

Host-Parasitoid Interactions of Two Invasive Drosophilid Species in Virginia Fruit Crops

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ABSTRACT (Academic)

1.) Sentinel traps were used to survey for parasitoids of frugivorous drosophilids in Virginia fruit cropping systems, and determine if parasitoids were attacking invasive flies *Drosophila suzukii* (Matsumura) and *Zaprionus indianus* Gupta (Diptera: Drosophilidae) in the field. Two parasitoids of frugivorous drosophilids, *Leptopilina bouvardi* (Barbotin, Carton, and Kelner-Pillault) (Hymenoptera: Figitidae) and *Pachycrepoideus vindemiae* (Rondani) (Hymenoptera: Pteromalidae), were reared, but only one *P. vindemiae* was reared from *D. suzukii*, and no parasitoids were reared from *Z. indianus*. Most parasitoids were reared from alternate host *Drosophila melanogaster* Meigen and other wild drosophilids. 2.) The ability of these parasitoids to attack *D. melanogaster*, *D. suzukii* and *Z. indianus* under controlled conditions was tested. Larval parasitoid *L. bouvardi* did not develop on *D. suzukii* or *Z. indianus*, just *D. melanogaster*. Pupal parasitoid *P. vindemiae* successfully developed on all three fly species, but also increased pupal fly mortality. 3.) Olfactometry was used to ascertain if *L. bouvardi* and *P. vindemiae* are selective about the type of fruit their hosts feed in. Results showed that among cherry, raspberry, blueberry, grape, and banana, *L. bouvardi* preferred raspberry and banana to cherry, and preferred grape least, but no fruit was most preferred. Insufficient data were obtained for *P. vindemiae*.

We conclude that parasitoids of Virginia are unlikely to provide effective biological control for *D. suzukii* or *Z. indianus*, and classical biological control should be investigated as a pest management option. Olfactometry results indicate tritrophic selectivity by *Drosophila* parasitoids, suggesting multiple parasitoids could be required for effective biological control.

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ABSTRACT (Public)

The spotted wing drosophila (henceforth SWD) is a globally invasive vinegar fly originating from southeast Asia, and is economically damaging to producers of small fruit, such as berries, cherries, and grapes. The African fig fly (henceforth AFF), a relative of SWD, is another recently invasive species to North America (originating from Africa) that often occurs simultaneously with SWD infestations, but its economic threat to North America is still unclear. With the economic threat posed by SWD, and potential threat posed by AFF, it is important to understand their relationships with other organisms in their environment, especially natural enemies, such as parasitoid wasps. Such information is integral for researchers to develop effective control methods, and will help determine if natural enemies can be used to our advantage as biological control agents. Biological control also helps to limit the use of chemical insecticides, mitigating the development of insecticide resistance in the pests.

This project employed unique field trapping methods and laboratory bioassays to investigate the relationships of SWD and AFF with parasitoid wasps in affected fruit cropping systems in southwestern Virginia. We discovered that parasitoids of vinegar flies are present in Virginia fruit cropping systems, but they do not help to control populations of SWD and AFF. The parasitoids that are present prefer to attack other fly species, are unable to attack SWD and AFF, or do not attack in high enough numbers to have an impact on SWD or AFF populations. Biological control success is more likely to come from parasitoid species that have co-evolved with SWD and AFF in their native ranges.

For my wife, Tessali.

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Preface

This project came into being after I summoned the courage to contact Loke Kok, the Head of the Virginia Tech Department of Entomology, about a possible graduate program involving invasive species and biological control, two of my professional interests. After reviewing my credentials, he told me, “Please apply for admission to graduate studies in our department as soon as possible. I will find funding for you.” (Luckily for me, there was a certain little fly that had begun wreaking havoc in small fruit production.) Thus started my experience of being a graduate student in Virginia Tech’s entomology program, which has been more rewarding and fulfilling than I had imagined. This was largely due to an atmosphere of camaraderie and mutual support that has made Price Hall not just a workplace, but also a second home. It has made the arduous process of graduate research and writing a thesis more bearable. I also could not have written this thesis alone, and I must again acknowledge my advisor, Doug Pfeiffer, and my graduate committee members, Scott Salom and Jayesh Samtani, for helping me along with this process. You will see their names as co-authors of some of the following chapters, because their comments, suggestions, and support were vital to the production of this manuscript.

– James C. E. Wahls

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Chapter 1

Introduction and Literature Review

James C. E. Wahls

Introduction

In 2008, a seemingly insignificant relative of the common vinegar fly (*Drosophila melanogaster* Meigen, Diptera: Drosophilidae) was discovered infesting some berries in California, and by 2009, it had spread into British Columbia. Initially thought to be inconsequential, the insect quickly demonstrated that it was no ordinary vinegar fly. This fly, characterized by one black spot on each wing of the male, was also discovered in Florida in 2009, and it was becoming a real problem on many different fruit crops (Hauser 2011). At this point, the fly had been recognized as *Drosophila suzukii* (Matsumura), now commonly known as spotted wing drosophila (SWD). Since 2009, SWD has become widespread throughout North America, and causes significant economic damage in fruit cropping systems such as cherries, caneberries, blueberries, and wine grapes (Walsh et al. 2011, Asplen et al. 2015).

In 2012, another unusual drosophilid, characterized by striking white longitudinal stripes, was observed in Virginia. This fly, *Zaprionus indianus* Gupta (Diptera: Drosophilidae), or the African fig fly (AFF), was often observed concurrently with SWD infestations in wine grapes (Pfeiffer 2012, Pfeiffer et al. 2012). AFF is a cosmopolitan tropical fruit pest that appeared in North America (FL) in 2005 (Steck 2005, van der Linde et al. 2006), and now appears to have some measure of competition with SWD in Virginia vineyards (Shrader, *unpublished data*).

SWD and potentially AFF can be very damaging for small fruit producers, and developing an IPM program for these species is a high priority. A commonly useful component of IPM is biological control using insect parasitoids. A plentiful parasitoid community is associated with SWD in its native range (Mitsui et al. 2007), and adaptive parasitoid species have been documented attacking both pest species in invaded regions (Marchiori et al. 2003,

Marchiori and Silva 2003, Silva et al. 2004, Rossi Stacconi et al. 2013, Rossi Stacconi et al. 2015).

The research described in this study examined the relationship between native hymenopteran parasitoids of Drosophilidae and the invasive drosophilids, SWD and AFF, in Virginia fruit cropping systems. The results of this study will help determine if conservation biological control will be a useful tactic against these pests in Virginia, and provide baseline information for future research into biological control of SWD and AFF.

Literature Review

Spotted Wing Drosophila. *Background.* SWD originates from Southeast Asia (Cini et al. 2012), but has become a global pest, arriving in both Europe and North America in 2008 (Hauser 2011, Calabria et al. 2012, Cini et al. 2012), and South America in 2013 (Deprá et al. 2014). The common name derives from the male of the species, which has a distinct black spot at the outside edge of each wing (Fig. 1.1). The female has no wing spots, but is distinguished by a large, serrate, sclerotized ovipositor, a unique characteristic among drosophilids (Walsh et al. 2011, Cini et al. 2012) (Fig 1.2). This structure allows the female to cut into the skin of ripe and ripening fruits and deposit her eggs within. SWD ovipositing and larval feeding damage fruit and create pathways for pathogens (Hamby et al. 2012, Ioratti et al. 2015), making fruit unmarketable. As a result, SWD can cause large amounts of crop damage and economic loss if populations are not kept in check (Bolda et al. 2010, Goodhue et al. 2011, Lee et al. 2011, Walsh et al. 2011).

SWD is known to oviposit on ripe and ripening fruits of caneberries, strawberries, blueberries, grapes, cherries, and others (Lee et al. 2011, Walsh et al. 2011). Assuming a 20%

yield loss from SWD on strawberries, blueberries, raspberries, blackberries, and cherries, Bolda et al. (2010) estimated that economic losses could be as high as \$511 million for the states of California, Oregon, and Washington. However, Goodhue et al. (2011) performed a more in-depth economic analysis with respect to SWD infestations of California strawberries and raspberries. The study accounted for increased prices due to yield loss, examined the benefits of management programs, and demonstrated that economic losses could be mitigated when effective management practices are in place (Goodhue et al. 2011).

Control Options. Research into chemical control of SWD is ongoing. After extensive laboratory bioassays and field trials, Bruck et al. (2011) showed that most pyrethroids, organophosphates, and spinosyns tested were effective at controlling SWD, providing 5-14 days of residual control in the field. However, these tests were performed along the west coast of the U.S., and different environmental conditions on the east coast may yield different results. As an alternative to chemical sprays, mass trapping and attract-and-kill strategies are also being explored. For example, experimental traps in blueberry fields have been shown to reduce infestation in berries distant from traps, so traps placed in a perimeter outside the growing area could reduce the number of flies entering the field (Hampton et al. 2014).

The traps used by Hampton et al. (2014) were red cups baited with a mixture of water, apple cider vinegar, yeast, and wheat flour, but trapping methods for drosophilids vary. Apple cider vinegar, cornmeal-yeast baits and mixtures of ethanol, acetic acid, and 2-phenylethanol are attractive to drosophilids, but may not be very selective (Hutner et al. 1937, Reed 1938, Zhu et al. 2003, Landolt et al. 2012, Lee et al. 2012). Apple cider vinegar, red wine, and yeasts are commonly used for trapping SWD specifically (Beers et al. 2011, Landolt et al. 2012, Lee et al. 2012), but baits using a mixture of apple cider vinegar and red wine are shown to be more

selective for SWD than using either product by itself (Landolt et al. 2012). Cha et al. (2012, 2014) have developed even more effective synthetic lures, based on SWD antennal reactions to headspace volatiles of Merlot wine and rice vinegar. Containers for *Drosophila* traps also vary, but are usually made up of modified plastic cups or containers, and dome traps (Landolt et al. 2012, Lee et al. 2012). Furthermore, in order to improve trapping techniques for SWD, Rice et al. (2016) investigated relative attractiveness of trap color, shape, and size, discovering that while shape appears irrelevant, SWD is most attracted to red or black spheres over other colored spheres, and larger sphere traps caught more SWD than smaller traps. In addition to mass trapping techniques and chemical sprays, forms of non-lethal chemical control are also being investigated, such as chemical deterrents (Wallingford et al. 2015), or edible coatings to impede oviposition (Swoboda-Bhattarai and Burrack 2014).

Although many chemical control options show promise, it is important to minimize the use of insecticides with an IPM program in order to reduce the cost of control, as well as the development of insecticide resistance. This is particularly important for a species like SWD, which has a short generation time and high fecundity—characteristics that predispose an insect species to quickly develop insecticide resistance. In order to develop an effective IPM program, all non-chemical control options must be explored. Some researchers are evaluating cultural and physical control measures for SWD, such as planting early-ripening blueberry cultivars to escape injury (Hampton et al. 2014), or planting raspberries within physical barriers such as high tunnels (Rogers et al. 2016). Additionally, another potentially important aspect of IPM is using insect parasitoids for biological control.

Biological Control. Research into parasitoids of SWD is progressing, and many parasitic wasps of SWD have already been identified. In Japan, larval parasitoids *Asobara japonica*

Belokobylskij and *A. tabida* (Nees) (Hymenoptera: Braconidae), and *Ganaspis* spp. (Hymenoptera: Figitidae) were reported to parasitize SWD, with *Ganaspis* showing the highest rate of parasitization (Mitsui et al. 2007, Mitsui and Kimura 2010, Kasuya et al. 2013). Ideo et al. (2008) also demonstrated that *A. japonica* successfully parasitized SWD in the laboratory. More recently, five additional species of *Asobara* have been reported to successfully attack SWD in the laboratory (Nomano et al. 2014). One of these species, as yet unidentified (documented as *Asobara* sp. TK1), may specialize on SWD because it has only been recorded from SWD in the field, making it a promising candidate for classical biological control (Nomano et al. 2014, Asplen et al. 2015), along with a “*suzukii*-associated” type of *Ganaspis xanthopoda* (Ashmead) (Kasuya et al. 2013, Nomano et al. 2014, Asplen et al. 2015). Exploration in South Korea and quarantine studies further supported *A. japonica* and *G. xanthopoda* as possible biological control candidates, and added *Leptopilina japonica japonica* Novković & Kimura (Hymenoptera: Figitidae) to the list. Other species of the genus *Leptopilina*, and pupal parasitoid *Trichopria drosophilae* (Perkins) (Hymenoptera: Diapriidae) are also reported to parasitize SWD in South Korea, Japan and Europe (Novković et al. 2011, Cini et al. 2012, Gabarra et al. 2015, Rossi Stacconi et al. 2015, Daane et al. 2016). In Europe, Oregon, and South Korea the cosmopolitan, generalist pupal parasitoid *Pachycrepoideus vindemiae* (Rondani) (Hymenoptera: Pteromalidae) has been reported to emerge from SWD pupae in the field (Rossi Stacconi et al. 2013, Gabarra et al. 2015, Rossi Stacconi et al. 2015, Daane et al. 2016, Mazzetto et al. 2016). Additionally, laboratory studies with European populations of *P. vindemiae* resulted in parasitization rates of up to 80% on SWD (Chabert et al. 2012, Rossi Stacconi et al. 2013, Asplen et al. 2015, Gabarra et al. 2015, Rossi Stacconi et al. 2015), raising the prospect of using *P. vindemiae* for augmentative biological control.

Augmentative biological control includes two general approaches: inoculative release and inundative release (Stinner 1977, Eilenberg et al. 2001). Inoculative release is when a relatively small number of biological control agents are released in intervals, generally starting early in the pest season when populations are low. The goal is to let the control agent's population build up naturally, and help suppress the pest population over a long-term period, so that the pest population does not reach economic injury levels. This is a preventive measure. Conversely, inundative release is a corrective measure, where a large number of biological control agents are released at one time, with the goal of overwhelming and immediately reducing the target pest population in the affected area (Stinner 1977, Eilenberg et al. 2001). Camacho (1998) described a successful case of augmentative biological control using *P. vindemiae* in an IPM program for Mediterranean fruit fly (*Ceratitis capitata* Weidemann, Diptera: Tephritidae) in Acosta County, Costa Rica. The methods described indicate inoculative releases, where 6,000 parasitoids per week per hectare were released during "the beginning of the program," length of time unspecified, as a preventive strategy. Additionally, these releases were in conjunction with the release of sterile flies (Camacho 1998). However successful this approach was, Guillén et al. (2002) expressed concern about non-target effects from using *P. vindemiae*, because *P. vindemiae* is hyperparasitic as well as generalist in nature. Wang et al. (2016) also studied interactions between *P. vindemiae* and *T. drosophilae*, another pupal parasitoid of SWD. When the two parasitoid species were present together, the study reported a negative effect by *P. vindemiae* on host suppression (Wang et al. 2016). Based on such literature, *P. vindemiae* may be a less appropriate candidate for SWD biological control, but further research is needed.

No records of SWD parasitoids have yet been published from eastern North America, although various parasitic wasps attacking frugivorous drosophilids have been reported from the

region, including *A. tabida*, *Ganaspis mellipes* (Say), *Leptopilina bouvardi* (Barbotin, Carton & Kelner-Pillault) and *L. heterotoma* (Thomson) (Carton et al. 1986). “Frugivorous” drosophilids are those whose larvae develop in fruit, such as SWD, *D. melanogaster*, *Drosophila simulans* Sturtevant, and many *Zaprionus* species, including AFF (Mitsui and Kimura 2010, Yassin and David 2010). These are distinguished from “fungivorous” drosophilids, whose larvae feed in fungi, such as *Drosophila phalerata* Hartig (Pannebakker et al. 2008).

African Fig Fly. AFF is a tropical to sup-tropical drosophilid species that originates from Africa and Asia, but it has become established in South America, and more recently North America. The species is easily identified by characteristic white longitudinal stripes on the head and thorax (Fig. 1.3). The first report of AFF in the Americas came from Brazil in 1999 (Vilela 1999). Since then it has spread through much of tropical and sub-tropical South America (Santos et al. 2003, Araripe et al. 2004, David et al. 2006), becoming a major competitor with native and introduced drosophilids (Tidon 2003, da Silva et al. 2005, Ferreira and Tidon 2005). It has also become an important pest on figs in S. America (Oliveira et al. 2013). AFF was found in Florida in 2005 (Steck 2005, van der Linde et al. 2006), and genetic analysis indicates the Florida population is more closely related to an African population than the South American population (Yassin et al. 2008), indicating a separate introduction rather than a range expansion from South America. AFF was also discovered in the western USA the following year, in 2006, which may have been a range expansion from Mexico (Castrezana 2007). In Virginia, AFF appeared in 2012 and has been observed on many fruit crops including grapes, caneberries, apples, and tomatoes (Pfeiffer 2012, Pfeiffer et al. 2012, personal observation).

