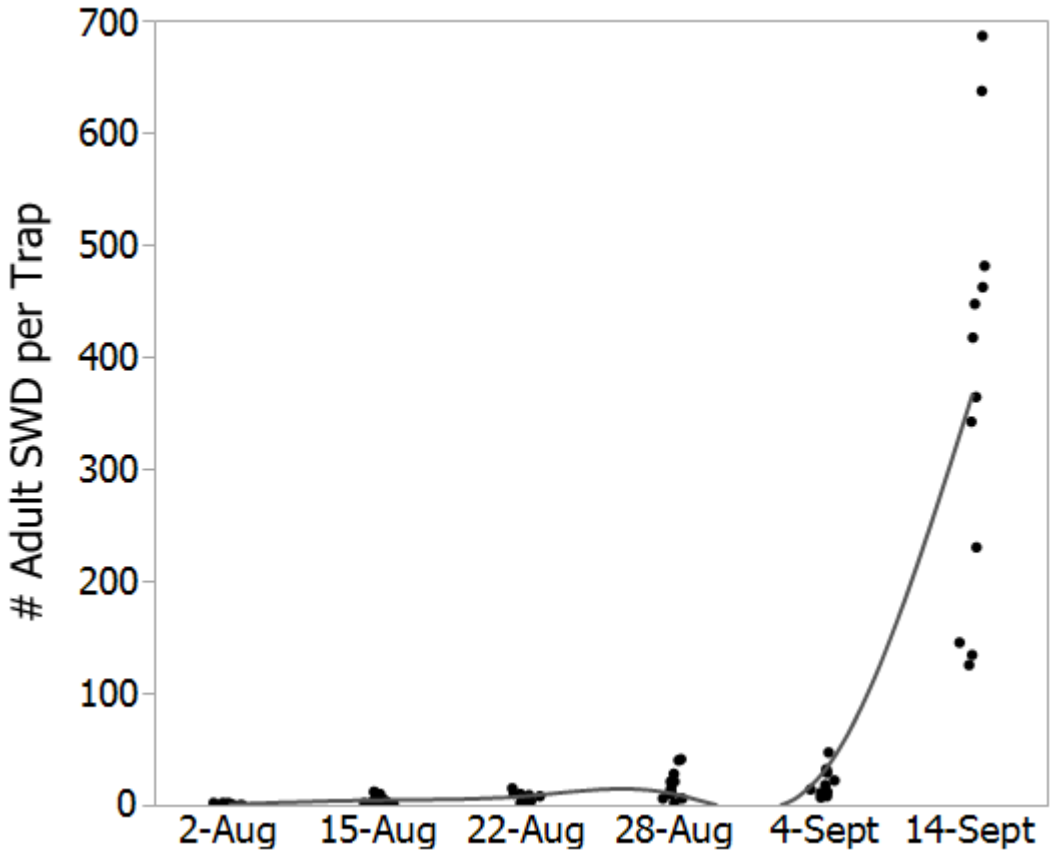
A randomized block design model, with year as a blocking factor, was utilized for analyzing the larval fruit infestation data obtained in both 2014 and 2015. Due to the instance of zeros in some cases, this analysis was accomplished by setting up a generalized linear model with a Poisson distribution and log-link function with protected vs unprotected being the categorical independent variable (JMP®, Version *12*.0.1 SAS Institute Inc., Cary, NC, 1989-2007).

Results

Preliminary Mass Trapping in Lowbush Blueberry

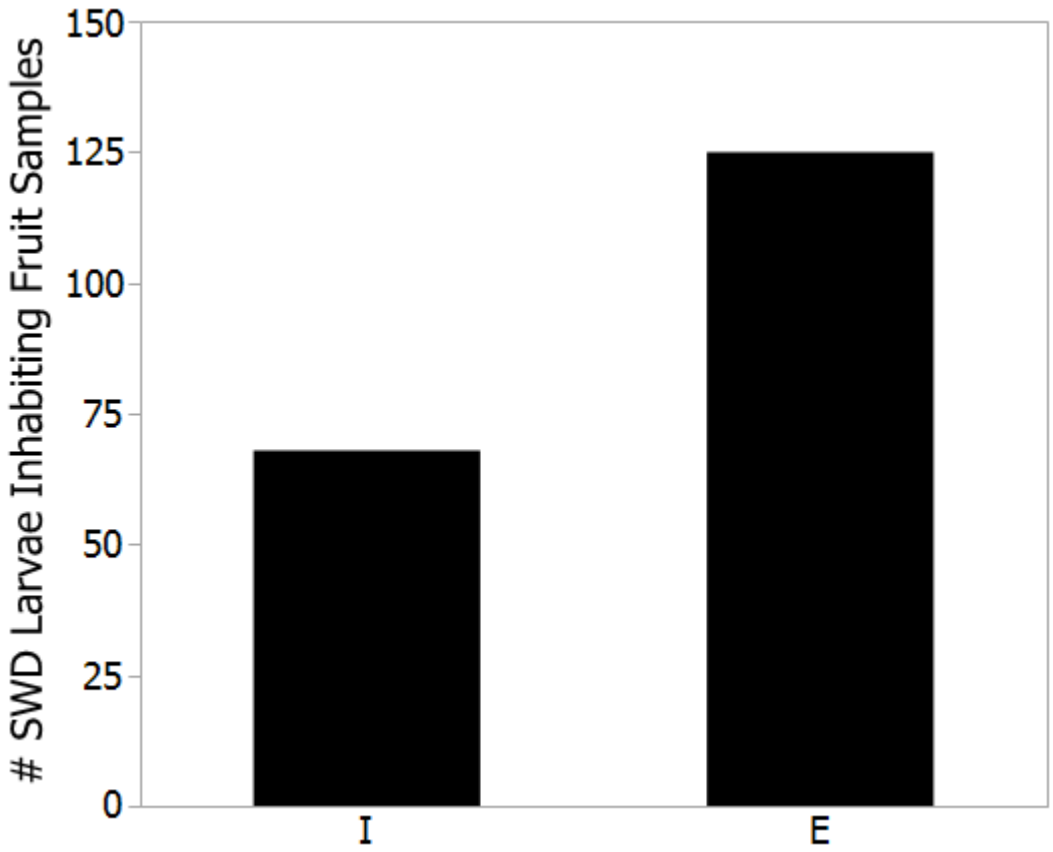
In 2013, there was a considerable increase in the abundance of SWD captured in baited traps toward the end of the experiment (Fig. 3.1). A maximum average capture rate of 18 ± 13 (SD) SWD larvae per trap were collected prior to the final sampling bout on 14-Sept. As shown in figure 3.1, however, a substantial increase in SWD captures per trap was observed on this date, with an

Figure 3.1 Abundance of SWD flies captured in each of twelve traps constituting a single trapping grid located in Jonesboro, ME during the summer of 2013.



average sampling rate of 373 ± 188 (SD) flies per trap. Interestingly, fruit samples collected within the trapping grid contained 68 SWD larvae in comparison to the 125 SWD larvae found infesting fruits obtained from externally located plant clones (Fig. 3.2). This corresponds to a 46% reduction in larval abundance, with a negligible 0.8 g difference in fruit sample weights of berries obtained from clones occupying internal vs external positions in relation to the trapping grid. This suggests that traps in this study were effective in reducing the infestation of berries by SWD.

