For: Biological Control

Title: Pupation behavior and larval and pupal biocontrol of *Drosophila suzukii* in the field

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Abstract

Biocontrol may play an important role in IPM of *D. suzukii*, a worldwide fruit crops pest. Predation on *D. suzukii* has been observed in the field, but rates have not been measured. Additionally, targeting juveniles may be more effective than targeting adults for reducing population sizes. The first objective of this study was to measure naturally-occurring levels of biocontrol on *D. suzukii* juveniles. Furthermore, *D. suzukii* may either pupate inside of fruit or emerge to pupate in the soil. Knowing the location of pupae can be important for developing biological and cultural control programs. The second objective of this study was to determine the proportion of *D. suzukii* pupating in fruit versus soil. Predator exclusion experiments were conducted to measure the ability of natural enemies to reduce *D. suzukii* larvae in strawberry and blueberry fruits, and pupae in the soil underneath strawberry, blueberry, and blackberry. Funnels were used to direct larvae dropping from infested blueberry and raspberry fruits into soil-filled containers. Significantly fewer *D. suzukii* larvae were extracted from fruits exposed to predators, representing a reduction in larval survival of 19–49%. Significantly fewer *D. suzukii* pupae were recovered from soil exposed to predators, representing a 61–91% decrease in pupae. In the pupation behavior experiment, 82–93% of pupae were found in soil, significantly more than were found in fruit. Ants and spiders were commonly observed in a variety of sampling methods, and ants were observed on video to remove pupae from the soil.

Keywords: spotted wing drosophila, biological control, natural enemies, pupation behavior

1. Introduction

Drosophila suzukii (Diptera: Drosophilidae), spotted wing drosophila, is a worldwide pest of fruit crops (CABI, 2016). A native of Asia, *D. suzukii* was first discovered in North America and Europe in 2008 (Rota-Stabelli et al., 2013), and South America in 2013 (Deprá et al., 2014). *D. suzukii* infests a number of commercially grown fruits, including raspberries, blackberries, strawberries, cherries, and blueberries (Lee et al., 2011a), and a variety of ornamental and wild fruits in the field (Kenis et al., 2016; Lee et al., 2015). Unlike most drosophilid flies, *D. suzukii* has a serrated ovipositor that allows it to lay eggs in ripening and ripe fruit, damaging fruit just as it is ready for harvest. This can lead to considerable yield losses (Bolda et al., 2010; Goodhue et al., 2011), or rejection of crops because of a zero tolerance for damaged fruit in the fresh fruit market (Bruck et al., 2011). Avoiding such economic losses necessitates costly management actions such as

an additional 5-8 insecticide sprays during a single growing season (Van Timmeren and Isaacs, 2013). Such intensive insecticide regimes may lead to insecticide residue levels that exclude fruit treated for *D. suzukii* from foreign markets (Haviland and Beers, 2012). Furthermore, there are few effective, organic insecticides for controlling *D. suzukii* (Beers et al., 2011; Bruck et al., 2011; Van Timmeren and Isaacs, 2013).

For homeowners who are reluctant to use chemical insecticides, and for organic growers who have few chemical control options, biological control may play an important role in *D. suzukii* management. Some entomopathogenic nematodes and fungi have been shown to induce mortality in *D. suzukii* adults (Naranjo-Lazaro et al., 2014; Woltz et al., 2015) and to reduce adult emergence from infested fruit (Gargani et al., 2013). However, none have demonstrated reduction in overall population growth (e.g. Woltz et al. 2015). The generalist pupal parasitoids *Pachycrepoideus vindemmiae* (Rondani) (Hymenoptera: Pteromalidae) and *Trichopria cf. drosophilae* Perkins (Hymenoptera: Diapriidae) are able to develop on SWD and cause significant mortality in the lab (Chabert et al., 2012; Gabarra et al., 2015). These two species are also the only two thus far to be recovered from *D. suzukii* in the field in the invaded range in North America and Europe (Gabarra et al., 2015; Rossi Stacconi et al., 2013). However, parasitism rates in the invaded range were too low to provide sufficient control (Miller et al., 2015). Parasitoids in the native range were found to exhibit up to 17% parasitism, and are currently being studied in quarantine (Daane et al., 2016).

Several laboratory feeding trials have been conducted to assess natural enemy willingness to consume *D. suzukii*. The earwig *Labidura riparia* (Dermaptera: Labiduridae) killed approximately 77% of exposed *D. suzukii* larvae and pupae offered in Petri dishes when starved (Gabarra et al., 2015). Two anthocorids have been similarly evaluated. *Orius insidiosus* Say (Hemiptera: Anthocoridae) decreased survival of *D. suzukii* larvae in blueberry fruits by 12-50% in lab trials but were not able to reduce populations in controlled field trials (Woltz et al., 2015). *Orius laevigatus* (Fieber) (Hemiptera: Anthocoridae) significantly increased mortality of *D. suzukii* eggs in strawberry fruits, but did not prey on larvae (Gabarra et al., 2015). The staphylinid *Dalotia* (= *Atheta*) *coriaria* Kraatz (Coleoptera: Staphylinidae) has also been evaluated in two studies. While individual *D. coriaria* did not reduce *D. suzukii* survival in infested blueberry fruits (Woltz et al., 2015), *D. coriaria* density was negatively related to adult *D. suzukii* emergence from rotting raspberry fruits (Renkema et al., 2015).