AFF has not noticeably become a pest in North America. It seems unable to survive cold winters, as it only appears later in the field season in Virginia, indicating that it travels up from

the warmer southern states every year. This is supported by a study from South America, which indicates AFF has a limited range due to the more temperate climates of higher latitudes, because it is only present in Uruguay during warmer months (David et al. 2006). Furthermore, Araripe et al. (2004) demonstrated that AFF males become infertile at lower growth temperatures (14°C), which is possibly a reason for the range restriction. Because AFF arrives late in the growing season of temperate areas, only late-season fruit crops may be vulnerable to AFF infestation. In addition to environmental limitations, AFF has a major physical limitation that prevents it from becoming a major pest. AFF is unable to oviposit in most intact fruit because, unlike SWD, its ovipositor is small and unsclerotized (Fig. 1.4). It is only able to attack intact figs because it can gain direct access to the flesh through the ostiole (Raga et al. 2003). These limitations make AFF a comparatively minor economic pest, especially when compared with SWD. The economic impact of AFF in North America is unclear, but Oliveira et al. (2013) report < US \$1 million economic losses due to AFF in Brazil, while SWD can potentially cause estimated economic losses of hundreds of millions of US \$, in the western US alone (Goodhue et al. 2011, Walsh et al. 2011).

Although AFF is a comparatively minor pest, it is a highly adaptable species, having demonstrated significant climatic niche shifts in India and America from its native range in Africa (da Mata et al. 2010), and it has been observed feeding on a wide variety of fruits in Africa, South America, and Florida (Vilela 1999, Santos et al. 2003, van der Linde et al. 2006). Although AFF does not have the ability to cut into intact fruit like SWD, recent research indicates AFF can take advantage of SWD oviposition wounds and infest fruit that way (Shrader, *unpublished data*). Additionally, AFF, along with other *Zaprionus* species, are known to be highly competitive and could potentially outcompete native drosophilid species if sharing the

same hosts (Gilpin et al. 1986, Tidon 2003, da Silva et al. 2005, Ferreira and Tidon 2005, Galego and Carareto 2005). Therefore, AFF has the potential to become an important pest in the United States, making it wise to research potential control options for this species, including biological control. Various hymenopteran parasitoids have already been documented parasitizing AFF in Brazil, including *L. boulandi*, *P. vindemiae*, and *Spalangia endius* Walker (Pteromalidae) (Marchiori et al. 2003, Marchiori and Silva 2003, Silva et al. 2004). A goal of this study is to determine if *Drosophila* parasitoids in Virginia can parasitize AFF, and with what success.

Challenges Facing Biological Control. Host Defenses. There are some challenges facing biological control with parasitic wasps, especially for SWD. One such challenge is immunological defenses. Studies have demonstrated that SWD is highly resistant to parasitization by many *Drosophila*-parasitic wasps, as compared with its relative *D. melanogaster* (Kacsoh and Schlenke 2012, Poyet et al. 2013). They show that SWD has more success encapsulating eggs and larvae of larval parasitoids because it produces considerably more hemocytes than *D. melanogaster* (Kacsoh and Schlenke 2012, Poyet et al. 2013). Furthermore, SWD may be even more resistant to parasitization in invaded regions. Native parasitoids have not evolved sympatrically with SWD, and so have not developed the ability to overcome SWD defenses, as demonstrated by Chabert et al. (2012). Additionally, populations of SWD in Europe exhibit even greater levels of hemocytes than SWD populations in the native range (Poyet et al. 2013)

Another difficulty facing biological control with parasitoids is behavioral modification of the host in order to avoid parasitization. For example, Kacsoh et al. (2013) showed that, in response to seeing larval parasitoid wasps, *D. melanogaster* and other *Drosophila* species began laying eggs in alcohol-rich food sources, which deter wasps, protecting hatched larvae from

infection. If such behavioral modifications evolved in other *Drosophila* species, it is possible that similar behavioral responses could have developed in SWD or AFF as well. However, in the case of SWD, one wonders if such an effective immune response would have made behavioral modification unnecessary.

Agricultural Practices. The use of insecticides in agroecosystems is another challenge. Insecticides can adversely affect insect predators and parasitoids, and hymenopteran parasitoids generally appear to be among the most susceptible, showing high levels of mortality and sterility (Theiling and Croft 1988, Cònsoli et al. 1998, Williams et al. 2003, Biondi et al. 2012). Spinosyns are used to control SWD, and this class is toxic to some hymenopteran parasitoids (Williams et al. 2003, Biondi et al. 2012). A review by Biondi et al. (2012) examined studies evaluating effects of spinosyns on beneficial arthropods, and 100% of reviewed laboratory studies on Hymenoptera showed positive lethal or sublethal effects. As a result of chemical toxicity to hymenopteran parasitoids, biological control agents may be less effective within non-organic, high-production systems that have to use insecticides for a variety of pests. The same may also be the case for some organic systems, because they may use organic insecticides, such as spinosad (a spinosyn). However, SWD has demonstrated a high range of alternate wild hosts (Lee et al. 2015), so biological control would be very important for wild ecosystems, or refugia, and could help reduce the number of individuals entering agricultural systems. Landscape surrounding an agricultural site can also influence the success of natural enemies, and natural biological control in agricultural systems can be more successful if the surrounding landscape is minimally disturbed, and structurally diverse and complex (Thies and Tschardtke 1999, Landis et al. 2000).

Tritrophic Interactions. Several studies have demonstrated that differing host crops, i.e.

host diets, may influence the parasitoids that attack a particular pest. The type of host diet may affect which parasitoid species will attack the host pest, and also the rate at which a particular parasitoid parasitizes the pest, because different parasitoids may prefer different plant volatiles released by pest feeding (Johnson and Hara 1987, van den Berg et al. 1990, Hoballah et al. 2002). It has been well established that plant volatiles, especially those released by host feeding, are important host-finding cues for parasitoids (Price et al. 1980, Powell and Zhi-Li 1983, Dicke et al. 1990, Turlings et al. 1991, Geervliet et al. 1994, Du et al. 1996, Takabayashi et al. 1998, Verheggen et al. 2008). This raises important questions: will parasitoids, which attack a pest with many different host crops, cue in on volatiles released by all the host crops or just certain ones? Additionally, if given a choice, will parasitoids orient toward certain crop types more often than others? In the case of *Cotesia marginiventris* (Cresson) (Hymenoptera: Braconidae), a parasitoid of Lepidoptera larvae, Hoballah et al. (2002) showed it preferred cowpea odors to maize odors, and even preferred odors of one maize variety to seven other maize varieties. Studies have also shown that host diet can have significant developmental effects on a parasitoid. For example, Eben et al. (2000) demonstrated significant host diet effects on size, longevity, fecundity, and sex ratio of *Diachasmimorpha longicaudata* (Ashmead) (Hymenoptera: Braconidae), a parasitoid of Tephritidae (Diptera). Therefore, it would be in a parasitoid's best interest to orient toward volatiles released by host feeding on a diet that would produce the fittest parasitoid offspring. This could explain why different parasitoid communities have been associated with different cropping systems affected by the same host pest (Johnson and Hara 1987, van den Berg et al. 1990).

Tritrophic interactions will be important for biological control practices in which parasitoids are mass released, because it may take several different parasitoid species to cover all

the different host crops of SWD or AFF. Moreover, it will be especially important for classical biological control. Government regulations require that introduced biological control agents be very host specific, which greatly limits the number of potential candidate species for introduction. Thus far, only a few parasitoid species of SWD, such as *Asobara* sp. TK1, *A. japonica*, *G. xanthopoda*, and *L. j. japonica*, have been discovered that could possibly meet the requirements, and those species remain largely unstudied (Kasuya et al. 2013, Nomano et al. 2014, Asplen et al. 2015, Daane et al. 2016). It is very possible that they will not attack SWD in each host plant.

The above challenges must be further investigated so that the most effective biological control program can be developed for SWD, and possibly AFF. The purpose of this project is not only to identify *Drosophila* parasitoids occurring in Virginia fruit cropping systems, but also to examine some of these challenges and provide baseline research for future biological control studies of SWD and AFF.

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Figures



Fig. 1.1. Adult male spotted wing drosophila (*Drosophila sukii*) exhibiting characteristic wing spots.



Fig. 1.2. Ovipositor of adult female spotted wing drosophila (*D. sukii*).



Fig. 1.3. Adult African fig fly (*Zaprionus indianus*) exhibiting characteristic white stripes.

Image: D. G. Pfeiffer

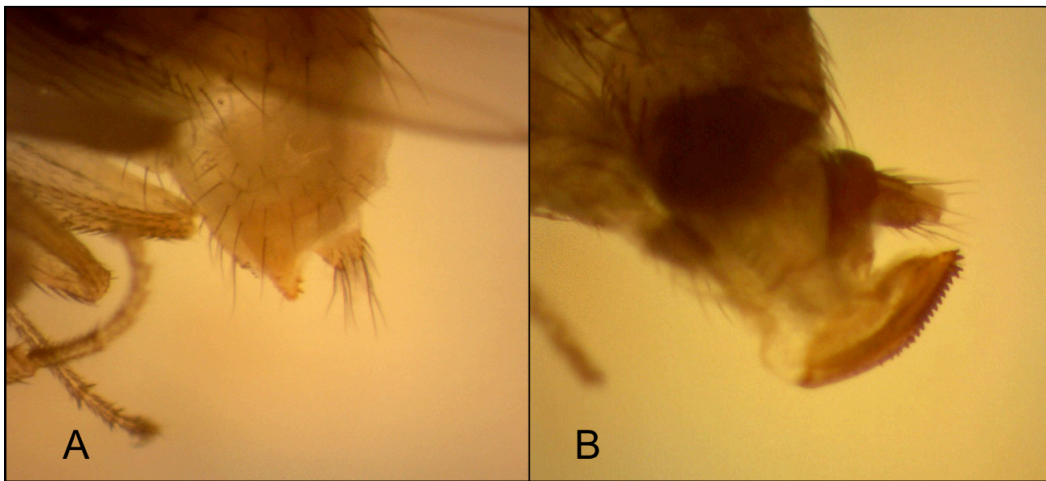


Fig. 1.4. Comparison of ovipositors of African fig fly (A) and spotted wing drosophila (B).

Notice the small size and lack of sclerotization and serration of AFF ovipositor. *Images: D. G.*

Pfeiffer

Chapter 2

Sentinel trapping for parasitoids (Hymenoptera) of exotic drosophilids in Virginia fruit cropping systems

James C. E. Wahls, Scott M. Salom, Jayesh B. Samtani and Douglas G. Pfeiffer

ABSTRACT: During the 2015 field season, sentinel traps were used to sample for hymenopteran parasitoids of frugivorous drosophilids in fruit cropping systems of southwestern Virginia. Sampled cropping systems included cherry, caneberry, blueberry, and grape. Traps were baited with banana or fruit corresponding the cropping system, and seeded with one of three frugivorous fly species (Diptera: Drosophilidae): *Drosophila melanogaster* Meigen, *D. suzukii* (Matsumura), or *Zaprionus indianus* Gupta. Only traps in the cherry orchard and caneberry field yielded parasitoids. From the cherry orchard, 674 *Leptopilina bouvardi* (Barbotin, Carton, and Kelner-Pillault) (Hymenoptera: Figitidae) were reared from *D. melanogaster* and other drosophilids that contaminated traps, 61 *Pachycrepoideus vindemiae* (Rondani) (Hymenoptera: Pteromalidae) were reared from *D. melanogaster*, and 1 *P. vindemiae* emerged from *D. suzukii*. Most *L. bouvardi* and all *P. vindemiae* were reared from banana-baited traps. From the caneberry field, 207 *Leptopilina clavipes* (Hartig) were reared solely from caneberry-baited traps, and likely developed on other contaminating drosophilids. In the cherry orchard and caneberry field, traps placed on the edge of the production-area tended to produce more parasitoids than interior traps. These results suggest that parasitization of exotic drosophilids, *D. suzukii* and *Z. indianus*, is negligible in southwestern Virginia fruit cropping systems, but trends in the data raise interesting questions about parasitoid behavior that could apply to biological control efforts.

KEY WORDS: invasive species, biological control, parasitoid, *Drosophila*, sentinel trap

The spotted wing drosophila, *Drosophila suzukii* (Matsumura) (Diptera: Drosophilidae), is a globally invasive pest of soft-skinned fruits, originating from Southeast Asia (Bolda et al. 2010, Hauser 2011, Cini et al. 2012, Deprá et al. 2014, Asplen et al. 2015). In 2008, spotted wing drosophila, henceforth SWD, first appeared in both N. America (CA) and Europe (Bolda et al. 2010, Hauser 2011, Calabria et al. 2012, Cini et al. 2012). In 2009, SWD spread up the west coast of the USA and was also detected in Florida (Hauser 2011). By 2011, SWD had spread to Virginia (Pfeiffer 2012, Pfeiffer et al. 2012), and is now widespread throughout the continental USA and temperate parts of Canada (Asplen et al. 2012). SWD was also discovered in Brazil in 2014 (Deprá et al. 2014). With a wide host range, high fecundity, short life cycle, and multivoltine life history, SWD is a major economic pest of many fruit cropping systems throughout the growing season (Bolda et al. 2010, Goodhue et al. 2011, Ioratti et al. 2015). SWD females use large, hardened, serrated ovipositors to cut into intact, ripe fruit where they deposit eggs, and the larvae then consume the flesh of the fruit (Hauser 2011). Affected crops include mainly cherries, strawberries, caneberries, blueberries, and grapes (Bolda et al. 2010, Goodhue et al. 2011, Asplen et al. 2015, Ioratti et al. 2015).

Due to the damage potential of SWD, it is necessary to formulate an effective integrated pest management (IPM) program for this pest. As a component of IPM, the use of biological control should be explored. Investigations of SWD parasitoids in invaded regions have discovered two species that can successfully parasitize SWD in the field: the generalist pupal parasitoid and hyperparasitoid *Pachycrepoideus vindemiae* (Rondani) (Hymenoptera: Pteromalidae), and the drosophilid-specific pupal parasitoid *Trichopria drosophilae* Perkins (Hymenoptera: Diapriidae) (Chabert et al. 2012, Rossi Stacconi et al. 2013). Both species are cosmopolitan, and have been discovered attacking SWD in western N. America, Europe, and

South Korea, but estimated parasitization rates in the field have been too low for adequate population control (Chabert et al. 2012, Rossi Stacconi et al. 2013, Daane et al. 2016, Wang et al. 2016b). No information on host-parasitoid interactions of SWD in eastern N. America has yet been published.

Another drosophilid that has recently invaded the Americas is the African fig fly, *Zaprionus indianus* Gupta, henceforth AFF. AFF originates from Africa and Eurasia, and has been invasive in S. America since 1999 (Vilela 1999, Santos et al. 2003, van der Linde et al. 2006). The species has a wide host range, but is known for being an economic pest of figs (Raga et al. 2003, van der Linde et al. 2006, Oliveira et al. 2013). AFF was first detected in N. America (FL) in 2005 (Steck 2005, van der Linde et al. 2006), and it was recorded in Virginia in 2012 (Pfeiffer 2012, Pfeiffer et al. 2012). AFF is reported to be intolerant of cold temperatures (Araripe et al. 2004, David et al. 2006), so it likely only survives year-round in the more subtropical regions of N. America (e.g. FL, TX, Mex.), then re-invades the more temperate regions every growing season. In support of this, AFF only appears in VA later in the growing season (Pfeiffer 2012, Pfeiffer et al. 2012). While AFF has not emerged as a significant pest in N. America, it is reported to be very adaptable and very competitive (Tidon 2003, da Silva et al. 2005, Ferreira and Tidon 2005, Galego et al. 2005, da Mata et al. 2010), and therefore threatens native drosophilid communities. Still, little is known about the ecology and impacts of AFF in N. America. In Virginia, AFF often appears in tandem with late-season SWD infestations of fruit crops (Pfeiffer 2012, Pfeiffer et al. 2012). This co-occurrence, combined with a lack of information and potential ecological threat, warranted the inclusion of AFF in this study.

The main objectives of this study were to determine which parasitoids of drosophilids are present in Virginia fruit cropping systems, and if parasitoids are successfully parasitizing *D.*

suzukii or *Z. indianus* in the field. As secondary objectives, we aimed to determine if trap placement (edge vs. interior) and type of fruit bait would affect the number of parasitoids reared from traps, or which species of parasitoids were reared from traps.