Figure 3.2 Abundance of SWD larvae inhabiting single fruit samples gathered internally (I) or externally (E) of a trapping grid located in Jonesboro, ME during the summer of 2013.



Mass Trapping in Lowbush Blueberry

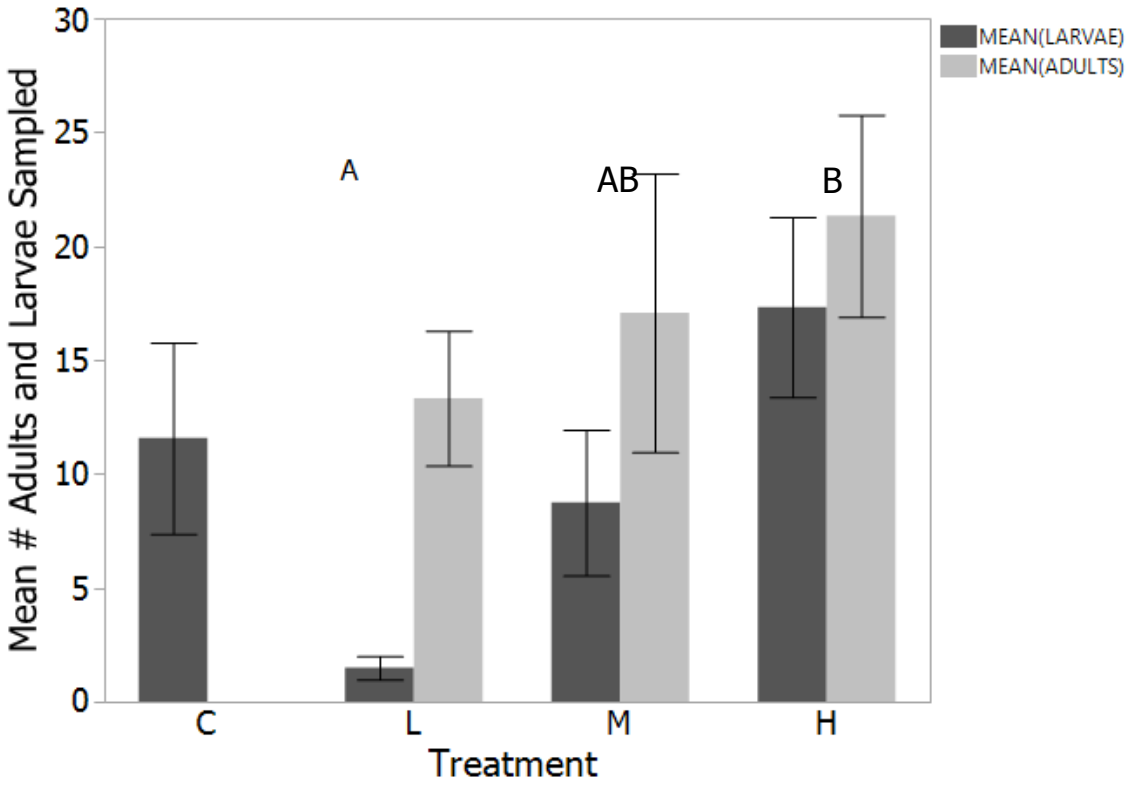
In the 2014 replicated field trapping study, comparing control grids (1.8 m trap spacing) with trapping grids of medium density spatial arrangement (1.8 m trap spacing), there was no statistically significant difference in larval abundance over time in blueberry samples (F= 0.12, df = 1,19; P = 0.97). No adult *D. suzukii* were captured in the unbaited control traps compared to 17 ± 21 (SD), SWD per trap captured in the medium density treatment. This suggests that attracting and capturing flies into baited traps in plots can still result in a

reduction of larvae, meaning that a considerable proportion of the flies coming into the baited trapped plots were killed in traps. Figure 3.3 shows that varying trap spacing of experimental grids did have a significant effect on the larval infestation in blueberry samples ($F = 15.00$; df = 1, 30; $P = 0.001$). However, there was no significant difference detected in the mean abundance of adults captured in traps across the range of trap-densities ($F = 0.74$; df = 1,30; $P = 0.40$). A trend in adults captured within plots does appear to correlate with the abundance of larvae (r = 0.63; $P < 0.0001$). Therefore, higher concentrations of traps tended to only increase the aggregation rate of flies to these areas, and did not effectively remove enough ovipositing females to reduce the resulting degree of fruit infestations.

Exclusion Netting on Lowbush Blueberry

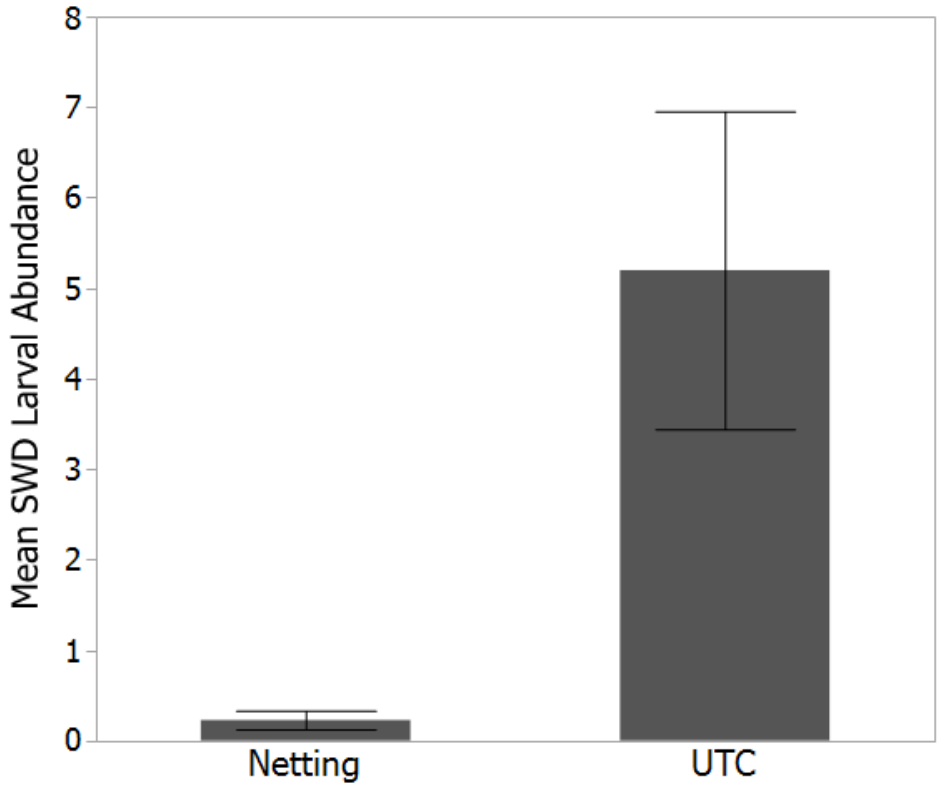
There was no considerable difference in mean larval samples of 7 ± 7 (SD) and 4 ± 1 (SD) gathered from unprotected blueberry fruits during the 2014 vs 2015 trials, nor was there any difference between mean counts of 0.2 ± 0.3 (SD) and 0.3 ± 0.3 (SD) SWD larvae obtained from net protected plots during 2014 vs 2015, respectively ($P = 0.93$). Moreover, the inclusion of a treatment and year interaction did not explain additional observed variation in mean larval counts taken from protected or unprotected blueberry fruits ($P = 0.75$). Overall, the exclusion netting provided a high degree of protection efficacy in comparison to unprotected fruits ($X^2_{(df = 3)} = 29.2$; $P < 0.0001$). An average of 5 ± 4 (SD) larvae were found infesting berries obtained from the uncovered crop, in

Figure 3.3 Mean abundance of individual *D. suzukii* captured in control trapping grids (C) and in low density (L), medium density (M) and high density (H) experiment trapping grids. Each mean represents the average of three replicates over four weeks of trapping. Control and medium density treatments were arranged such that traps were spaced 1.8 m from one another. Letters above larval abundance columns represent Tukey post-hoc results, with bars displaying dissimilar letters signifying statistical significance. Error bars were constructed using one standard deviation of the mean.



comparison to 0.2 ± 0.2 (SD) inhabiting samples protected with exclusion netting (Fig. 3.4).

Figure 3.4 Mean abundance of *D. suzukii* larvae inhabiting five blueberry samples (160 mL each) from uncovered crops (UTC), or crops protected with exclusion netting. The measurements presented here represent the combined data from two consecutive trials conducted in 2014 and 2015. Error bars represent one standard deviation of the mean.



Discussion

After experimentation with mass trapping and insect netting in lowbush blueberry, only preventative exclusion of adult *D. suzukii* with netting demonstrated management potential, based on the low relative abundance of larvae sampled in protected vs unprotected study areas over the two years. These results complement the findings of Cormier et al. (2015) who reported zero *D. suzukii* adult emergences from blueberry fruits grown under net-protected plots. Furthermore, this approach is thought to maintain a high degree

of SWD exclusion efficacy in other cultivated, small-fruit producing plants such as raspberry (Schattman 2015). To date, all positive results with this technique have been obtained from studies conducted under limited space where the area of the study plots represents only a fraction of crop coverage necessary in large scale production operations. Scaling up would substantially increase the necessary netting product, labor and maintenance inputs required.

Practical Implications of Insect Exclusion Netting & SWD Management

Excluding the presumed labor requirements for seasonal installation and maintenance, the deployment of exclusion netting on a single hectare would cost roughly US$ 4,600 according to product pricing by Gardeners Supply co.®. According to Chen et al. (2015), from 2010 – 2015 organic lowbush blueberry cultivation in Maine generated annual net revenue of US $3,724 per hectare. In a single year, therefore, the financial inputs of purchasing netting units alone would exceed the total amount of generated revenue. However, more economically justifiable cost estimates are derived when considering the repeated use of netting units. Assuming a degree of material durability and longevity spanning 2, 3, 4 and 5 years, the respective proportional cost decreases to roughly 62%, 41%, 31%, and 25% of total revenue generated from organically produced lowbush blueberry fruits. Given the high degree of control obtained with exclusion netting reported in this and other studies, it might be more feasible to deploy this technique if the percentage of yield damage incurred by SWD exceeds the proportional cost estimates of using a

single netting unit repeatedly over time. Presumably, this disparity will increase over longer time periods. Unfortunately, monetary losses due to SWD infestation have not yet been quantified in the lowbush blueberry crop system. With respect to the projected value of this preventative technique, the theoretical SWD damage thresholds may constitute a limiting piece of information, as well as the additional required labor costs for maintenance and installation not discussed here.

Insect exclusion netting has demonstrated considerable success with phytopathogen vectors, preventing their physical contact with host plants in order to suppress insect-mediated disease transmission. Stansly et al. (2004) demonstrated the potential for using this technique to limit the spread of tomato yellow leafcurl virus (TYLCV) among greenhouse tomato plants. Plants displayed TYLCV symptoms less frequently when protected with netting for exclusion of the pathogen's insect vector, the whitefly *Bemisia tabaci* (Gennadius). Another relevant phenomenon has been described by Louise et al. (1996) for *Drosophila melanogaster* and the dispersal of its microbial symbiont *Botrytis cinerea* Pers.: Fa, a necrotrophic fungus responsible for the occurrence of grape rot in vinyards. External localization of fungal spores on fly integuments, in conjunction with internal occupancy of the flies' alimentary canal is thought to facilitate passive spore dispersal throughout vinyards harboring *D. melanogaster* populations (Louise et al. 1996).

The symbiotic association of SWD with *B. cinerea* or alternative pestiferous microbes has not been documented, nor have the risks of introducing microbial symbionts of SWD into recently colonized agroecosystems been addressed in the literature. However, considering the described mechanisms of passive grape rot transmission by *D. melanogaster*, it is possible that intimate endosymbiotic associations within SWD may diversify the pest pressures imposed on certain plant species that further reduce fruit marketability, perhaps even leading to the inadvertent introduction of additional pestiferous microbes to the agroecosystem.

The exclusion efficacy of netting in preventing SWD flies from contacting viable fruit hosts has been demonstrated in multiple crop systems. Coupled with the theoretical cost-efficiency in the context of Maine lowbush blueberry production, it is justifiable to consider exclusion-netting as a viable avenue for further evaluation as a component of developing IPM programs for SWD. However, one potential limitation to this method of SWD prevention includes the alteration of microclimates located within netting units. If the accumulation and retention of moisture within the space encapsulated by the netting material effectively alters crop and pest growth conditions in relation to ambient environmental conditions, the consequent humidification could create a habitat in which phytopathogenic fungi flourish (Ciancio and Mukerji 2008). Although not yet tested for, this is a necessary consideration to address should the technique

be considered for SWD management in large-scale crop systems where fungal pests may become problematic and require damage control.