Materials and Methods

Sentinel Traps. *Insects.* Species used in these experiments included *Drosophila melanogaster*, *D. suzukii* (SWD), and *Z. indianus* (AFF). The laboratory colony of *D. melanogaster* was acquired from stock colonies maintained in the Departments of Entomology and Biological Sciences at Virginia Tech. Colonies of SWD and AFF were raised from individuals collected in southwestern Virginia. Flies were maintained on a molasses-based diet formula (Nutri-Fly™ MF, Genesee Scientific Corp., San Diego, CA) in 178-ml, square-bottom polypropylene drosophila stock bottles (Genesee Scientific Corp., San Diego, CA). Colonies were reared in an environmental chamber at 23.3° C and 14 h day length (18 W “cool white” fluorescent bulbs).

Trap Design. Sentinel traps (Fig. 2.1) were created using 1.4 L plastic deli containers. An opening of about 5 × 4 cm was cut into both the front and back of each container for odor dispersal and insect access. Fifty-two smaller access holes of 0.5 cm were also cut into all sides of each container, and were placed symmetrically so that opposite sides had the same number and distribution. Each container was inlaid with ~2 mm mesh aluminum screening to exclude larger insects. To minimize desiccation within the traps, the container lids were painted with an undercoat of black for shade and a topcoat of white for sunlight reflection. Each trap was also outfitted with a string for hanging in the field. Placed within each trap was a 9-cm Petri dish, which would hold the bait. The bait for each trap was ~50 g of fruit infested with larvae of one of

the three fly species. Fruit used in the bait was either the same crop as produced by the cropping system (see “Experimental Design”) or banana. Banana was used as the alternate fruit type because banana is common bait used for drosophilids and their parasitoids (Carson 1951, Carson and Stalker 1951, McKenzie 1974, Allemand et al. 2002, Mitsui et al. 2007, Rossi Stacconi et al. 2013).

Bait preparation. Five to seven days prior to setting traps, adult flies of the species to be used for bait were transferred into fresh rearing bottles with new food media. The bottles were placed in the environmental chamber to allow flies to reproduce. On the day of trap placement, fresh fruit to be used in the bait was purchased from the local supermarket. Fruit was rinsed with water before use. For each trap to be set, ~50 g of fruit was measured out and placed in a Petri dish, then sliced and macerated so that it fit into the dish with the lid on (the lid had to be on during transportation). Once the fruit was allocated to the dishes, larvae were harvested from the aforementioned rearing bottles. Larvae were collected from a bottle by filling it with ~3 cm of lukewarm water to encourage larvae to come to the top of the food media, swirling the bottle to get the larvae up in the water column, and then dumping it over a fine mesh net to strain out the larvae. Larvae were then scooped from the net and placed into one of the Petri dishes with fruit, so that each bait dish ended up with an estimated 100-200 larvae, ranging from 1st-3rd instar. For *D. melanogaster* and AFF, 1 bottle usually sufficed for 4 dishes. For SWD, 1 bottle was usually enough for 2 dishes. Once the baits were completed, the dishes were capped, labeled, and transported to the field where they were placed in a trap.

Experimental Design. During the 2015 field season, sentinel traps were placed in four different fruit cropping systems: cherry, caneberry, blueberry, and grape (Table 2.1). These systems were chosen because they are the most affected by SWD in the local region (Pfeiffer

unpublished data). The cherry orchard was located in Patrick Co., the caneberry field was in Montgomery Co., the blueberry plantation was in Giles Co., and the two vineyards were in Montgomery Co. and Amherst Co. At the beginning of trapping, only *D. melanogaster* and SWD were used for baits. AFF is only naturally present in Virginia during the late harvest season (Pfeiffer 2012, Pfeiffer et al. 2012), so baits containing AFF larvae were not deployed until AFF had appeared in the area. *Drosophila melanogaster* was chosen as an alternate host species because it is naturally occurring in southwestern Virginia, is closely related to SWD, and is known to be more susceptible to parasitization (Kopp and True 2002, Kacsoh and Schlenke 2012). Therefore, if local parasitoids are unsuccessfully attacking SWD, the same species might successfully attack *D. melanogaster* and still develop from the sentinel traps.

Trapping surveys in each cropping system were considered separate experiments. As such, each survey was a $3 \times 2 \times 2$ factorial experiment. Infesting fly species was one factor, with 3 levels: *D. melanogaster*, SWD, and a control with no flies. Fruit type was the second factor, with 2 levels: banana and corresponding fruit crop (e.g. sweet cherry for cherry orchard, mix of raspberries and blackberries for caneberry field, blueberries for blueberry plantation, or black table grapes for vineyard). Trap placement was the last factor, where the 2 levels were field edge and interior. Therefore, 12 traps were placed in each cropping system, with 6 traps on the field edge and 6 on the field interior. Each group of six traps contained every possible fruit/fly combination. Traps were placed ≥ 20 m apart in random order. Each trapping session lasted 3-4 d, and 6-7 trapping sessions were completed in each cropping system, so that 21-24 trapping days were accumulated for each experiment.

For the last two trapping sessions in caneberry, blueberry, and grape cropping systems, four additional traps containing baits with AFF were included, and were distributed to account

for the experimental factors described above. These traps were not included in the cherry orchard because cherry is an early season crop, while AFF only occurs during the late season in Virginia. Additionally, because traps infested with AFF were only out for two trapping sessions, results from those traps were analyzed separately from the traps containing SWD and *D. melanogaster*.

At the end of each trapping session, Petri dishes were collected from traps and returned to the laboratory, where they were individually enclosed within rearing containers, and insects were allowed to complete development. Rearing containers were created from 1 L plastic deli cups, and the lids were modified with a hole covered in cloth to allow for airflow but prevent escapes. Rearing containers were monitored for fly and parasitoid emergence 2-3 times per week, for 1 month after collection. All flies and parasitoids emerged within 1 month. Emerged insects were collected, preserved in 70% ethanol, and counted. Samples of parasitoid specimens were sent away for professional identification. Data were analyzed using descriptive statistics.

Results

Cherry Orchard. Of the six sentinel-trapping sessions in the cherry orchard, the last three sessions (date range 6/8–6/26) produced parasitoids. Two parasitoid species were reared: the larval endoparasitoid *Leptopilina boulardi* (Barbotin, Carton and Kelner-Pillault) (Hymenoptera: Figitidae), and the pupal ectoparasitoid *Pachycrepoideus vindemiae* (Rondani) (Hymenoptera: Pteromalidae), which only emerged from the 4th trapping session. Additionally, it was not unusual for traps, including control traps, to produce adult drosophilids that were not initially infesting the bait, indicating that wild flies were contaminating the traps. Therefore, the host on which the parasitoids developed was sometimes difficult to distinguish.

A total of 674 *L. bouleardi* and 62 *P. vindemiae* were reared from sentinel traps. All individuals of *L. bouleardi* were reared from either *D. melanogaster* or ‘other’ drosophilids that contaminated the traps. ‘Other’ drosophilids were defined as any drosophilids other than SWD, AFF, or *D. melanogaster*. One *P. vindemiae* was reared from SWD and all other *P. vindemiae* were reared from *D. melanogaster*. Most *L. bouleardi* and all *P. vindemiae* were reared from traps baited with banana (Fig. 2.2), and most parasitoids of both species were reared from traps placed on the edge of the orchard (Fig. 2.3). However, because a large proportion of traps produced no parasitoids, only descriptive statistics could be used to interpret the data.

Caneberry Field. Only the first three trapping sessions (date range 7/6–7/26) in the caneberry field yielded parasitoids, and only one species emerged: the larval parasitoid *Leptopilina clavipes* (Hartig). A total of 207 parasitoids emerged, and only from traps baited with caneberry (Fig. 2.2). Most *L. clavipes* were reared from traps placed on the edge of the field (Fig. 2.3). Additionally, these parasitoids only emerged from traps that were contaminated with ‘other’ drosophilids, and mainly from control traps, i.e. traps that had no host larvae supplied, but were apparently colonized by wild drosophilids. Again, only descriptive statistics could be used to interpret the data, because most of the traps did not yield any parasitoids.

Blueberry Plantation and Vineyard. Three *Leptopilina* individuals were collected from a trap pre-infested with SWD in the blueberry plantation, which was active during the third trapping session (8/20–8/23). However, the specimens were heavily damaged and stuck within dried blueberries when they were discovered, so the species could not be identified. No other parasitoids were reared from traps placed in the blueberry plantation. Additionally, no parasitoids were reared from traps placed in vineyards.

Discussion

Parasitoid Species. Although the sentinel traps yielded three parasitoid species, the results are not promising for biological control of SWD. *Leptopilina bouleardi* is a known parasitoid of frugivorous *Drosophila* (Carton et al. 1986, Dubuffet et al. 2009, Kacsoh and Schlenke 2012), but Kacsoh and Schlenke (2012) demonstrated that SWD is resistant to parasitization by *L. bouleardi*, as well as several other parasitoid species, due to a high hemocyte load. Mazzetto et al. (2016) also demonstrated that *L. bouleardi* in Italy could not develop on SWD. Therefore, it makes sense that *L. bouleardi* was not reared from SWD in the sentinel traps. Follow-up laboratory studies will confirm whether this strain of *L. bouleardi* is capable of parasitizing SWD (Chapter 3).

Leptopilina clavipes was only reared from traps producing ‘other’ drosophilids, especially control traps. Therefore, it likely preferred the other drosophilids to *D. melanogaster* or SWD. This is supported by the literature, which indicates *L. clavipes* is more associated with fungivorous drosophilids, rather than frugivorous drosophilids (Vet 1983, Carton et al. 1986, Driessen and Hemerik 1991, Pannebakker et al. 2008). Indeed, the raspberries used in the trap baits often became moldy, especially the control traps, so perhaps the ‘other’ drosophilids were fungal-feeding species (many of the ‘other’ drosophilids that emerged resembled known fungal-feeders *Drosophila phalerata* Hartig and *Drosophila subobscura* Collin (Driessen and Hemerik 1991, Pannebakker et al. 2008), but identification has not been confirmed). As a natural parasitoid of fungivorous species, *L. clavipes* would not be appropriate for biological control of SWD or AFF.

The presence of pupal parasitoid *P. vindemiae* was to be expected, because *P. vindemiae* is a cosmopolitan species, and a generalist of several schizophoran families including

Drosophilidae (Nøstvik 1954, Carton et al. 1986, Goubalt et al. 2004, Marchiori et al. 2013). Furthermore, Rossi Stacconi et al. (2013) reported *P. vindemiae* as a parasitoid of SWD in Europe and Oregon, and Daane et al. (2016) reported the same in South Korea. It was somewhat surprising that only one *P. vindemiae* was reared from SWD throughout this study, and that *P. vindemiae* was only reared from one trapping session. However, the sentinel traps were only seeded with fly larvae, not pupae, so pupae would have been present for a shorter length of time. Because of that, the traps may have been attractive to *P. vindemiae* for a more limited time. Conversely, perhaps *P. vindemiae* is simply less abundant in Virginia. Regardless, because *P. vindemiae* can successfully attack SWD, it seems somewhat more promising as a potential candidate for biological control of SWD. In Costa Rica, *P. vindemiae* has been used as an augmentative biological control agent in an IPM program against *Ceratitis capitata* Weidemann (Diptera: Tephritidae), and with marked success (Camacho 1998). However, there is a valid concern about non-target impacts because of the generalist and hyperparasitic behavior of *P. vindemiae* (Guillén et al. 2002, Wang and Messing 2004, Wang et al. 2016a). The use of *P. vindemiae* as a biocontrol agent for SWD needs to be further explored. Still, several studies have identified potential candidates for classical biological control of SWD that might prove more beneficial (Kasuya et al. 2013, Nomano et al. 2014, Asplen et al. 2015, Daane et al. 2016).

No parasitoids were reared during the first three weeks of trapping in the cherry orchard. That early in the season, insect populations may have still been recovering from winter, so fly hosts and therefore parasitoids may have been less abundant. Additionally, fruit were unripe, so the cherry orchard may have been less of a beacon to drosophilids and their parasitoids.

No parasitoids were reared from AFF in the sentinel traps, but that does not mean AFF escapes parasitization completely. Overall, the AFF-seeded traps were out for a much more

limited time than the other traps, so there were fewer opportunities for parasitoids to find AFF larvae or pupae. It must also be noted that parasitoids were not reared from any of the sentinel traps during the time in which AFF-seeded traps were active (Aug.–Oct.). Perhaps the trapping sessions did not coincide with the seasonal phenology of the parasitoids, or parasitoid abundance was low, or alternate host sources were more attractive to parasitoids. In addition, the blueberry and grape growers had been using insecticides to combat SWD, which might have reduced any parasitoid presence in the area. Follow-up laboratory experiments will determine if *P. vindemiae* or *L. boulandi* will parasitize AFF under controlled conditions (Chapter 3).

Data Trends. While there were not enough overall data for an accurate and meaningful statistical analysis, interesting trends were still observed in the cherry orchard and caneberry field. In the cherry orchard, considerably more parasitoids were reared from banana-baited traps than cherry-baited traps (Fig. 2), suggesting the type of fruit containing the host may be an important factor in parasitoid host-finding behavior. Plant odors released by host feeding activity are known to be important olfactory cues for parasitoid host-finding ability (Price et al. 1980, Geervliet et al. 1994, Du et al. 1996). Furthermore, it has been shown that some parasitoids are selective about which type of plant their host feeds on (Johnson and Hara 1987, van den Berg et al. 1990, Hoballah et al. 2002). Perhaps this is the case with parasitoids of frugivorous drosophilids, and such behavior could be important for biological control efforts of SWD or AFF. An olfactometry study investigating the relative attractiveness of different fruit odors to *Drosophila* parasitoids, such as *L. boulandi* and *P. vindemiae*, would be enlightening.

Another apparent trend in the data is that parasitoids were reared more from traps placed on the edge than on the interior (Fig. 2.3). Several factors could contribute to such an effect. Assuming the parasitoids enter the fruit production area from surrounding habitat, individuals

would not have to venture further into the area if the edge already supplies their needs. There could also be a similar effect occurring with the host insects, with more hosts available on the edge than on the interior. If there is a higher host population on the edge, the edge might be a more attractive location for parasitoids than the interior. In addition, microclimatic conditions could have been more conducive to parasitoid presence on the edge than on the interior. The cherry orchard and caneberry field were situated within or directly adjacent to woods, so that the edges where traps were placed were less exposed to direct sunlight than the interiors. To examine this theory, temperature data were recorded throughout July 2016 in the caneberry field, in the same general locations that traps were placed in 2015. Average daytime high temperatures were consistently higher in the interior of the field than the edge (Fig. 2.4), indicating that microclimates were indeed different between the two areas. Assuming this difference occurs every year, and that parasitoids of frugivorous drosophilids prefer the cooler edge habitat to the warmer interior, it could help explain the observed difference in parasitoid emergence.

Design Limitations. Although some data trends were observed, the high zero count for parasitoid emergence should be addressed. While such results may be attributed to pesticide usage at field sites, parasitoid phenology, trap placement and microclimates, one cannot rule out potential design flaws of the traps themselves. Rossi Stacconi et al. (2013) used red delta traps for their sentinel traps, and reared more parasitoids over the season, especially *P. vindemiae*. Color can be an important attractive component of insect traps (Hoback et al. 1999, Campbell and Hanula 2007), so perhaps the color red is more attractive to parasitoids of *Drosophila* than white, the color of our traps. Furthermore, another factor could be the manner in which the bait was “infested” with larvae before placement in the field. The larvae were simply dumped directly onto fresh fruit immediately before trap placement, so the bait would not have had the

same odors as if the larvae had developed within the fruit for a few days prior. Specifically, the parasitoids could be attracted to the yeast and vinegar odors associated with *Drosophila* larvae, and those odors might have taken a while to develop within the bait. Consequently, the baits may not have been attractive to parasitoids for as long as they should have. It may have been better to directly expose the fruit bait to adult flies for several days before placement, so that larvae would develop within the fruit, and the proper odors would be present at the time of placement.

Conclusions and Next Steps. The results indicate that parasitization of SWD and AFF in southwestern Virginia is negligible, and that none of the reared parasitoid species would be effective biological control agents for SWD or AFF. However, *L. boulandi* and *P. vindemiae*, the two species reared that parasitize frugivorous drosophilids, must still be assessed in the laboratory to determine if they can parasitize SWD or AFF under controlled conditions. The results also raise further questions: How does AFF compare with SWD and *D. melanogaster* with respect to parasitization resistance? Are parasitoids of frugivorous drosophilids selective about what type of fruit their host feeds in? These inquiries will be pursued.

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Tables and Figures

Table 2.1. Date ranges (2015) of sentinel trapping sessions for each fruit cropping system in this study.