Future Mass-Trapping Efforts

Another component of this investigation evaluated mass-trapping as a protocol for reducing the quantity of larvae infesting berries. In comparison to control study grids with traps containing water, deployment of traps containing an ethanol emitting bait and topical boric acid application failed to reduce the abundance of larvae infesting blueberry samples. It is worth noting that the quantity of larvae sampled in medium density experiment plots was significantly greater than larval counts of fruit samples from low density experimental plots. However, despite the apparently low relative infestation observed in low-density vs control plots, no statistical comparison was conducted due to dissimilar methodology in the spatial arrangement of traps.

Manipulating trap spacing did not result in lower adult SWD captures per trap. This seems to explain the observed reductions of larvae inhabiting fruits collected from low density trapping grids. The study therefore provided evidence that spatially concentrating traps within lowbush blueberry study plots attracted an overall greater number of flies to the study area. This aggregation effect, whereby the number of actively ovipositing females in the proximity of trapping grids increases proportionally with trap density, would have consequently increased the frequency of contact between ovipositing females and healthy blueberry fruits due to the substantial percentage (approximately 84 % ± 8 %

(SD) according to the results of Alnajjar (2016)) of flies not drowning in traps. The capture efficacy of a comparable trap design was assessed by Hampton et al (2014) who found variable capture rates ranging from 10 % - 30 % in traps baited with a combination of acetic acid and ethanol. Although the impact of boric acid was not quantified in this study and may have negated the fitness of some uncaptured individuals, it is also possible that the presence of borate in the sugar solution acted as a repellent. Regardless, its combined deployment with ethanol baited mass-trapping did not achieve satisfactory control levels. Hampton et al. (2014) suggested the incorporation of insecticides directly on the external surface of traps, and/or on proximal plants as exposure reservoirs. Doing so might eliminate the attracted, uncaptured adults given the high efficacy of various insecticides against SWD (Bruck et al. 2011, Collins and Drummond 2016a, b). The results of Hampton et al. (2014) also imply an inverse relationship between distance from trap and the probability of *D. suzukii* infestation. It has therefore been proposed that trapping grids be deployed on the periphery of managed fields in order to confine invasions within trap cropping reservoirs in these areas so they can be effectively treated and prevented from further dispersal into the fields during pre-harvest intervals.

Additional evaluations on SWD bait preference and the chromatic attractiveness of traps have provided insights for the development of trap designs that enhance *D. suzukii* fly attraction and capture rates. The physical localization of SWD flies on the exterior portion of traps is believed to occur more

frequently on dark pigmented surfaces. Basoalto et al. (2013) reported double the observed fly capture rates in traps wielding a black painted strip enveloping entry holes for the flies. The implementation of this trap coloration protocol in SWD monitoring may lead to earlier and more accurate detection of flies in agricultural landscapes (Basoalto et al. 2013) and also enhanced proficiency in the capture rates of individual traps within mass-trapping grids. The overall alluring power of traps, however, is mainly influenced through primary physiological attraction of *Drosophilid* flies to naturally occurring volatile semiochemicals, given the innate implications of nutritional, copulatory and reproductive rewards (Landolt et al. 2011). Potentially then, developing a visually appealing trap to *D. suzukii* flies, combined with baiting of optimal chemical mixtures for spatially expansive and powerful attraction, could constitute a more effective mass-trapping management tactic with greater SWD control potential than the protocols tested here.

Field trials on the preference of *D. suzukii* with various volatile compounds by Landolt et al. (2011 & 2012) suggest that the maximum positive chemotactic response of adult *D. suzukii* occurs during concurrent emission of ethanol and acetic acid, as indicated by a greater abundance of adults captured in traps emitting both baits in comparison to those containing either compound independently. However, the *D. suzukii* chemosensory niche specialization for reproductive exploitation of fresh or ripening fruits in nature appears to be in stark contrast to the mechanics of physiological stimulation in *D. melanogaster*.

This latter species displays a consistent positive chemotactic response upon detection of volatile fermenting byproducts emitted by decaying plant material. Keesey et al. (2015) conducted a laboratory bioassay in which olfactory attraction cues for *D. suzukii* and its close relative, *Drosophila biarmipes*, were uniquely found to positively correlate with the volatile isoprenoid ß-cyclocitral believed to have developed a novel association with *D. suzukii* neuronal receptors. This volatile semiochemical is associated with the host plant leaf tissue and is therefore suspected of significantly influencing the degree of *D. suzukii* attraction toward ripening fruits occupying stem positions on an otherwise healthy plant. Taken together, evaluating the exploitation of ß-cyclocitral and other as of yet unknown phytochemicals in mass-trapping of SWD should consider the preparation of chromatically appealing trap exteriors for increasing the capture frequency, and also consider the arrangement of traps at relatively low concentrations for decreasing the probability of mass fly aggregation and maximizing the degree of management capability that can be achieved with this technique. For the purposes of trap cropping, however, it may be the case that maximizing the concentration of flies in trap crops is more desirable given that a greater quantity of flies can be targeted with a single insecticide treatment.

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APPENDIX A

PRELIMINARY SWD BIOCONTROL ASSESSMENTS

Selection of Concentration Assay Exposure Surfaces

A preliminary *D. suzukii* adult-conidia contact assessment was conducted to evaluate the surface inoculation efficacy of two different types of filter paper. *Beauveria bassiana* conidia are approximately 2.0 μm in length. In comparison to *M. anisopliae,* which produces conidia measuring about 5 μm, the localization and isolation of *B. bassiana* conidia within surface pores could therefore act as a constraining factor in designing a single protocol for both fungal conidia. This prompted the selection of *B. bassiana* as the model pathogen in this exploratory analysis.