Crop	May	June	July	Aug	Sept	Oct
Cherry	■					
Caneberry			■		■	■
Blueberry				■	■	
Grape				■	■	



Fig. 2.1. An example of the sentinel traps used in this study.

Legend for Figs. 2.2 and 2.3:

■ *Leptopilina*

■ *P. vindemiae*

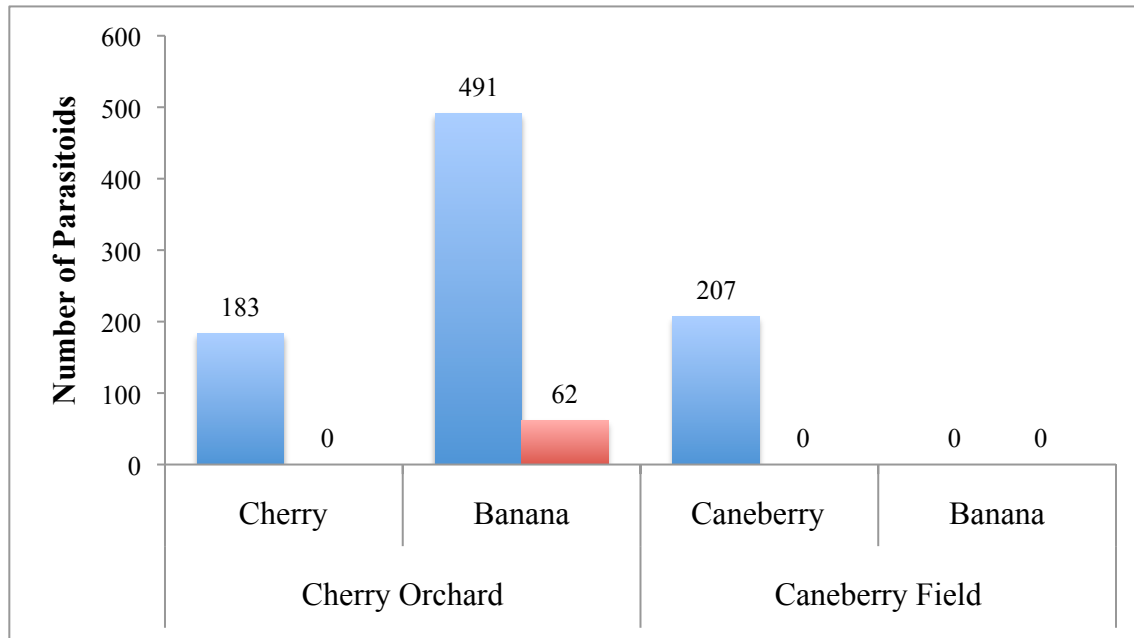


Fig. 2.2. Comparison of parasitoid emergence from sentinel traps, with respect to the type of fruit used to bait the trap. *Leptopilina* bars represent *L. boulandi* for the cherry orchard, and *L. clavipes* for the caneberry field.

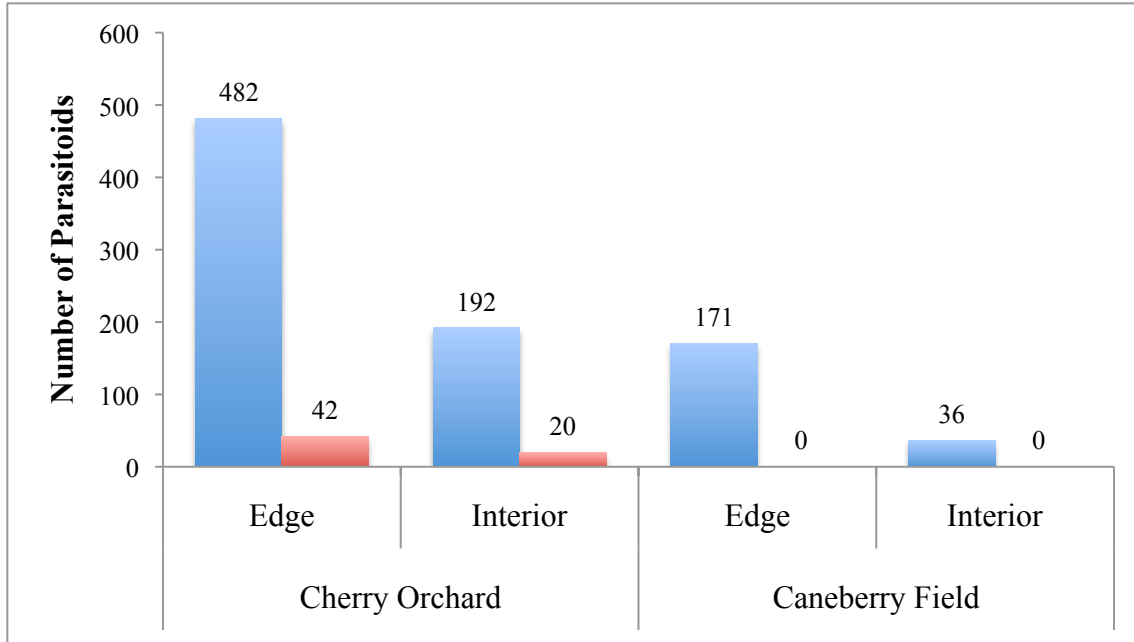


Fig. 2.3. Comparison of parasitoid emergence from sentinel traps, with respect to trap placement.

Leptopilina bars represent *L. boulardi* for the cherry orchard, and *L. clavipes* for the caneberry field.

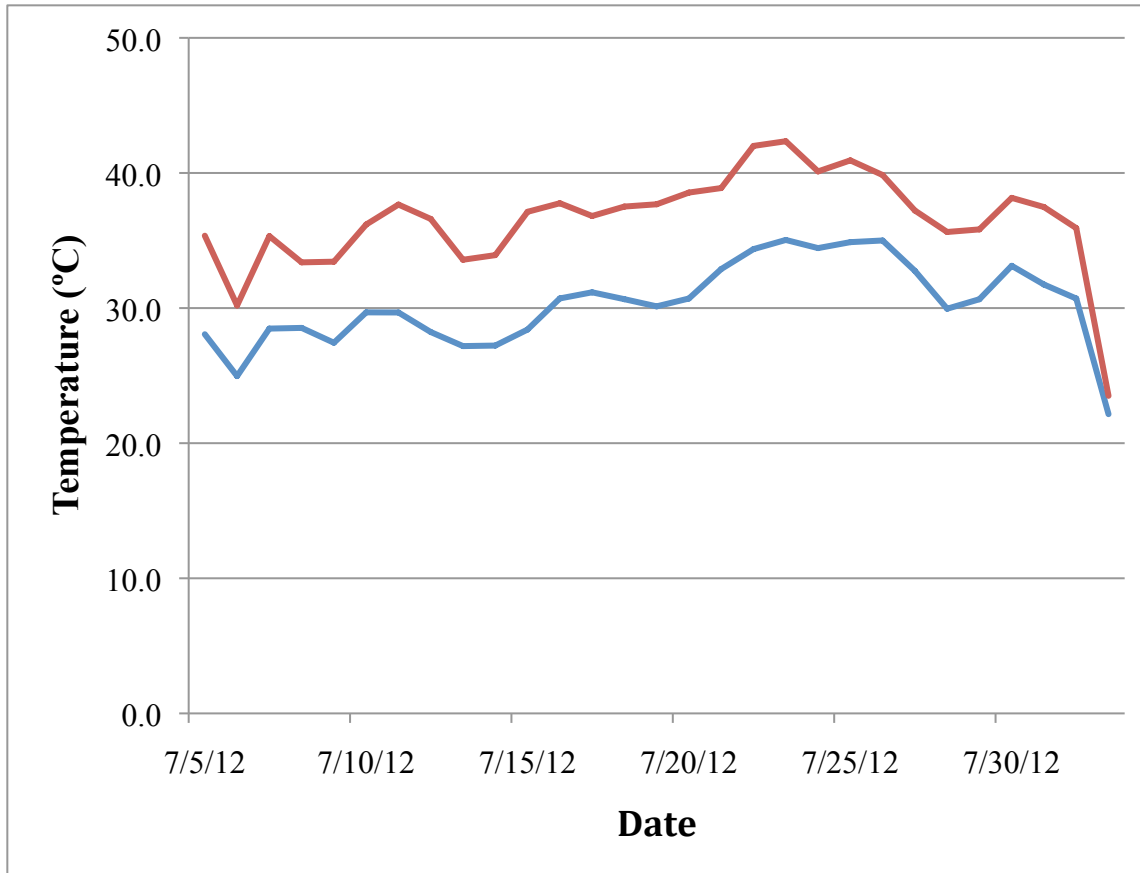


Fig. 2.4. Average daily high temperatures during summer 2016 for edge and interior of caneberry field. Temperature data were recorded by HOBO[®] Pro v2 data loggers – 4 on the edge and 3 on the interior.

Chapter 3

**Ability of two parasitoids in southwestern Virginia to attack invasive vinegar flies,
Drosophila suzukii (Matsumura) and *Zaprionus indianus* Gupta (Diptera: Drosophilidae)**

James C. E. Wahls and Douglas G. Pfeiffer

ABSTRACT: The ability of Virginia parasitoids, *Leptopilina boulardi* (Barbotin, Carton and Kelner-Pillault) (Hymenoptera: Figitidae) and *Pachycrepoideus vindemiae* (Rondani) (Hymenoptera: Pteromalidae), to parasitize invasive vinegar flies (Diptera: Drosophilidae), *Drosophila suzukii* (Matsumura) and *Zaprionus indianus* Gupta, was tested. The encapsulation responses of *Drosophila melanogaster* (Meigen), *D. suzukii*, and *Z. indianus* to parasitization by the Virginia strain of *L. boulardi* were also examined. Larvae of each fly species were exposed to female *L. boulardi* for 72 h, and wandering larvae and pupae of each fly species were exposed to female *P. vindemiae* for 72 h. After 72 h, a sample of larvae exposed to *L. boulardi* were dissected for encapsulated wasp eggs, and remaining larvae were allowed to complete development. No significant difference was observed among encapsulation rates of the three fly species (ANOVA, $p > 0.05$), but *Z. indianus* was attacked at a significantly lower rate than the other two fly species. Zero *L. boulardi* adults emerged from *D. suzukii* or *Z. indianus* puparia, and could only complete development on host *D. melanogaster*. *Pachycrepoideus vindemiae* was able to complete development on each fly species, but exposure to *P. vindemiae* also increased pupal mortality of each fly species. These results indicate that the Virginia strain of *L. boulardi* is not a viable biological control agent for *D. suzukii* or *Z. indianus*. *Pachycrepoideus vindemiae* could be considered for augmentative biological control, but restraint in its use is recommended because of its reported generalist and hyperparasitic behavior.

KEY WORDS: *Drosophila suzukii*, *Zaprionus indianus*, biological control, parasitoid

Two exotic species of vinegar fly have recently invaded North America: the spotted wing drosophila, *Drosophila suzukii* (Matsumura) (Diptera: Drosophilidae) of southeast Asia, and the African fig fly, *Zaprionus indianus* Gupta (Drosophilidae) (Steck 2005, van der Linde et al. 2006, Bolda et al. 2010, Walsh et al. 2011). Since the initial detections of *D. suzukii* in California and Europe in 2008, the species has spread rapidly and become a global economic pest of small fruits, due to its ability to cut into ripe, intact fruit with a serrated ovipositor (Bolda et al. 2010, Cini et al. 2012, Asplen et al. 2015). In Virginia, *D. suzukii* has been an important pest of small fruit production since 2011 (Pfeiffer 2012, Pfeiffer et al. 2012).

Zaprionus indianus has been invasive in South America since the late 1990s, where it became a pest of fig production (Vilela 1999, Raga et al. 2003, Santos et al. 2003, Oliveira et al. 2013). In 2005, *Z. indianus* was discovered in Florida (Steck 2005, van der Linde et al. 2006), and was first detected in Virginia in 2012, where it has often been observed concurrently with late-season *D. suzukii* infestations (Pfeiffer 2012, Pfeiffer et al. 2012). Unlike *D. suzukii*, *Z. indianus* does not have a large, serrate ovipositor, and so cannot puncture intact fruit during oviposition (Fig. 3.1). Only previously damaged or overripe fruit would be susceptible to *Z. indianus* infestation (Shrader unpublished data). Additionally, cooler climates and winter temperatures may limit the spread of *Z. indianus* (Araripe et al. 2004, David et al. 2006). Therefore, the chance of *Z. indianus* becoming a major agricultural pest in North America is low, with the exception of fig-producing areas such as California. While there may be geographic limitations to this pest, *Z. indianus* has a wide host range (Vilela 1999, Santos et al. 2003, van der Linde et al. 2006), and is reported to be highly adaptable and highly competitive (Tidon et al. 2003, da Silva et al. 2005, Ferreira and Tidon 2005, Galego and Carareto 2005, da Mata et al.

2010), suggesting it has potential to have a major impact on drosophilid communities in invaded regions.

In the search for an effective integrated pest management program for spotted wing drosophila, biological control research has been gaining ground, particularly with hymenopteran parasitoids. Kacsoh and Schlenke (2012) examined the immune responses of *D. suzukii* and its relative *Drosophila melanogaster* (Meigen), after being attacked by 24 different strains of parasitoid wasps, which represented four hymenopteran families, and at least 14 species. The study demonstrated that *D. suzukii* is far more effective than *D. melanogaster* at neutralizing wasp eggs via melanotic encapsulation, owing to a much higher hemocyte load than *D. melanogaster* (Kacsoh and Schlenke 2012). This augmented resistance of *D. suzukii* to parasitization was corroborated by Poyet et al. (2013).

In spite of the remarkable immune system of *D. suzukii*, several potential candidates for classical biological control have been discovered in Asia, which include species in the genera *Asobara* (Hymenoptera: Braconidae), *Ganaspis* (Hym.: Figitidae), and *Leptopilina* (Hym.: Figitidae) (Kasuya et al. 2013, Nomano et al. 2014, Asplen et al. 2015, Daane et al. 2016). Yet, as a prerequisite for classical biological control, the ability of parasitoids in invaded regions to attack *D. suzukii* must be investigated. Such research has already been performed in Europe and the west coast of North America, where only the pupal parasitoids *Pachycrepoideus vindemiae* (Rondani) (Hym.: Pteromalidae) and *Trichopria drosophilae* (Perkins) (Hym.: Diapriidae) are able to parasitize *D. suzukii* with some success (Chabert et al. 2012, Rossi Stacconi et al. 2013, Gabarra et al. 2015, Rossi Stacconi et al. 2015, Mazzetto et al. 2016, Wang et al. 2016a, 2016b). No such research has yet been published from eastern North America.

In the case of *Z. indianus*, there is currently no information on its relationship with natural enemies in North America. Research in Brazil, though, has documented pupal parasitoids *P. vindemiae* and *Spalangia endius* (Walker) (Hym.: Pteromalidae), and larval parasitoid *Leptopilina bouvardi* (Barbotin, Carton & Kelner-Pillault) developing on *Z. indianus* (Marchiori et al. 2003, Marchiori and Silva 2003, Silva et al. 2004).

Sentinel trapping surveys in 2015 identified larval endoparasitoids *Leptopilina bouvardi* and *L. clavipes* (Hartig), and pupal ectoparasitoid *Pachycrepoideus vindemiae* as parasitoids of frugivorous drosophilids in southwestern Virginia (Wahls *unpublished data*). Laboratory colonies of *L. bouvardi* and *P. vindemiae* were successfully developed from individuals reared from sentinel traps, using host *D. melanogaster*. The primary objective of this study was to investigate whether these parasitoids could successfully develop on *D. suzukii* or *Z. indianus* in the laboratory. A second objective was to examine and compare the larval encapsulation responses of *D. melanogaster*, *D. suzukii*, and *Z. indianus* after exposure to the Virginia strain of *L. bouvardi*. The purpose of these objectives is to determine if the Virginia strains of *P. vindemiae* and *L. bouvardi* could be useful for augmentative or conservation biological control of *D. suzukii* or *Z. indianus*.

Materials and Methods

Insects. This study involved three species of vinegar flies, *Drosophila melanogaster*, *D. suzukii*, and *Z. indianus*, and two parasitoid species, *Leptopilina bouvardi* and *Pachycrepoideus vindemiae*. The laboratory colony of *D. melanogaster* was developed from existing stock colonies in the Virginia Tech Departments of Biological Sciences and Entomology. Colonies of *D. suzukii* and *Z. indianus* were developed in laboratory from individuals wild-caught in

southwestern Virginia. Fly colonies were maintained on molasses-based food media (Nutri-Fly™ MF, Genesee Scientific Corp., San Diego, CA) in 178-ml, square-bottom polypropylene drosophila stock bottles (Genesee Scientific Corp, San Diego, CA), and kept in an environmental chamber with 14 h daylength (18 W “cool white” fluorescent bulbs) and temperature at a constant 23.3°C. Both laboratory colonies of parasitoids were developed from individuals collected in small fruit cropping systems in southwestern Virginia. Parasitoids were maintained on host *D. melanogaster* from the aforementioned laboratory colony, and kept in an environmental chamber with 14 h daylength, day temperature at 26°C and night temperature at 23°C.

Experimental Design. *Larval parasitoids.* Three days prior to experimentation, newly eclosed *L. boulandi* were collected from the laboratory colony and placed in a stock bottle with fresh food medium but no fly larvae, returned to the environmental chamber, and left to mate during that time. On the day of experimentation, 50 1st- and 2nd-instar larvae of *D. melanogaster* were placed in a 35 mm Petri dish with ~1 mm depth of food media. The Petri dish was then enclosed in a rearing bottle with three mated females and one male of *L. boulandi* (females have short antennae and males have long antennae). For the control experiment, another Petri dish was prepared the same way and enclosed in a bottle with no parasitoids. The bottles were then placed in an environmental chamber with 14 h daylength (18 W “cool white” fluorescent bulbs), 26°C day temp, 23°C night temp, and left for 72 h. After 72 h, the parasitoids were removed, and 10 larvae were collected from the dish that had been exposed to parasitoids. These larvae were placed in 70% ethanol and observed under a microscope. When placed in ethanol, the integument of the larvae becomes nearly transparent and internal structures can be observed, especially encapsulated parasitoid eggs/larvae (Fig. 3.2). For each larva, the number of eggs laid and

number of encapsulated eggs was recorded, in order to determine encapsulation rate.

Encapsulation rate was calculated as the number of encapsulated eggs divided by the number of eggs laid. Attack rate by *L. bouleardi* was also determined based on the number of larvae that contained at least 1 wasp egg. The remaining larvae were allowed to complete development in the environmental chamber, and the number of emerged flies and parasitoids was recorded to determine level of survival, parasitization, and overall mortality. Mortality was measured as the number of insects that did not complete development. Emerged flies were also observed for signs of attempted parasitization, i.e. encapsulated parasitoid eggs/larvae, which were still quite visible in adult flies (Fig. 3.3). This experiment had six replicates, and was repeated once with host *D. suzukii*, and once with host *Z. indianus*. Methods were adapted from Kacsoh and Schlenke (2012).

Pupal Parasitoids. Three days prior to experimentation, newly eclosed *P. vindemiae* were collected from the laboratory colony and placed in a stock bottle with fresh food medium but no flies, returned to the environmental chamber, and left to mate during that time. On the day of experimentation, 50 late 3rd-instar larvae and newly-formed puparia of *D. melanogaster* were placed in a 25 × 95 mm polystyrene drosophila rearing vial (Genesee Scientific Corp., San Diego, CA) with ~2 mm depth of food medium, and a paper strip for a pupariation surface. Next, three mated female and one male *P. vindemiae* were placed in the vial (females have pointed abdomens, males have rounded abdomens). A second vial was prepared with no parasitoids as a control. The vials were then placed in the environmental chamber for 72 h. After 72 h, the parasitoids were removed, and the larvae were allowed to complete development. The number of emerged flies and parasitoids were recorded to determine rates of survival, parasitization, and

overall mortality. This experiment was replicated six times, and repeated using hosts *D. suzukii* and *Z. indianus*. Methods were adapted from Kacsoh and Schlenke (2012).

Statistical Analyses. The *L. bouleardi* encapsulation experiments were analyzed using ANOVA and Tukey's multiple comparison, comparing differences in attack rate and encapsulation rate among the three fly species. For parasitization experiments with *L. bouleardi* and *P. vindemiae*, ANOVA and Tukey's multiple comparison were used to compare survival/mortality, and parasitization rates among the three fly species. Additionally, in the *L. bouleardi* parasitization experiments, the same analysis was used to compare the amount of emerged adult flies containing encapsulated wasp eggs/larvae. To determine if the presence of parasitoids influenced mortality, a Student's t-test was used to compare the difference between mean control mortality and mean experimental mortality for each fly species in each experiment.

Results

Encapsulation response to *Leptopilina bouleardi* attacks. After 72 h exposure to females of *L. bouleardi*, an average of 4.8 out of 10 *D. melanogaster* larvae showed signs of attempted parasitization, for an attack rate of ~48%. Based on the number of eggs laid, and the number of eggs/larvae encapsulated, encapsulation rate was calculated at 80% (Fig. 3.4). *Leptopilina bouleardi* attacked an average of 5.7 out of 10 *D. suzukii* larvae, for an attack rate of ~57%. The observed encapsulation rate of *D. suzukii* was ~64%. Also, an average of 1.2 out of 10 *Z. indianus* larvae were attacked, for an attack rate of ~12%, and the observed encapsulation rate of *Z. indianus* was 90%. The attack rate on *Z. indianus* was significantly less than the attack rates on *D. melanogaster* and *D. suzukii*, but attack rates on *D. melanogaster* and *D. suzukii* were not significantly different from one another (ANOVA and Tukey's multiple comparison: D.f. =

2, 15, $F = 17.787$, $p < 0.05$) (Fig. 3.4). No significant difference was observed among encapsulation rates of the three species (D.f. = 2, 14, $F = 2.588$, $p > 0.05$).

***Leptopilina boulardi* Parasitization Trials.** After fly larvae exposed to *L. boulardi* completed development, significant differences in emergence and mortality were observed among the three fly species (Fig. 3.5). Following exposure to *L. boulardi*, the mean numbers of flies emerging for each species were significantly different from the other two, with *D. melanogaster* emergence the lowest, and *Z. indianus* emergence the highest (D.f. = 2, 15, $F = 103.46$, $p < 0.05$). An average of 26 wasps emerged from *D. melanogaster* for a 65% parasitization rate. No wasps emerged from *D. sukukii* and *Z. indianus*. Additionally, the number of emerged flies containing at least 1 encapsulated wasp egg or larva was significantly higher in *D. sukukii* than in *D. melanogaster* and *Z. indianus* (D.f. = 2, 15, $F = 75.067$, $p < 0.05$). Mortality was also significantly higher in *D. sukukii* than in the other two species (D.f. = 2, 15, $F = 13.858$, $p < 0.05$), but the same was observed in control mortality (D.f. = 2, 15, $F = 10.101$, $p < 0.05$). No significant difference was observed between mortality and control mortality for each species (Table 1).

***Pachycrepoideus vindemiae* Parasitization Trials.** After fly pupae exposed to *P. vindemiae* completed development, *Z. indianus* again had the highest mean number of adult flies emerge and was significantly higher than that of *D. melanogaster* (D.f. = 2, 15, $F = 3.429$, $p < 0.05$) (Fig. 3.6). The number of adult *D. sukukii* flies emerging was between that of *D. melanogaster* and *Z. indianus*, and was not significantly different from either. *Pachycrepoideus vindemiae* was able to parasitize each fly species, but no significant difference was observed among the number of adult wasps emerged from each fly species. Also, no significant difference

was observed among experimental mortality of each species. However, for each species, experimental mortality was significantly greater than control mortality (Table 3.1).

Discussion

The attack rates of *L. bouleardi* on *D. melanogaster* and *D. suzukii* were quite similar, while *Z. indianus* seemed much less appealing to *L. bouleardi*. The two *Drosophila* species are both within the *melanogaster* species group (Kopp and True 2002), likely making them similar with respect to physiology and olfactory cues. *Z. indianus* is more distantly related to the two *Drosophila* species (DeSalle 1992, Remsen and O'Grady 2002, van der Linde 2010), so the differences in physiology and scent might make it a less suitable and less attractive host to *L. bouleardi*.

The observed encapsulation rates of *D. melanogaster* and *D. suzukii* were not significantly different, which initially seems surprising due to what we know about the high hemocyte load of *D. suzukii* (Kacsoh and Schlenke 2012, Poyet et al. 2013). However, Kacsoh and Schlenke (2012) reported a similar situation with another strain of *L. bouleardi*, specifically LbG486, where both *D. melanogaster* and *D. suzukii* showed a comparably high level of encapsulation. The observed encapsulation rate of *Z. indianus* in this study was also not significantly different from either *Drosophila* species, but one must keep in mind that the sample size for *Z. indianus* was quite low, due to the low number of larvae that were actually attacked. Still, it can be said that the encapsulation response of *Z. indianus* is certainly not lacking, and a comparative analysis of its hemocyte load with that of *D. suzukii* and *D. melanogaster* would be of interest.

When the adult emergence results are compared with the larval encapsulation results, some inconsistencies become apparent. For example, although a high encapsulation rate was observed in larvae of all three fly species, *L. boulandi* was still able to complete development on *D. melanogaster*. Kacsoh and Schlenke (2012) reported a similar lack of correlation between encapsulation and emergence, and gave explanations that apply here. They rationalized that even if a larva had encapsulated a wasp egg, the larva still may have been super-parasitized and perhaps not all infesting wasp eggs were killed (Kacsoh and Schlenke 2012). Blumberg (1997) also explains that if parasitoid eggs/larvae are only partially encapsulated, they can still complete development. Such may have been the case here. Additionally, upon performing larval dissections, we discovered that non-encapsulated wasp eggs within a fly larva are naturally more difficult to identify than encapsulated eggs, due to similarities in coloration with internal structures. Therefore, it is possible that some non-encapsulated eggs were missed during larval dissections.

Another inconsistency was that zero parasitoids emerged from *D. suzukii* and *Z. indianus*, even though the observed encapsulation rates did not reach 100%. Again, Kacsoh and Schlenke (2012) reported similar results with *D. suzukii*, and explained that even though some wasp eggs may not have been encapsulated by the time larval dissections occurred, the eggs might have been encapsulated and killed at a later point. This is why it is also important to observe the number of emerged adult flies that contained encapsulated wasp eggs.

By examining the number of emerged flies containing encapsulated wasp eggs, one can gain a better understanding of the wasp's ability to parasitize the flies, and their ability to resist parasitization (Fig. 5). For *D. melanogaster*, a small number of adult flies emerged compared to the number of wasps that emerged, and most flies that emerged did not have encapsulated wasp

eggs, or “capsules”. This, combined with the larval encapsulation results, shows that the Virginia strain of *L. bouleardi* is able to somehow overcome the encapsulation response of *D. melanogaster*, and that most of the emerged flies probably avoided attack. For *D. suzukii*, a comparatively larger number of adult flies emerged with zero wasps, and a majority of the emerged flies contained capsules, showing that the Virginia strain of *L. bouleardi*, like other strains, cannot overcome the encapsulation response of *D. suzukii* (Chabert et al. 2012, Kacsoh and Schlenke 2012, Mazzetto et al. 2016). Because *Z. indianus* had such a high level of fly survival, no wasp emergence, and very few adult flies with capsules, these results are consistent with the larval encapsulation results, and indicate that *Z. indianus* is simply not an attractive host for this strain of *L. bouleardi*. However, *L. bouleardi* has been reported to attack *Z. indianus* in Brazil (Marchiori et al. 2003), suggesting that the susceptibility of *Z. indianus* to parasitization may vary depending on the strain of *L. bouleardi*.

Based on the lack of difference between experimental and control mortality levels, one can conclude that *L. bouleardi* did not affect mortality for each fly species tested. However, *D. suzukii* showed higher levels of mortality in both the experimental and control assays, indicating that the environmental conditions may have been less conducive for *D. suzukii* survival.

Interestingly, evidence of cannibalism was observed in some of the *D. suzukii* puparia (Fig. 7), so perhaps cannibalism also played a role in the higher mortality levels.

Emergence results from the *P. vindemiae* trials showed that the Virginia strain of *P. vindemiae* could successfully develop on each fly species tested in the laboratory. Moreover, attack by *P. vindemiae* appeared to cause a significant increase in total mortality for the three fly species. These results are consistent with reports of other *P. vindemiae* strains attacking *D. melanogaster* and *D. suzukii* (Chabert et al. 2012, Rossi Stacconi et al. 2013, Rossi Stacconi et

al. 2015, Mazzetto et al. 2016, Wang et al. 2016a), so it is not surprising that a similar result was seen with host *Z. indianus*. While *P. vindemiae* has repeatedly been reported as a natural parasitoid of *D. suzukii* (Chabert et al. 2012, Rossi Stacconi et al. 2013, Daane et al. 2016), and once as a parasitoid of *Z. indianus* (Silva et al. 2004), parasitization rates by *P. vindemiae* on these pests in the field were so low that it would make an insignificant impact on population levels. For example, Silva et al. (2004) reported a 3.5% parasitization rate of *P. vindemiae* on *Z. indianus* in Brazil, and Rossi Stacconi et al. (2013) estimated only a 1% seasonal parasitization rate on *D. suzukii* in Oregon, and even less in Italy. In Virginia, parasitization of *P. vindemiae* on *D. suzukii* in the field has also been observed as negligible, and it has yet to be observed at all on *Z. indianus* (Wahls *unpublished data*). Such low parasitization rates make sense, because *P. vindemiae* is known to be a generalist that hosts on many species within many different schizophoran families (Nøstvik 1954, Carton et al. 1986, Goubalt et al. 2004, Marchiori et al. 2013). Therefore, one should not expect *P. vindemiae* to seek out drosophilids over other fly hosts.

Due to the generalist behavior of *P. vindemiae*, and the low parasitization rates observed in the field, it is clear that *P. vindemiae* will not be an effective conservation biological control agent. However, should it be used for augmentative biological control? *P. vindemiae* has been mass released in Costa Rica to control Mediterranean fruit fly (*Ceratitidis capitata* Weidemann, Diptera: Tephritidae) in oranges, and, in conjunction with the release of sterile fruit flies, was purportedly very successful (Camacho 1998). Yet, serious problems arise when considering this strategy to control *D. suzukii*. Mainly, the invasion of *D. suzukii* is on a far greater scale, affecting at least three different continents (Asplen et al. 2015). Even if enough wasps could be mass reared in captivity, one has to consider the environmental consequences for

such large-scale releases. Not only is *P. vindemiae* a generalist, it is also a hyperparasitoid (van Alphen and Thunnissen 1983), and researchers have expressed concern about the non-target impacts of mass-releasing this species, with respect to native dipteran species as well as parasitoid species (Guillén et al. 2002, Wang and Messing 2004). Therefore, the authors do not recommend using *P. vindemiae* for augmentative biological control of *D. suzukii* or *Z. indianus*, at least on a large scale, because the risk to non-targets is too high.

Conclusions. Overall, the results of this study are consistent with previous published research concerning the ability of *L. boulandi* and *P. vindemiae* to parasitize *D. suzukii*. Under the laboratory conditions of this study, the Virginia strain of *L. boulandi* cannot successfully attack pest *D. suzukii* due to its enhanced encapsulation response, and is not attracted to *Z. indianus* as a host. Consequently, the Virginia strain of *L. boulandi* is not a viable candidate for conservation or augmentative biological control of these pests. While *P. vindemiae* can parasitize both pest species, its generalist and hyperparasitic nature will likely make *P. vindemiae* ineffectual for conservation biological control. The authors also recommend extreme caution if considering *P. vindemiae* for augmentative biological control.

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Tables and Figures

Table 3.1. Student's t-test results comparing experimental mortality in *Drosophila melanogaster* (*D.m.*), *D. suzukii* (*D.s.*), and *Zaprionus indianus* (*Z.i.*) exposed to parasitoid females, versus control mortality in unexposed flies.

T-test Results	<i>D.m.</i>	<i>D.s.</i>	<i>Z.i.</i>
<i>L. boulardi</i> Trials			
Mean Experimental Mortality	7	14.5	5.5
Mean Control Mortality	8.6667	17.5	4
t	-0.6817	-0.8321	0.6311
D.f.	5	5	5
<i>p</i>	0.5257	0.4433	0.5557
<i>P. vindemiae</i> Trials			
Mean Experimental Mortality	20	17.3333	17.5
Mean Control Mortality	4	2.1667	8
t	-8.7636	-4.8711	-3.3075
D.f.	5	5	5
<i>p</i>	0.0003*	0.0046*	0.0213*

Mortality was measured as the number of individuals that did not complete development. Asterisks indicate a significant *p* value.