Methods & Analyses

Two *B. bassiana* conidia concentrations were prepared in suspensions of approximately 1×10^6 and 1×10^8 conidia/mL in 0.01% Tween®. Eighteen petri dishes were lined with moistened filter paper, nine of which received an additional layer of 0.22μm GV millipore filter paper. Including a 0.01% Tween® control, six total treatments were prepared and replicated three times each. A Berkard® computer controlled spray machine with a click setting of 6 and pressure of 10psi was utilized for the application of suspensions onto surfaces. Liquid droplets were allowed to evaporate after spraying to prevent drowning of flies upon introduction to the enclosures. Laboratory cultures of SWD were immobilized by steadily releasing gaseous CO_2 from a canister (regulator setting

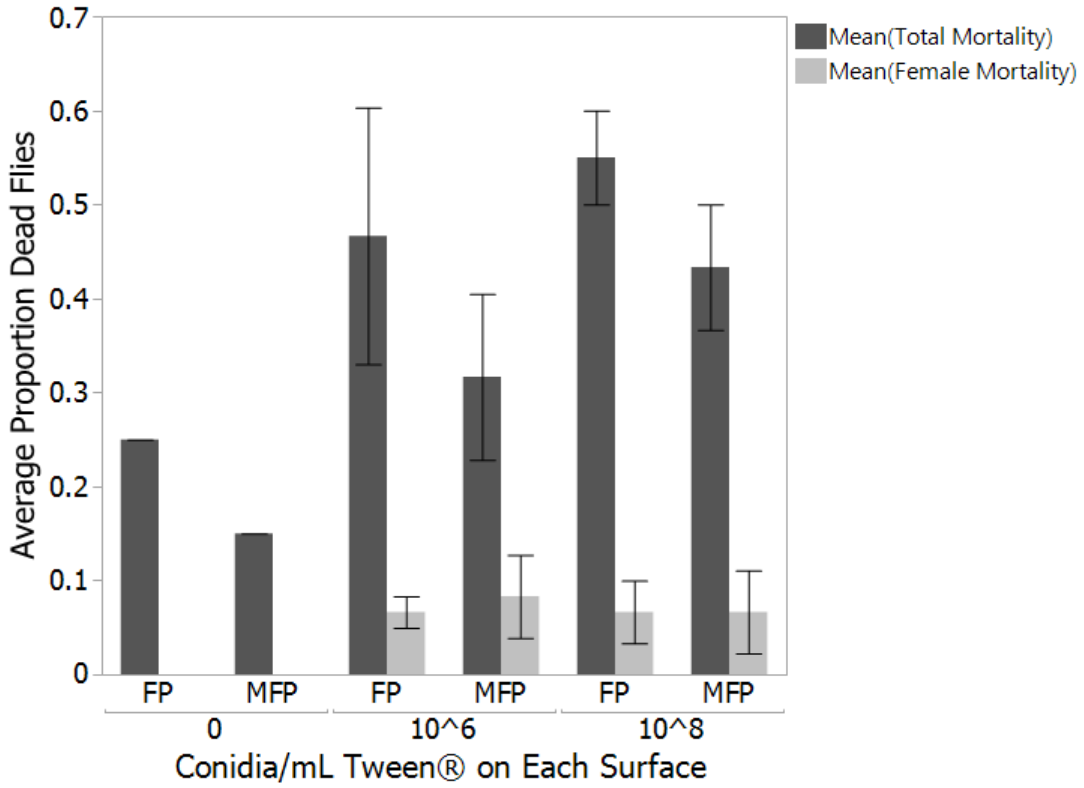
= 5 psi) into the vial, and then tapping flies out onto a petri dish over ice. Ten male and 10 female flies were then introduced to each pathogen inoculated surface and held in the dark at 25 ± 1°C for 24 hours. The next day, SWD were again CO_2 immobilized and placed in 50 mL culture vials containing Carolina: Formula 4-24® instant drosophila media. The remainder of the study was carried out at 25 ± 1°C with a 12h L/D cycle; mortality was measured daily for five days following initial contact of flies with entomopathogenic conidia. The impact of pathogen dose and surface type on adult SWD mortality was analyzed via nominal logistic regression (JMP®, Version 12.0.1 SAS Institute Inc., Cary, NC, 1989-2007).

Results & Conclusion

The dose and surface interaction was not significant for either total ($X^2_{(2, n=20)}$ = 0.13, P =0.94) or female ($X^2_{(2, n=10)}$ = 0.428, P =0.807) fly mortality. Both models were rerun by omitting the interaction term for dose and surface type. The results suggest that greater *B. bassiana* concentrations increased total ($X^2_{(2, n=20)}$ = 24.23, P <0.0001) and female ($X^2_{(2, n=10)}$ = 12.27, P =0.002) fly mortality. However, a surface effect was only detected when looking at the response of total ($X^2_{(1, n=20)}$ = 6.25, P = 0.012) and not female flies ($X^2_{(1, n=10)}$ = 0.33, P =0.57).

Figure A.1 shows average mortality measurements of control and infected *D. suzukii* adults over the five-day observation period. A disproportionate gender

Figure A.1. Average % mortality of total (male and female) SWD after exposure to two *B. bassiana* conidia concentrations on millipore filter paper (MFP) or regular porosity filter paper (FP). Error bars are 1 standard error.



response suggests that male survival may have been jeopardized by unidentified variables, or that males are more susceptible to *B. bassiana* induced mycosis. Therefore with respect to surface selection, comparisons were restricted to female mortality. It was concluded that, regardless of conidia coverage, inoculated millipore filter paper caused no difference in mortality than inoculated filter paper. Despite the apparent homogeneous inoculation efficacy, millipore filter papers were designated as the exposure surface to be utilized in subsequent laboratory bioassays. Unlike the alternative exposure medium, the low relative permeability of millipore filter papers is believe to more closely represent functional resemblance to the epicuticular waxy layer of fruit and leaf

integuments that work in part to retain moisture. In addition, the observed control fly mortality in this investigation prompted the utilization of young, newly emerged adult SWD in subsequent laboratory experiments as a standard.

Oocyte Development in Teneral SWD Females

Examining the maturation of oocytes within *B. bassiana* infected adult SWD necessitated a preliminary ovarian development assessment in order to address three key questions: (1) do the ovaries of teneral females contain fully developed oocytes, (2) if not, what is the duration of adult maturation required to complete oocyte development, and (3) is copulation necessary to stimulate oviposition behaviors in reproductively mature females?