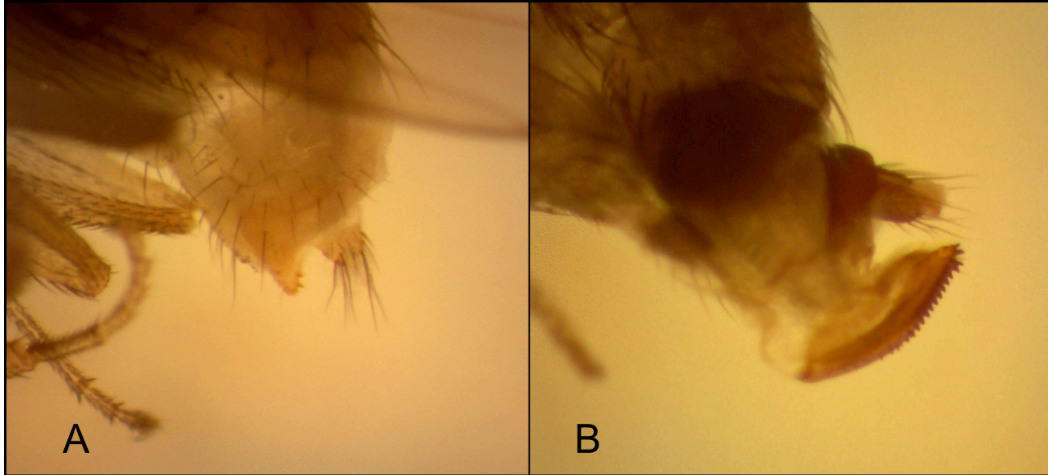


Figure 3.1. Comparison of ovipositors of *Zaprionus indianus* (A) and *Drosophila suzukii* (B). The ovipositor of *Z. indianus* lacks the size, sclerotization, and serration necessary to puncture the skin of intact fruit, contrary to *D. suzukii*. Images: D. G. Pfeiffer

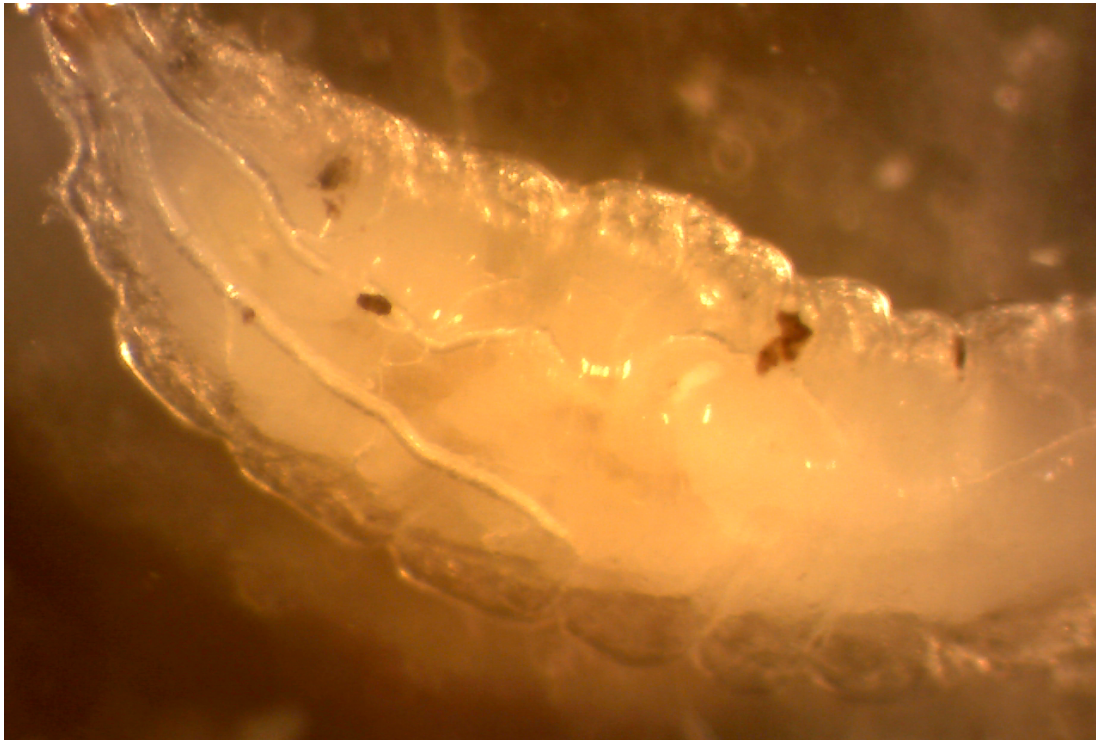


Fig. 3.2. *Drosophila suzukii* larva in 70% ethanol. Dark spots within fly larva demonstrate melanotic encapsulation of eggs of *Leptopilina boulardi*.



Fig. 3.3. Adult *Drosophila suzukii* exhibiting melanized parasitoid eggs/larvae within abdomen.

Legend for Figs. 3.4, 3.5, and 3.6

- *D. melanogaster*
- *D. suzukii*
- *Z. indianus*

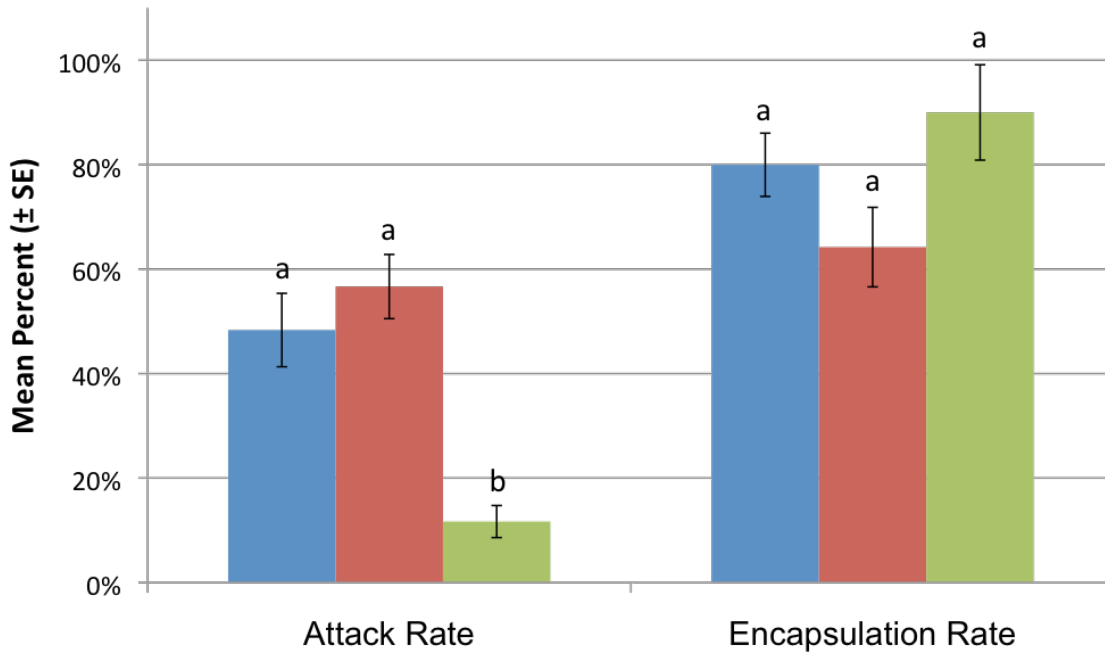


Fig. 3.4. Mean percent (\pm SE) attack rates of *Leptopilina boulardi* on larvae of *Drosophila melanogaster*, *D. suzukii*, and *Zaprionus indianus*, and encapsulation rates of wasp eggs by fly larvae. Attack rate was measured as (mean number of larvae attacked) / N , where $N = 10$ larvae. Encapsulation rate was measured as the mean of (no. encapsulated wasp eggs) / (no. wasp eggs laid). Within each cluster, columns with different letters are significantly different, based on Tukey's multiple comparison ($p < 0.05$).

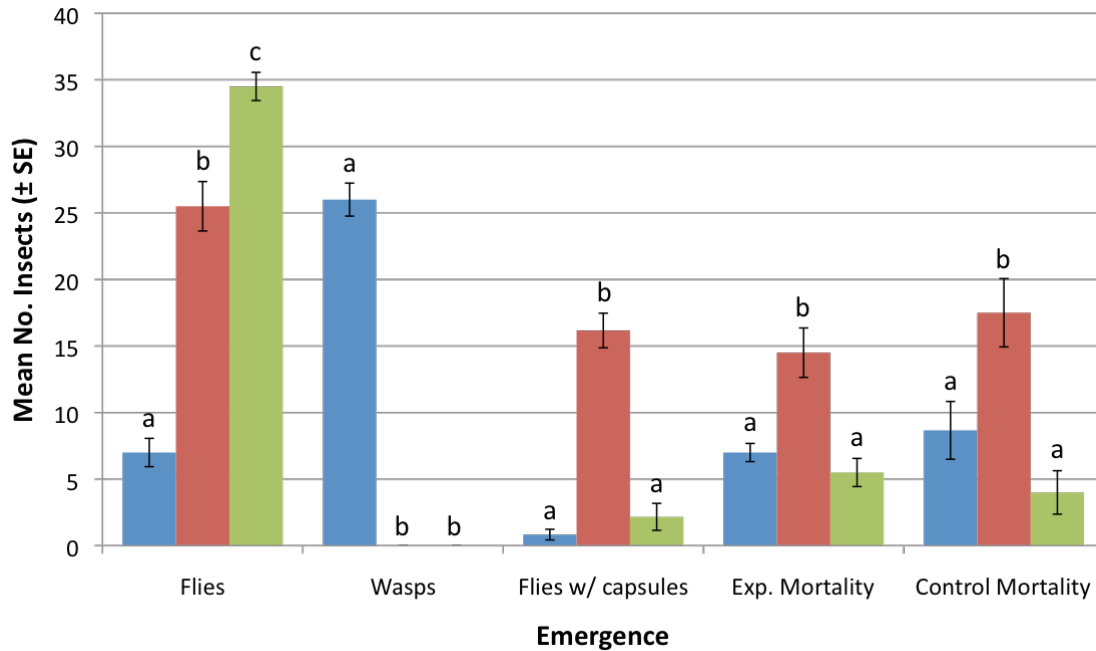


Fig. 3.5. Emergence and mortality results after *Drosophila melanogaster*, *D. suzukii*, and *Zaprionus indianus* larvae were exposed to *Leptopilina boulardi* females for 72 h. “Flies w/ capsules” refer to adult flies containing encapsulated wasp eggs/larvae (Fig. 3.3). “Experimental mortality” refers to individuals that did not complete development after exposure to parasitoids. “Control mortality” refers to individuals that did not complete development and were not exposed to parasitoids. Within each cluster, columns with different letters are significantly different, based on Tukey’s multiple comparison ($p < 0.05$).

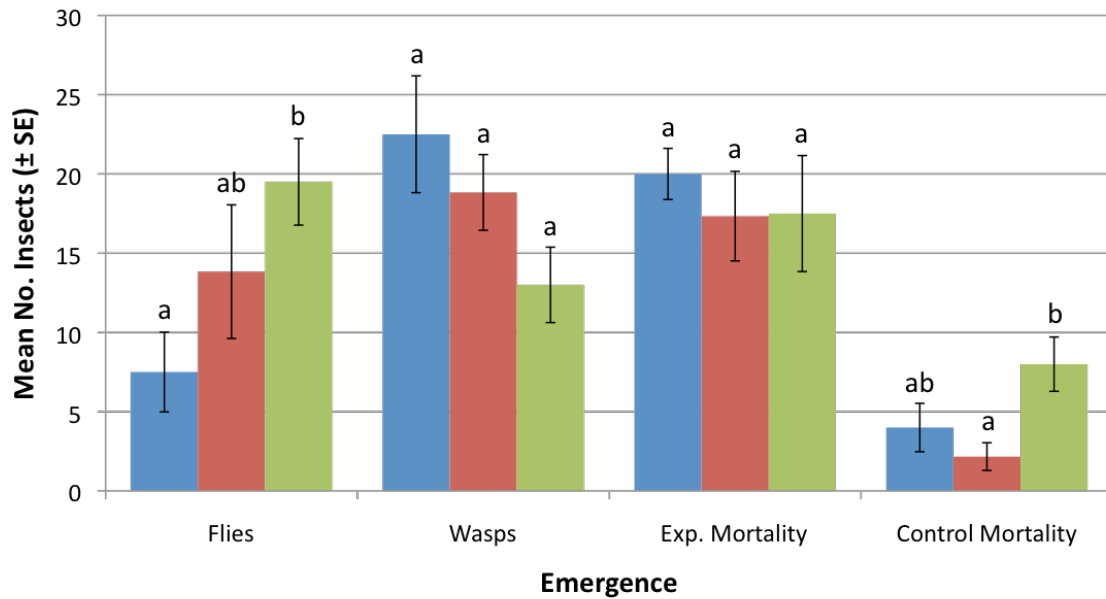


Fig. 3.6. Mean (\pm SE) emergence and mortality results after 3rd instar larvae or pupae of *Drosophila melanogaster*, *D. sukikii*, and *Zaprionus indianus* were exposed to *Pachycrepoideus vindemiae* females for 72 h. “Experimental mortality” refers to individuals that did not complete development after exposure to parasitoids. “Control mortality” refers to individuals that did not complete development and were not exposed to parasitoids. Within each cluster, columns with different letters are significantly different, based on Tukey’s multiple comparison ($p < 0.05$).

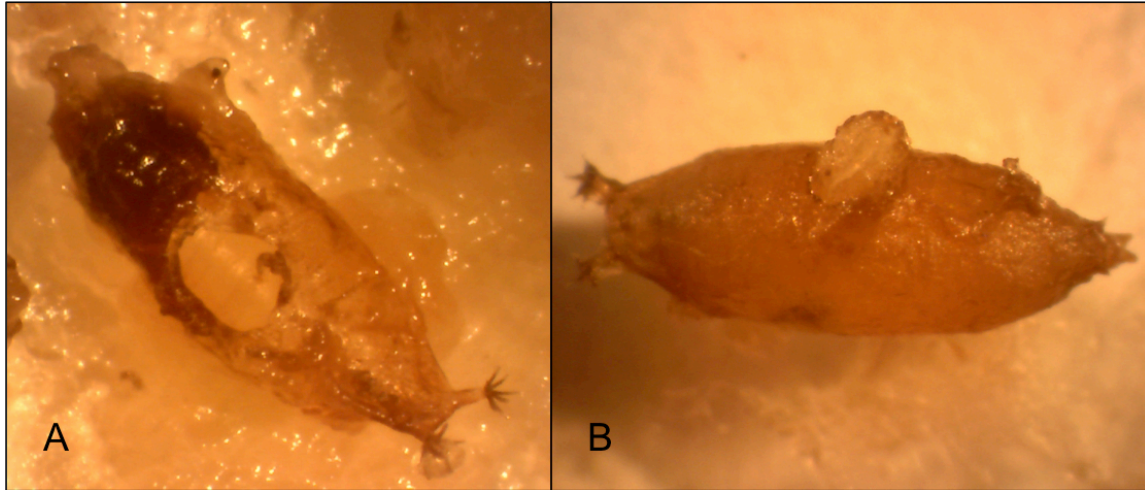


Fig. 3.7. A.) A cannibalized *D. suzukii* puparium from the *Leptopilina boulandi* parasitization experiment. The large hole in the side of the puparium clearly shows where another larva cut through in order to eat the pupa within. B.) Another instance of cannibalism in *D. suzukii*. Here, the antagonist larva is still feeding with its posterior visibly protruding from the hole in the puparium. These individuals were taken directly from the laboratory colony, and were never exposed to parasitic wasps, so the hole in the side cannot be attributed to predation by a wasp.

Chapter 4

Investigating fruit search preference of a larval and pupal parasitoid (Hymenoptera) of frugivorous drosophilids

James C. E. Wahls, Scott M. Salom, and Douglas G. Pfeiffer

ABSTRACT: A Y-tube olfactometer was used to examine the behavior of mated female parasitoids of frugivorous drosophilids when presented with odors of different fruit types infested with host *Drosophila melanogaster* (Meigen) (Diptera: Drosophilidae), including sweet cherry (*Prunus avium* L., Rosales: Rosaceae), red raspberry (*Rubus idaeus* L., Rosaceae), blueberry (*Vaccinium* sp., Ericales: Ericaceae), black table grape (*Vitis vinifera* L., Vitales: Vitaceae), and banana (*Musa acuminata* Colla, Zingiberales: Musaceae). Two parasitoid species were tested: the larval parasitoid *Leptopilina bouvardi* (Barbotin, Carton and Kelner-Pillault) (Hymenoptera: Figitidae), and the pupal parasitoid *Pachycrepoideus vindemiae* (Rondani) (Hymenoptera: Pteromalidae). Results showed that when given a choice between infested fruit and clean air, *L. bouvardi* had a significant preference toward infested fruit (χ^2 test, $p < 0.05$). When infested fruit were paired against each other, *L. bouvardi* significantly preferred banana and raspberry to cherry, and grape was least preferred. Differences in mean response time of *L. bouvardi* to make a choice for each odor source were not significant, based on two-sample t-tests. *Pachycrepoideus vindemiae* did not respond well to treatments, so data could not be presented for this species. Results of the *L. bouvardi* trials imply host diet may be important for *Drosophila* parasitoids, with regard to orientation to hosts. The importance of researching tritrophic interactions associated with frugivorous drosophilids is discussed, with respect to biological control of the invasive spotted wing drosophila (*Drosophila suzukii* (Matsumura), Diptera: Drosophilidae).

KEY WORDS: Tritrophic interactions, *Drosophila suzukii*, parasitoid, biological control, olfactometer

Since the breakthrough that plants can influence the behavior of their herbivores' natural enemies (Price et al. 1980), tritrophic interactions among insect herbivores, their host plants, and their natural enemies have become a widely studied area of entomology (Powell and Zhi-Li 1983, Dicke et al. 1990, Turlings et al. 1991, Geervliet et al. 1994, Du et al. 1996, Takabayashi et al. 1998, Verheggen et al. 2008). Natural enemies such as parasitoid wasps can be selective about which plant volatiles or odors they orient toward when multiple host plants are involved (Johnson and Hara 1987, Hoballah et al. 2002). Such selectivity can likely be attributed to effects of different host diets on the parasitoid, such as size, longevity, fecundity, and sex ratio, as demonstrated by Eben et al. (2000). However, there remains insufficient information regarding tritrophic interactions associated with vinegar flies (Diptera: Drosophilidae).

Within the past decade, the spotted wing drosophila, *Drosophila suzukii* (Matsumura) (Diptera: Drosophilidae), henceforth SWD, has been introduced into three continents with devastating effects. This has led to intense pressure to develop effective management programs for this pest, including use of biological control (Hauser 2011, Walsh et al. 2011, Cini et al. 2012, Deprá et al. 2014, Asplen et al. 2015). Therefore, it is imperative to examine the tritrophic interactions associated with frugivorous drosophilids, especially SWD. The selectivity of parasitoids for host diet will be important for determining which and how many biological control agents should be used to effectively control SWD populations.

Recent sentinel trapping surveys in southwestern Virginia found two parasitoid species attacking frugivorous drosophilids: the larval endoparasitoid *Leptopilina bouvardi* (Barbotin, Carton and Kelner-Pillault) (Hymenoptera: Figitidae), and the pupal ectoparasitoid *Pachycrepoideus vindemiae* (Rondani) (Hymenoptera: Pteromalidae) (Chapter 2). Although *L. bouvardi* is unable to successfully parasitize SWD (Chabert et al. 2012, Kacsoh & Schlenke

2012, Chapter 3), its relative, *L. japonica japonica* Novković & Kimura, has been discovered parasitizing SWD in South Korea (Daane et al. 2016). Furthermore, other members within Figitidae have been reported to successfully attack SWD in Asia, including *Ganaspis brasiliensis* (Ihering), and a population of *Ganaspis xanthopoda* (Ashmead) that may specialize on SWD, making *G. xanthopoda* a candidate for classical biological control (Kasuya et al. 2013, Asplen et al. 2015, Daane et al. 2016). *Pachycrepoideus vindemiae* is a cosmopolitan, generalist parasitoid and it has been found attacking drosophilids, including SWD, wherever surveys have occurred, such as in USA, Europe, Japan, and South Korea (Chabert et al. 2012, Rossi Stacconi et al. 2013, Daane et al. 2016, Mazzetto et al. 2016, Chapter 2). Due to its ability to successfully attack SWD and its widespread distribution, *P. vindemiae* has potential as a conservation biological control agent. Also, if *P. vindemiae* can be mass reared in captivity, augmentative biological control with this species may also be possible.

The objective of this study was to determine if *L. bouleardi* and *P. vindemiae*, parasitoids of frugivorous drosophilids in Virginia, exhibit a preference for certain host diet odors over others. Additionally, this study discusses implications of tritrophic interactions for SWD biological control, and aims to instigate further tritrophic research with candidate biological control agents for SWD.

Materials and Methods

Insects. Insects used for this study included *Drosophila melanogaster* (Meigen) (Diptera: Drosophilidae), and the parasitoids *L. bouleardi* (Fig. 4.1) and *P. vindemiae* (Fig. 4.2). The *D. melanogaster* laboratory colony was acquired from stock colonies in the Departments of Entomology and Biological Sciences at Virginia Tech. The colony was maintained in 178-ml,

square-bottom polypropylene drosophila stock bottles (Genesee Scientific Corp., San Diego, CA) on a diet of molasses-based rearing formula (Nutri-Fly™ MF, Genesee Scientific Corp., San Diego CA). Parasitoid colonies were developed from wild-collected individuals, which were caught in southwestern Virginia. Parasitoid colonies were reared in the same type of rearing bottles with the same molasses-based formula, using *D. melanogaster* as host. Insect colonies were housed in environmental chambers with 14 h daylength (18 W “cool white” fluorescent bulbs), 26° C day temp and 23° C night temp.

Olfactometer Bioassays. *Equipment.* To determine if parasitoids of frugivorous drosophilids in Virginia have a preference for host diet, parasitoids were run through a Y-tube olfactometer apparatus. The Y-tube was glass with an inner diameter of 0.5 cm. The main tube was 5.2 cm in length, and each arm was 5.5 cm in length. The entrance chamber was created using 0.6-cm diam Tygon® tubing, and was blocked from behind with glass wool, so that the length of the entrance chamber from the glass wool barrier to the Y-tube was 0.5 cm. A small hole for insect insertion was fashioned at the top of the entrance chamber, and was closed using a modified 1-ml syringe. To reduce external visual stimuli, the Y-tube and entrance chamber were situated within a lidless box (12.7 x 12.7 x 4.2 cm) inlaid with white paper (Fig. 4.3), and a 60W, 120V incandescent soft white light (650 lumens) was situated 30 cm directly above the Y-tube. A white sheet of paper was also situated in front of the box to block other light sources (i.e. windows). Odor sources were contained within 50-ml glass filtering flasks, which were connected to the Y-tube arms via polytetrafluoroethylene (PTFE) tubing. Air was filtered through activated carbon before flowing through the flasks. Air was pulled through the apparatus at a rate of 150 (\pm 10) ml/min using an 115V dual air pump (Second Nature Whisper® 500). The room temperature was maintained at ~25° C.

Preparation. Five host food substrates were used in this experiment: sweet cherry (*Prunus avium* L., Rosales: Rosaceae), red raspberry (*Rubus idaeus* L., Rosaceae), blueberry (*Vaccinium* sp., Ericales: Ericaceae), black table grape (*Vitis vinifera* L., Vitales: Vitaceae), and banana (*Musa acuminata* Colla, Zingiberales: Musaceae). Cherry, raspberry, blueberry, and grape were selected because they are often highly affected by the invasive SWD (Walsh et al. 2011, Asplen et al. 2015, Ioratti et al. 2015). Banana was selected because it has historically been used as trap bait for drosophilids and their parasitoids (Carson 1951, Carson and Stalker 1951, McKenzie 1974, Allemand et al. 2002, Mitsui et al. 2007, Rossi Stacconi et al. 2013). Fruit was acquired from the local supermarket.

Prior to experimentation, fruit to be used was processed and placed in an empty drosophila stock bottle, filling the bottle to the 50 ml mark. Fruit was processed by cutting, slicing, or squishing, so that it could easily fit in the bottle and be easily accessed by *Drosophila* larvae. A cohort of adult *D. melanogaster* (50+) was then placed in the bottle, which was kept in an environment chamber for 5 d. After 5 d, the fruit was well infested with *D. melanogaster* larvae ranging from 1st to 3rd instar, and was ready for use in the *L. bouhardi* olfactometer trials. For *P. vindemiae* olfactometer trials, an extra 2 d were required for infested fruit to be ready, so that pupae were also present with the fruit. Immediately prior to running trials, prepared infested fruit was placed in one of the odor-source flasks of the olfactometer.

Only mated female parasitoids were tested in the olfactometer, based on the assumption that mated females would be more inclined to seek out *Drosophila* larvae or pupae for oviposition. In order to ensure the females were mated, newly eclosed adult male and female parasitoids were collected from the laboratory colonies, and then placed together in a stock bottle with fresh molasses-based food medium and no flies. These parasitoids were allowed to mate in

an environmental chamber for 5 d before experimentation. Parasitoids were kept in a different environmental chamber than the one containing fruit, so they were not exposed to fruit volatiles before experimentation. On the day of the assay run, the mated parasitoids were anesthetized with CO₂, and the females were collected and contained within an empty rearing vial.

Bioassays. In order to verify that mated females of a parasitoid species were attracted to each type of infested fruit, preliminary olfactometer trials were performed using infested fruit as one odor source and clean air as the other. If results showed that parasitoids were indeed attracted to the infested fruit odors, then trials comparing one type of infested fruit to another were performed, until every paired combination was tested. Note that the experiment tested the parasitoids' relative attraction to *D. melanogaster* larvae infesting different fruit types, not the parasitoids' relative attraction to the different fruit types themselves.

Each trial consisted of ≥ 30 runs. To begin a run, one individual was aspirated out of the vial containing mated female parasitoids. The parasitoid was anesthetized with CO₂ and inserted into the olfactometer entrance chamber using a camelhair brush, and then the insertion hole was sealed using the modified syringe. The parasitoid was allowed 5 min in the entrance chamber to revive and enter the Y-tube. If the parasitoid did not enter the Y-tube after 5 min, 'no choice' was recorded. If the parasitoid entered the Y-tube, it was allowed 3 min to make a choice. A choice was defined as the first time a parasitoid walked 1 cm up an arm past the Y-junction. The response time from entering the Y-tube to making the choice was also recorded. If the parasitoid did not choose an arm within 3 min after entering the Y-tube, 'no choice' was recorded. After every 5 runs, the odor sources were swapped to eliminate directional bias. The Y-tube was also flipped 180° so that each odor source had the same corresponding Y-tube arm, in order to prevent intermingling of odors within one arm. Each trial was performed until 30 individuals had made a

choice. Before a new trial began, the Y-tube, flasks, and tubing were cleansed with hexane to eliminate residual odors.

Statistical Analyses. For each trial, a chi-square test was used to determine if parasitoids preferred one odor source to another, i.e. if parasitoids walked toward one odor source significantly more often ($p < 0.05$) than the other. Differences in mean response time to choose an odor source were analyzed using a two-sample t-test. The efficacy of the entire experiment was determined by using a paired t-test and Bonferroni correction to analyze the mean number of individuals making a choice vs. not choosing.

Results

***Leptopilina bouvardi* Trials.** In each of the preliminary trials (infested fruit vs. air), *L. bouvardi* mated females clearly preferred the infested fruit odors to clean air, with chi-square p values < 0.05 (Figs. 4.4). Based on results and chi-square analyses of bioassays comparing two infested fruit odor sources at a time, *L. bouvardi* mated females preferred cherry, raspberry, blueberry, and banana to grape, i.e. grape was the least preferred fruit. Banana and raspberry were also significantly more attractive than cherry. When considering response times to the odor sources in each trial, there was generally no significant difference observed based on the two-sample t-test, with the exception of the cherry/raspberry trial (Table 4.1). Overall, the olfactometer experiment with *L. bouvardi* was highly efficient, where the mean number of individuals that made a choice was significantly greater than the mean number of individuals that did not make a choice, based on a paired t-test (d.f. = 14, $t = -27.275$, $p = 1.549e-13$) (Table 4.2). The trial in which the most individuals did not make a choice was air vs. grape – the least preferred fruit.

***Pachycrepoideus vindemiae* Trials.** Under the same olfactometric conditions as the *Leptopilina* trials, the *P. vindemiae* trials were unsuccessful. During preliminary testing of infested fruit vs. clean air, *P. vindemiae* individuals did not respond to the olfactometer apparatus as well as *L. boucardi* individuals. The *P. vindemiae* females often did not leave the entrance chamber or did not make a choice after entering the Y-tube. Additionally, those that made a choice did not show an observable trend toward either the infested fruit or clean air. Y-tube angle and airflow rate were adjusted several times in an attempt to increase parasitoid responses within the olfactometer, but no such increase was observed. Due to the reluctance of *P. vindemiae* to leave the entrance chamber and make a choice, it was considerably more time consuming to test most individuals, and the goal of 30 choices made for each trial was not reached within time constraints.

Discussion

Larval Parasitoids. The choice test results show that a larval parasitoid of frugivorous drosophilids orients to host larvae better in some fruit olfactory environments than others. Although a specific preference was not demonstrated, *L. boucardi* had a markedly reduced affinity for infested grape than other fruit, and to a lesser extent sweet cherry. These results have important implications for biological control efforts against drosophilid pests using hymenopteran parasitoids. The analyses of response times of *L. boucardi* individuals to make a choice indicate that the relative attractiveness of different fruit odors did not influence the time it took for an individual to make a choice. However, in cases where an odor source was more attractive than the other, the sample size of individuals choosing the less preferred odor source

was so small that the statistical analyses cannot be considered meaningful. Therefore, this discussion is based largely on the choice results.

It is well known that frugivorous drosophilids have a wide host range. For example, SWD is an important invasive pest on a wide variety of fruit crops such as cherries, raspberries, blackberries, blueberries, strawberries, grapes, and others (Goodhue et al. 2011, Hauser 2011, Walsh et al. 2011, Ioratti et al. 2015), and has also been reported on a wide range of non-crop hosts (Lee et al. 2015). Previous studies have shown that in cases where a pest attacks multiple host crops, the associated parasitoid community can vary among those cropping systems (Johnson and Hara 1987, van den Berg et al. 1990). Indeed, some parasitoid species have demonstrated preference for certain host plant volatiles over others, such as the aphid parasitoids *Aphidius uzbekistanicus* (Luzhetski) and *A. ervi* (Haliday) (Powell and Zhi-Li 1983), and the Lepidoptera parasitoid *Cotesia marginiventris* (Ashmead) (Hoballah et al. 2002). As a result, it may take several different parasitoid species to generate effective biological control of a pest attacking multiple crop and non-crop hosts.

Conversely, a review by Denoth et al. (2002) questioned the use of multiple biological control agents. In this review, they examined previous classical biological control efforts and assessed whether releasing multiple biological control agents improves the likelihood of successful control. They actually showed that, of 108 projects, more successful control agent establishments occurred in single-agent projects than in multi-agent projects, possibly as a result of negative interactions among control agents. They also showed that in most of the projects, only one agent was necessary for effective control, and so suggest restraint in releasing multiple control agents (Denoth et al. 2002). However, the review did not address the host ranges of the

pests in the examined biological control projects. Therefore, one wonders if the pests that did require multiple control agents were pests that also had wide host ranges.

In the case of SWD, ongoing research indicates that classical biological control with larval parasitoids is likely to be more effective than conservation biological control using parasitoids of invaded regions (Chabert et al. 2012, Kasuya et al. 2013, Rossi Stacconi et al. 2013, Nomano et al. 2014, Asplen et al. 2015, Daane et al. 2016, Mazzetto et al. 2016, Wahls *unpublished data*). This is likely because of SWD's enhanced ability to resist parasitization by larval parasitoids, especially those that have not evolved sympatrically with SWD (Kacsoh and Schlenke 2012, Poyet et al. 2013). However, recent research in Japan and South Korea has identified hymenopteran larval parasitoids within genera *Leptopilina*, *Ganaspis* (Figitidae), and *Asobara* (Braconidae) as possible candidates for classical biological control, because they appear to specialize on SWD as a host (Kasuya et al. 2013, Nomano et al. 2014, Daane et al. 2016). If these species prefer to attack SWD on certain host plants, like *L. bouhardi* did for *D. melanogaster* in this study, it would suggest that more than one biological control agent might be required for effective suppression of SWD. Such tritrophic interactions should be an important concern when vetting classical biological control agents for SWD. The results would help determine if one species establishment could be effective, or if multiple species are required for control.

Pupal Parasitoids. Although insufficient data were obtained to determine any host food preference of *P. vindemiae*, the reluctance of *P. vindemiae* individuals to enter the Y-tube from the entrance chamber is telling. It is entirely possible, if not probable, that the olfactometry conditions used in this study were unsuitable for *P. vindemiae* individuals to comfortably make a choice. Perhaps red lighting or complete darkness would have been more suitable. However, one

must consider that the odor sources used might not have been very attractive. *P. vindemiae* is a known generalist, hosting on species in several cyclorrhaphous dipteran families besides Drosophilidae, including Tephritidae, Tachinidae, Calliphoridae, Sarcophagidae, Muscidae, Fanniidae, and Anthomyiidae (Carton et al. 1986, Goubalt et al. 2004, Marchiori et al. 2013). As such, *P. vindemiae* may have more general responses to odors than a specialist like *L. bouhardi*, especially considering the very wide range of diets among its hosts. Perhaps *P. vindemiae* individuals are equally attracted to odors of any type of decay, or perhaps olfactory cues are less significant for *P. vindemiae* host-finding. In any case, further experiments are required if conclusions are to be made about odor preferences of *P. vindemiae*.