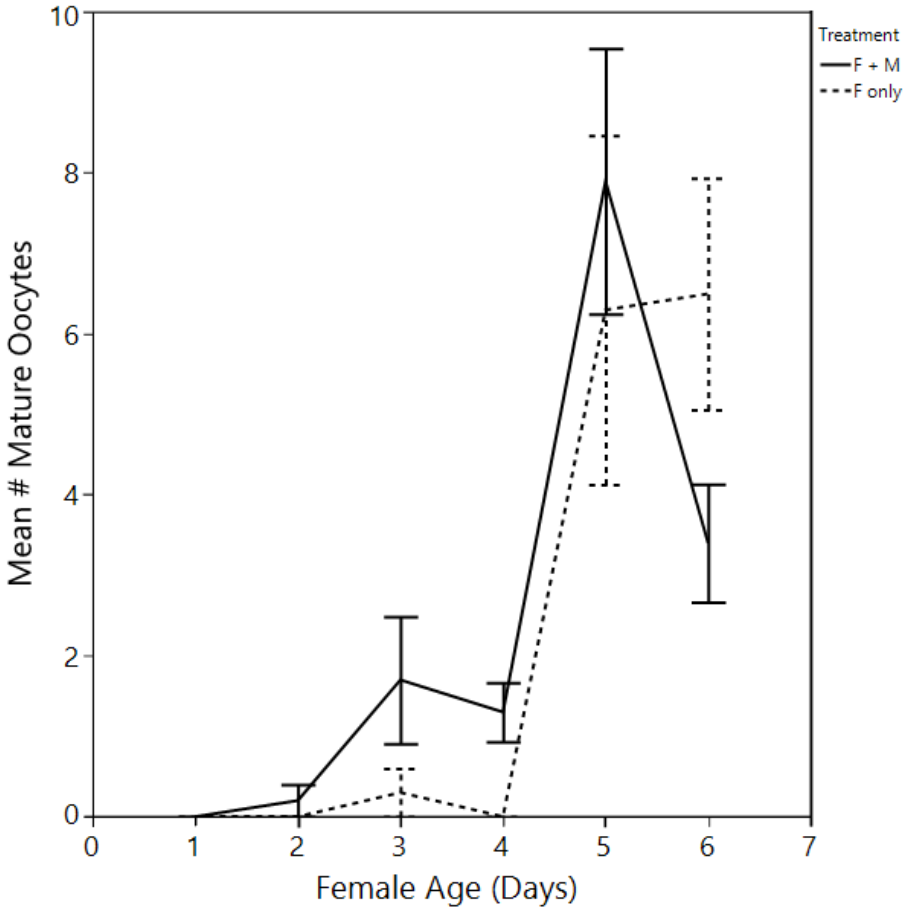
Methods & Analyses

After a twenty-hour eclosion period, 10 teneral female *D. suzukii* flies were placed in each of 12, 50 mL culture tubes containing Carolina: Formula 4-24® instant drosophila media. Thirty adult males displaying prominent wing spots were obtained from laboratory stock colonies and divided equally into six of the female samples. The following six days, one group of mated and unmated females were euthanized in 70% ethanol and dissected in order to quantify mature oocyte abundance in each individual. These count data were analyzed via a generalized linear model due to the high frequency of immature oocytes recorded in young female flies. The cumulative effects of copulatory stimulation and days of adult maturation were modeled as predictors of female fecundity.

Results & Conclusion

Females in the proximity of males were observed engaging in mating behaviors. A time sensitive impact of male presence on female oocyte abundance was indicated by the generalized linear model ($X^2_{(df=1,\ n=10)} = 16.01$; $P < 0.0001$). Figure A.2 compares the average fecundity of mated and virgin females over time. The data show that teneral females sustain ovarian development throughout the first two to three days of adulthood, and virgin females appear to produce and mature eggs until at least six days after pupal eclosion. The statistical results and trends observed in figure A.2 suggest that oocyte development occurs more rapidly in immature female SWD in the presence of male flies (presumably due to reproductive stimuli through molecular or physical contact with male pheromones and copulatory behaviors). Therefore, I concluded: 1) ovarian development progresses in the absence of mating, and 2) virgin SWD females appear to retain eggs until at least six days of adult maturation. Collectively, these results support the implementation of protocols that prevent mating in *D. suzukii* ovarian development assessments to prevent induction of female oviposition behaviors. This will aid in reducing the extent of inherent experimental error of the study methods.

Figure A.2. Abundance of fully developed SWD oocytes over time in young female flies. Each mean represents the average egg abundance of ten individual female flies. Oocytes of virgin females developed less quickly and appear to accumulate in the ovaries. Error bars are 1 standard error of the mean. Dashed line is for females without exposure to males and solid line represent oocyte development trends for females in the presence of sexually mature male flies.



APPENDIX B

PESTICIDE SYNERGY IN THE COMMON EASTERN BUMBLE BEE

Susceptibility of Individual *B. impatiens* Workers

The risks of exposure to pesticides on wild and domestic bee populations are thought to include synergistic mechanisms between different toxin classes that affect dissimilar yet interconnected components of an organism's physiology. For example, increased mortality responses to the neonicotinoid insecticide, acetamiprid, have been observed in individual honey bee (*Apis meliffera* Linnaeus (1758)) workers after direct topical exposure to propiconazole, a DMI fungicide that inhibits cytochrome P450 (Iwasa et al. 2004). Given the importance of wild and domestic bee species in maximum fruit yield production through plant cross-pollination mechanisms, this study was conducted in order to test the effects of toxin synergy in the common Eastern Bumble Bee (*Bombus impatiens* Cresson (1863)) workers after indirect topical exposure to filter papers inoculated with acetamiprid and/or propiconazole.

Methods & Analyses

Previous *A. meliffera* pesticide risk assessments were consulted to obtain the estimated acetamiprid and propiconazole LD_{50} values for individual worker bees; 14.5 µg bee^{-1} and 100 µg bee^{-1}, respectively (Thoreby 2011, Blacquière et al. 2012). Acetamiprid was used as the formulated product Assail® in granular form, and diluted to an LD_{50} suspension with 1.99g L^{-1} dH_2O. Propiconazole was used as the formulated product Tilt® in viscous liquid form and was diluted to its

full LD_{50} potency at 2.34mL L^{-1} dH_2O. Each was utilized as a stock suspension to prepare additional dilutions corresponding to 0.1x and 0.5x the pesticide's LD_{50}. A dH_2O suspension was also prepared and utilized as a control. In addition to three independently applied toxin dosages of each chemical, one treatment entailed concomitant exposure of the propiconazole LD_{50} with the lowest concentration of acitamiprid. Control, propiconazole, acetamiprid, and cumulative exposure treatments were replicated five, four, three and four times, respectively.

Two live *B. impatiens* hives were obtained from Koppert, Inc. (Romulus, MI) as Natupol® colonies. The colonies were held at 3 ± 1°C for three hours prior to toxin exposure. During this time, each chemical suspension was prepared and applied to a layer of dry filter paper. Surfaces were sprayed in petri dishes with a Burkard® computer controlled sprayer (settings: click = 6, psi = 10). Surfaces were then installed on the base of cylindrical metal hardwire cloth enclosures occupying a volume of 6.9 m^3, with about 9 hardwire gaps per cm^2. Two dispenser vials were positioned at the top of each exposure chamber as a food source (25% sugar syrup) for the insect test subjects.