Conclusions. A larval parasitoid specializing on drosophilids, *L. bouhardi*, demonstrated a low preference for *Drosophila*-infested grape odors relative to other infested fruit odors that were tested. Therefore, further research exploring tritrophic interactions with larval parasitoids of drosophilids is warranted, especially for those being considered for classical biological control against SWD. Such research will provide valuable insight as to the potential efficacy of those parasitoids on different SWD host plants. Because our olfactometric methods were not effective with *P. vindemiae*, similar experiments with different methods should be pursued for this species, which will help determine the importance of *Drosophila*-infested fruit odors as host-finding cues for *P. vindemiae*, and further clarify whether *P. vindemiae* would be an appropriate augmentative biological control agent for SWD.

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Tables and Figures

Table 4.1. Results of two-sample t-tests conducted on mean response times of *Leptopilina* mated females in 15 Y-tube olfactometer trials.

Trial Group 1/Group 2	Mean Response Time (sec)		Conf. Int. ($\mu_1 - \mu_2$)		d.f.	p-value
	Group 1	Group 2				
Raspberry/Air	45.46	23.5	-41.83	85.76	2.0119	0.278
Banana/Air	44.14	76.5	-70.696	5.981	2.0074	0.06823
Blueberry/Air	45.14	28.5	-47.87	81.16	1.7813	0.3503
Grape/Air	48.3	32.67	-25.03	56.29	4.5903	0.3605
Cherry/Air	61.12	35	-20	72.24	7.3681	0.2246
Cherry/Raspberry	14.33	22.04	-13.127	-2.281	17.732	0.007988*
Cherry/Blueberry	40.11	42.25	-28.79	24.51	15.676	0.8669
Cherry/Banana	26.25	40.04	-40.37	12.79	8.6351	0.2691
Cherry/Grape	35.6	28.4	-15.33	29.73	7.7094	0.4803
Blueberry/Grape	50.55	42.12	-19.36	36.2	22.912	0.5367
Grape/Banana	27.2	43.4	-36.181	3.781	9.6239	0.1006
Raspberry/Grape ^a						
Raspberry/Banana	24.88	35.08	-28.561	8.171	18.583	0.2593
Blueberry/Banana	46.94	29.64	-7.381	41.971	22.905	0.1606
Blueberry/Raspberry	39.53	49.6	-40.47	20.34	26.286	0.5023

Response time was the time it took for an individual to make a choice after entering the Y-tube. Mean response times were considered significantly different if confidence intervals of $\mu_1 - \mu_2$ did not include 0, and if $p < 0.05$.

Asterisk indicates a significant difference.

^a The raspberry/grape trial was unable to be analyzed because only one individual chose grape.

Table 4.2. Paired t-test comparing number of *Leptopilina* mated females that made choice vs. those that did not choose in Y-tube olfactometer trials.

Trial	# Choices	# Non-choices
Air v. Che	30	2
Air v. Ras	30	6
Air v. Blu	30	5
Air v. Gra	30	13
Air v. Ban	30	10
Che v. Ras	30	1
Che v. Blu	30	1
Che v. Gra	30	4
Che v. Ban	30	4
Ras v. Blu	30	0
Ras v. Gra	30	2
Ras v. Ban	30	0
Blu v. Gra	30	2
Blu v. Ban	30	0
Gra v. Ban	30	3
Mean	30	3.5333
Paired T-test Results		
t	-27.275	
d.f.	14	
p	1.549e-13*	

T-test result was compared with desired $p = 0.05$ with Bonferroni correction, where $p = 0.05 / m = 0.05 / 15 = 0.003$. Therefore, the result shows that the means are significantly different ($p < 0.003$), and the experiment was highly efficient.



Fig. 4.1. *Leptopilina boulardi*. A larval parasitoid specializing on fruit-feeding *Drosophilidae*.

Photo Credit: Paul Marek.



Fig. 4.2. *Pachycrepoideus vindemiae*. A generalist pupal parasitoid of Diptera, known to attack Drosophilidae. Photo Credit: Paul Marek.

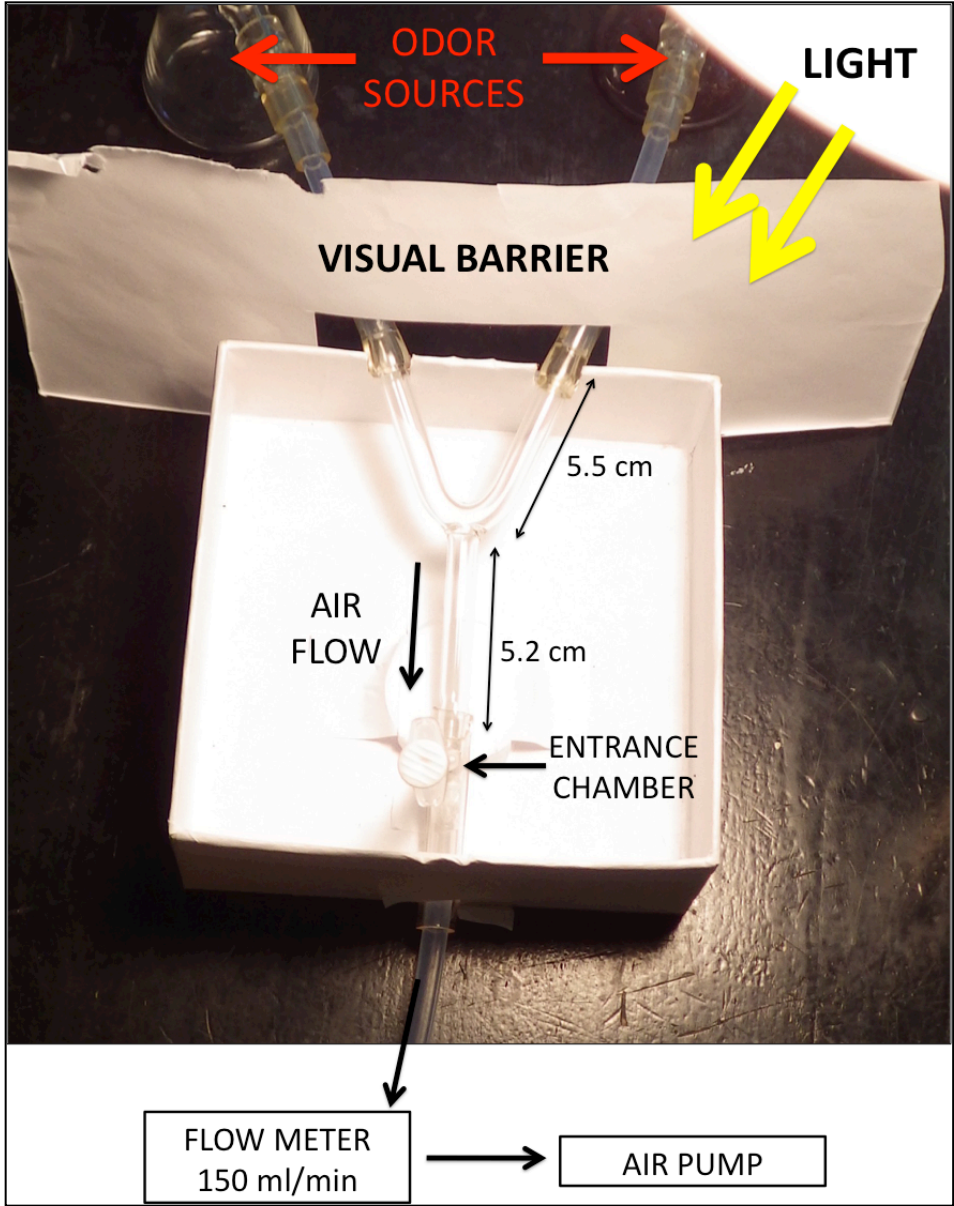


Fig. 4.3. Diagram of the olfactometer apparatus used in this study. Entrance chamber was blocked from behind with glass wool to prevent insects from walking the wrong direction.

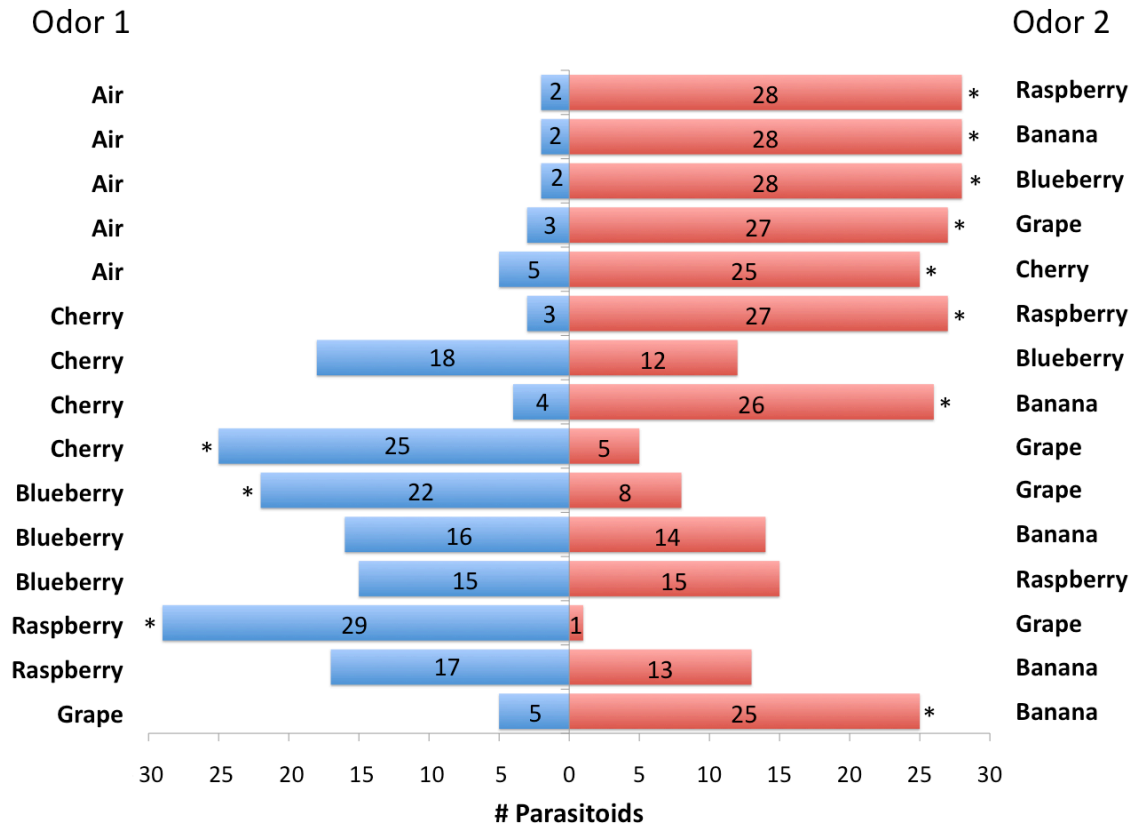


Fig. 4.4. Number of mated female *L. boulandi* choosing odor sources in a Y-tube olfactometer, where each fruit odor source was infested with larvae of *D. melanogaster*. Bars with asterisks are significantly greater than the alternate odor source, based on χ^2 analysis ($p < 0.05$).

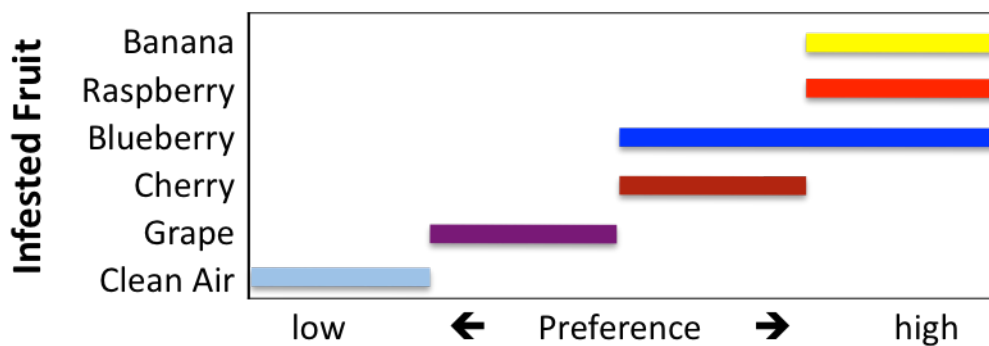


Fig. 4.5. Relative preference of mated female *L. boulandi* for *D. melanogaster* larvae infesting different types of fruit (and a clean air control) based on Y-tube olfactometer results.

Conclusions

Several important conclusions can be made from the research conducted in this project. First, let us sum up the findings from each study:

Chapter 2. Sentinel trapping surveys discovered three parasitic wasp species attacking drosophilids in Virginia cherry and caneberry cropping systems, including larval parasitoids *Leptopilina bouleardi* and *L. clavipes* (Hym.: Figitidae), and generalist pupal parasitoid *Pachycrepoideus vindemiae* (Hym.: Pteromalidae). However, *L. clavipes* appears to be more associated with fungivorous drosophilids versus frugivorous drosophilids, so is not relevant to biological control of *Drosophila suzukii* (spotted wing drosophila, henceforth SWD) or *Zaprionus indianus* (African fig fly, henceforth AFF). The other larval parasitoid, *L. bouleardi*, did not develop on SWD or AFF in the sentinel traps, just *D. melanogaster* or other contaminating drosophilids. That *L. bouleardi* did not develop on SWD was consistent with previous findings in the literature. The pupal parasitoid, *P. vindemiae*, did successfully develop on SWD on one occasion, so *P. vindemiae* can and will develop on SWD in Virginia. This result was also consistent with previous findings in the literature, except that other studies reared more *P. vindemiae* from SWD in the field. Because only one parasitoid was reared from SWD and none were reared from AFF, conservation biological control for these species is unlikely to be effective in Virginia.

Chapter 3. To follow up the sentinel trapping results, the ability of *L. bouleardi* and *P. vindemiae* to parasitize *D. melanogaster*, SWD, and AFF was examined in laboratory. Under controlled conditions, results were consistent with our sentinel trapping results and previous studies from the literature. Specifically, *L. bouleardi* could successfully parasitize *D. melanogaster* but not SWD. Results also suggest that AFF is not an attractive host for this strain

of *L. boulandi*. Therefore, *L. boulandi* should not be considered for biological control of SWD or AFF. Conversely, *P. vindemiae* was able to successfully parasitize each of the tested fly species, demonstrating its generalist behavior. Because *P. vindemiae* can overcome the defenses of SWD and can also parasitize AFF, *P. vindemiae* might be considered as an augmentative biological control agent. However, its generalist and hyperparasitic nature described in the scientific literature raise concerns about reduced control efficiency and non-target effects.

Chapter 4. An olfactometer study was used to determine if host-finding behavior of parasitoids of frugivorous drosophilids (*L. boulandi* and *P. vindemiae*) is influenced by the type of fruit their host infests. Among cherry, raspberry, blueberry, grape, and banana (all infested with *D. melanogaster*), *L. boulandi* oriented to grape the least, and oriented to banana and raspberry more than cherry. Unfortunately, *P. vindemiae* did not respond well to the olfactometer set-up, so insufficient data was collected for analysis. The results show that *L. boulandi* is at least partially selective about which fruit its host is feeding in, and perhaps this behavior extends to other specialist larval parasitoids that are being considered for biological control of SWD. Such tritrophic selectivity suggests that biological control may be somewhat more effective in some cropping systems than others. If this is the case with parasitoids of SWD, it means that effective biological control of SWD may require more than one biological control agent to cover the affected crops. Further investigation of such tritrophic interactions is required.

Overall, we can conclude that conservation biological control will not be suitable for controlling SWD or AFF in Virginia, and biological control with the Virginia strain of *L. boulandi* will be ineffectual. Augmentative biological control with *P. vindemiae* might be possible, but may also be inefficient or too great a risk. Consequently, successful biological

control for SWD will more likely come from a classical biological control program, using specialist parasitoids—perhaps several species—that have evolved sympatrically with SWD. The same might be true for AFF, but the pest status of AFF in North America remains uncertain, so it is unclear whether biological control will be necessary.