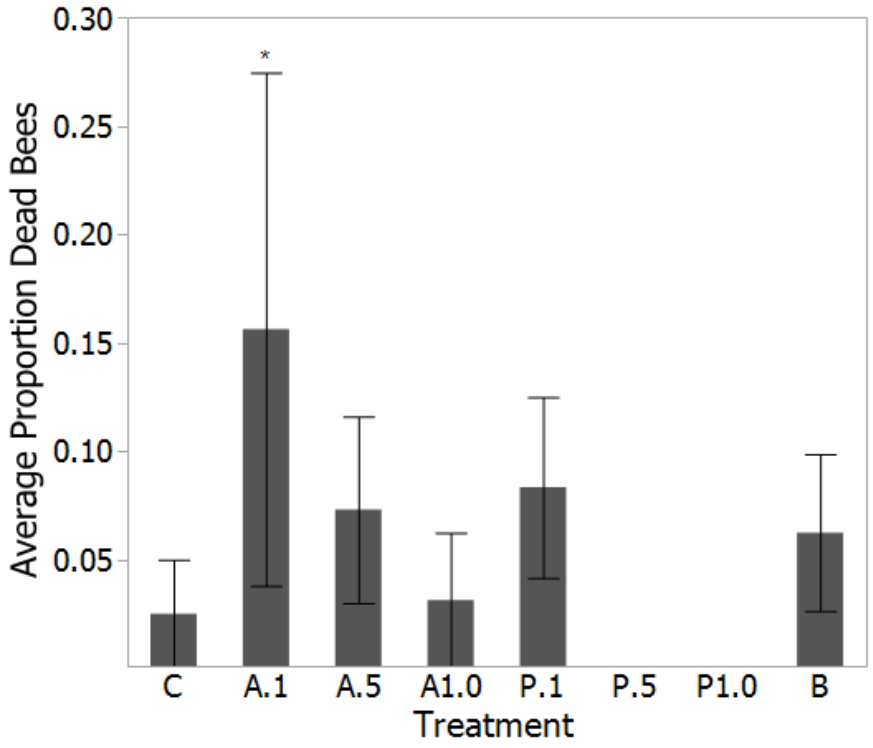
Utilizing a red headlamp in the dark at 3°C, eight worker bees were transferred to each exposure chamber with four of the bees randomly selected from each hive. All chambers were then secured with rubber bands and held in a growth chamber at 25 ± 1°C under a 12 h L/D cycle. The chambers were inspected daily and mortality was recorded over a seven day period. Death was

defined as little or no observable kinetic response to physical disturbance. It was noted that on the first day of mortality observations, a maximum of two bees had escaped from five enclosures. Statistical analyses were conducted on proportional mortality data, and these sample size reductions were observed in only one replicate. Designating a control mortality threshold of 20%, the statistical analysis utilized nominal logistic regression of treatment and replicate on the mortality response of *B. impatiens.* Pairwise comparisons ($P < 0.05$) between experimental and control group mortality data were then used to assess differences between individual treatments (JMP®, Version 12.0.1 SAS Institute Inc., Cary, NC, 1989-2007.).

Results & Conclusions

Bees exposed to dH_2O exceeded the analytical control mortality restriction (20%) over the 7-day observational period. Therefore, only the immediate 24 h response of bees to toxin exposure was compared statistically. In comparison to control mortality, figure B.1 suggests that only acetamiprid exposure at 0.1x ($X^2_{(df = 5,\ n = 68)} = 17.44$; $P = 0.004$) LD_{50} potency resulted in significant mortality of *B. impatiens* workers. Interestingly, greater concentrations of acetamiprid did not elevate mortality rates of *B. impatiens* workers. In fact, the opposite trend was observed; on average, over the 24 h period fewer bees died after exposure to greater acetamiprid concentrations. It is worth noting the wide range of variation observed for the 0.1 x acetamiprid dose, implying that this statistical result

Figure B.1 Average proportional mortality of domestic *B. impatiens* workers after indirect topical exposure to varying concentrations of acetamiprid (A) or propiconazole (P). Toxins were provided to bees independently, or concomitantly (B) at the lowest chemical concentrations tested in the study (0.1x LD_{50}). Toxins were delivered through topical exposure after spraying of pesticide suspensions onto filter paper. The (*) signifies a statistically significant difference in mortality upon comparison with the control treatment (C). Error bars are one standard error of the mean.



should be interpreted with caution. For comparison, Iwasa et al. (2004) also conducted an indirect topical exposure assessment utilizing the alfalfa plant cuticle as an exposure medium of *A. meliffera* workers to acetamiprid and triflumizole, another DMI fungicide. However, no significant mortality response was reported from independent or concomitant contact with pesticides, in comparison to that of the control group.

In a subsequent and independently conducted *D. suzukii* laboratory study, relative humidity was recorded at 17% under identical growth chamber conditions implemented in these pesticide risk assessments. In relation to SWD adults, *B. impatiens* workers are relatively robust with a low surface area to volume ratio. However, it is possible that in the absence of an adequate moisture retention protocol, insect tissues were unable to sustain osmotic potentials critical to functioning biological systems. Cellular desiccation would have inevitably resulted in death of the organism, and could have exacerbated the observed mortality of control bees.

Sub-Lethal Susceptibility of Bumble Bee Colonies to Oral Pesticide Exposure

No published risk assessments on pesticide synergy in bee species investigated at the colony level have been reported in the literature. Given the reductions of enzymatic detoxification capabilities induced by a DMI-fungicide in *Apis meliffera* (Linnaeus, 1758) workers (Iwasa et al. 2004), the synergistic toxicity of different pesticide classes has received more attention in pollinator conservation and agroecosystem research. Previously, I tested the lethal susceptibility of individual *B. impatiens* workers to pesticide exposure through an indirect topical contact mechanism. While the results did not provide substantial empirical evidence suggesting synergistic impacts of acetamiprid and propiconazole on individual bee survival, no chronic responses were investigated in the experiment. It is possible that the sub-lethal aggregation of xenobiotics in

honey reservoirs may induce non-lethal responses in colony health. Thus, the objective of this project was to quantify *B. impatiens* fluctuations in colony health in response to the consumption of syrup inoculated with acetamiprid and propiconazole.

Methods & Analyses

Twenty *Bombus impatiens* hives (Koppert. Inc.; Romulus, MI) were obtained during the spring of 2015. Colonies arrived in sets of four and were numbered by quad occupancy. With an approximation of 200 adult *B. impatiens* per colony, predetermined hive syrup consumption rate estimations (roughly 1.3 x 10^6 mg $colony^{-1}$) were obtained to calculate the necessary acetamiprid and propiconazole treatment concentrations based on the LD_{50} estimation for each pesticide; 14.5 µg bee^{-1} and 100 µg bee^{-1} for acetamiprid and propiconazole, respectively (Thoreby 2011, Blacquière et al. 2012). Chemicals were obtained from the commercial pesticide products Assail® and Tilt®. Prior to being infused with pesticides, the quantity of syrup filling each bag was determined by taking the average weight of rations and subtracting by the mass of an empty dispenser pouch. All four hives in each quad were individually provided syrup reservoirs containing one of the following treatments: no toxin, acetamiprid alone at 1.0x LD_{50} (9.0 ng mg^{-1} syrup) or 0.1x LD_{50} (0.9 ng mg^{-1} syrup), propiconazole alone at 0.1x LD_{50} (6.0 x 10^{-2} nL mg^{-1} syrup), or a mixed concentration corresponding to 0.1x the LD_{50} of both toxins. After mixing, the actual amount of syrup

administered to each hive was weighed in order to provide an initial measurement for determining % consumption after treatment exposures.

After replacing commercially provided syrup rations with pesticide infused commercial syrup, bees were held in an air conditioned laboratory and prevented from foraging for 14 days in order to induce toxin consumption. Following the two-week period hives were placed underneath of an outdoor trailer and allowed to forage for four weeks. Treatments were grouped together by block, with blocks positioned a minimum of 2 m from one another. All colony boxes were closed on the evening prior to data collection, and held at 3 ± 1°C for three hours the following morning. Colonies and syrup remnants were separately weighed (g) and adult *B. impatiens* mortality in each hive was categorized as such: none (0 dead), low (1-5 dead), medium (6-25 dead) and high (> 25 dead). All hives were then euthanized via freezing in order to obtain larval, pupal and adult counts. Relative adult composition was obtained by determining the proportion of small (<1 cm), medium (1 – 2 cm) and large (>2 cm) bees in each colony based on qualitative observation of the length of individuals.

Categorized mortality observations were converted to ordinal data and analyzed via logistic regression. An ANOVA was utilized for percent data, and also for square root transformed count data. All ANOVA were concluded with a Tukey post-hoc test for pairwise comparisons between treatments (JMP®, Version 12.0.1 SAS Institute Inc., Cary, NC, 1989-2007).

Results & Conclusion

There was no statistically significant difference in adult mortality observed among treatments ($X^2_{(df= 4;\ n = 20)}$ = 4.61; P = 0.33). The addition of quad number as a covariate (rank of spatial proximity of colonies during foraging) did not provide a better explanation for the variation observed in total adult abundance (F = 0.70; df = 8, 19; P = 0.72) or the overall size of colonies (F = 0.70; df = 8, 19; P = 0.71). There was also no impact of treatment on the abundance of individuals in hives (F = 2.01; df = 15, 19; P = 0.14). As shown in Table B.1, the addition of pesticides to syrup had no impact on resulting syrup consumption rates (F = 0.32; df = 15, 19; P = 0.86) or hive weight changes (F = 1.47; df = 15, 19; P = 0.26). The statistical analysis revealed a notable difference in the number of gynes (P = 0.049) inhabiting colonies exposed to the 1.0x acetamiprid LD_{50} vs 0.1x propiconazole LD_{50}. However, wide variation in gyne rearing was observed in hives exposed to the former treatment, and neither pesticide considerably changed gyne production upon comparison with control colonies (Table B.1). On average, all colonies experienced positive growth and were not deterred from feeding on pesticide contaminated food sources. Despite the expectation that the occurrence of xenobiotics in floral nectaries would induce a negative chemotactic response in bee foragers if more chemically pure resources are available, Kessler et al. (2015) reported contrary observations in laboratory bioassays; both *A. meliffera* and *Bombus (Bombus) terrestris subsp. audax* (buff-tailed bumblebee) foragers displayed a significant preferences for sucrose solutions infused with neonicotinoid insecticides over unlaced sucrose

Table B.1 Estimated mean ± standard deviation of each dependent variable by LD_{50} potency applied (A: Acetamiprid, P: Propiconazole). Only the abundance of gynes appears to have responded to treatment; hives provided syrup with 1.0x acetamiprid LD_{50} reared significantly more gynes than hives treated with 0.1x propiconazole LD_{50}.

TMNT	Total Abundance (N)	Adults	*Gynes	Med. Adults	Sm. Adults	Pupae	Larvae	Honey Pots	% Inc. Hive Wgt	% Change Syrup Wgt
Control	616 ± 103	180 ± 26	2.5 ± 1.3	115 ± 25	62 ± 44	211 ± 101	225 ± 95	62 ± 19	52% ± 24%	67% ± 15%
0.1x A	693 ± 234	157 ± 53	3 ± 1.4	110 ± 42	44 ± 14	290 ± 118	246 ± 112	68 ± 20	42% ± 18%	69% ± 17%
1.0x A	638 ± 97	172 ± 51	*5.5 ± 4.5	117 ± 47	49 ± 10	225 ± 34	241 ± 88	72 ± 41	46% ± 11%	58% ± 16%
0.1x P	1021 ± 260	248 ± 45	*1 ± 0.8	173 ± 55	74 ± 11	369 ± 108	405 ± 112	79 ± 36	70% ± 27%	58% ± 37%
0.1x A + 0.1x P	938 ± 480	230 ± 92	2.3 ± 0.5	174 ± 64	54 ± 29	420 ± 262	288 ± 149	49 ± 9	72% ± 30%	72% ± 22%

solutions. However, a negative chemotactic response has also been described by Laycock et al. (2012) with an inverse relationship between neonicotinoid concentration and *per capita* buff-tailed bumblebee worker consumption rates of syrup and pollen during no-choice pesticide risk assessments.

Although the results of this assessment suggest a neutral response of *B. impatiens* workers to neonicotinoid contamination in the hive food supply, it will be necessary to accurately define the species' behavioral response to oral detection of xenobiotics to determine how colonies respond to the localization of pollutants in food sources. Overall, the results presented here indicate that sub-lethal oral exposure of *B. impatiens* to acetamiprid and propiconazole may not pose immediate chronic hazards to colony health. While the variation in gynes was significant among two of the experimental treatments, the ambiguous statistical result suggests that this discrepancy should be interpreted with caution, possibly serving to promote additional risk assessments on colony gyne rearing and queen overwintering physiology in response to the accumulation of antagonistic pesticide contaminants in hive resources.

BIOGRAPHY OF THE AUTHOR

Gabe was born and raised on the outskirts of Baltimore, Maryland. After graduating high school in 2007 he enrolled in the biological sciences program at the University of Maryland in Baltimore County. As an undergraduate, Gabe participated in various ecological research programs as a laboratory and field assistant in the hopes of exposing himself to a spectrum of topics in conservation biology. After earning a bachelor's degree, he began working as a naturalist for Oregon Ridge Nature Center in Cockeysville, MD. During that year-long hiatus from school, Gabe constructed and led educational programs aimed at connecting the public with nature. Since that time, he's been fortunate enough to pursue career aspirations in applied conservation research through the school of biology and ecology at the University of Maine. He is a candidate for the Master of Science degree in Ecology and Environmental Sciences from the University of Maine in August 2016.