In the field, a number of predatory hemipterans have been found to occur in *D. suzukii*-infested fruit, including *Dicyphus tamaninii* Wagner (Hemiptera: Miridae), and three anthocorids (Hemiptera: Anthocoridae): *Orius laevigatus* (Fieber), *Cardiastethus nazarens* Reuter, and *C. fasciventris* Garbiglietti (Arnó et al., 2012; Gabarra et al., 2015). *Orius* sp. were also observed eating *D. suzukii* larvae (Walsh et al., 2011). However, nothing is known about the potential for natural enemies to reduce *D. suzukii* populations in the field. Identifying other natural enemies and understanding background consumption levels can aid in the development of conservation biological control programs. Additionally, *D. suzukii* populations are characterized by a large proportion of eggs, larvae, and pupae, and a relatively small proportion of adults (Wiman et al., 2014). As a consequence, targeting juvenile stages of *D. suzukii* should be much more effective at reducing overall populations than targeting adults only (Wiman et al., 2014, 2016). Therefore, the first objective of this study was to measure naturally-occurring levels of biocontrol on *D. suzukii* larvae and pupae.

Furthermore, while it is well known that *D. suzukii* eggs are laid in fruit and that larvae develop in fruit, pupation behavior is more ambiguous. *D. suzukii* may either pupate inside of fruit with spiracles protruding through the fruit skin, or they may emerge from fruit as larvae before pupating in the surrounding medium (Kanzawa, 1939). The pupation behavior of *D. suzukii* impacts the potential for biocontrol as pupae inside of fruit may be more protected from predation than pupae that have emerged from fruit to pupate in the soil. Furthermore, a different set of natural enemies forages on foliage than those that forage on or in the soil. An understanding of what percentage of individuals emerge from fruit to pupate, and how this varies by crop, can help in the development of conservation biological control programs by concentrating focus on either foliar or ground predators. Cultural control measures can also be targeted to specific locations where pupae are more likely to be found. Thus, the second objective of this study was to determine the proportion of *D. suzukii* that pupate in fruit versus the soil.

2. Materials and Methods

Drosophila suzukii used in experiments were from a laboratory colony started from infested fruits collected in Oregon. Wild *D. suzukii* were added to the colony each year. The colony was fed diet comprised of 45 g agar, 125 g cornmeal, 200 g sugar, 70 g nutritional yeast, 4.7 l dH₂O, 17.7 ml propionic acid, 3.3 g methyl paraben, 33.3 ml 95% ethanol, and a sprinkle of baker's yeast on the surface. Flies were provided with water via a wet cotton pad held in a petri dish. Flies were maintained at 22°C, 16L: 8D, and 60% RH.

2.1 Larval biocontrol

Field experiments and natural enemy sampling were conducted at the USDA-ARS experimental farm in Corvallis, OR, (44°33'14.20"N, 123°13'09.57" W). Ambient biocontrol of *D. suzukii* larvae was assessed in 'Shuksan' and 'Rainier' strawberries, potted 'Liberty' blueberries, and planted 'Elliott' blueberries.

Straw- to blush-colored strawberry fruits were enclosed in a 38 cm long x 50 cm diameter bag made from white organza (Jo-Ann Stores, LLC., Hudson, OH) to prevent naturally occurring *D. suzukii* infestation. A drawstring at the top of each bag was pulled tight around a 3 x 3 cm sponge placed next to the plant branch, to make a tight seal. Three strawberry fruits located close to one another were enclosed in a single bag and treated as a "cluster." At fruit ripening, five 2 wk-old mated female *D. suzukii* were enclosed in each bag for 24 h to standardize infestation levels to the extent possible. All infestations were made between 30 May and 12 June 2014 and between 18 May and 3 June 2015. *D. suzukii* were provided with a wet sponge and a 30% sucrose solution wick. Flies were removed after 24 h. At that time, half of the bags were removed to create a "Predator Access" treatment, and the other half remained bagged to create a "Predator Exclusion" control. Clusters were distributed randomly throughout the strawberry rows. Dataloggers (Onset HOBO dataloggers, model U23-002, Onset Computer Corporation, Bourne, MA, USA) were attached to one predator access and one predator exclusion cluster on each day that infestations were made, and left to record temperature and RH until those clusters were collected.

Six-year-old Liberty blueberry plants in 20-L pots were spaced 1 m apart in a 5 x 12 grid underneath shade cloth. Eleven-year-old Elliott blueberry plants were part of a 0.25 ha commercially-managed research plot. Plants were spaced 0.76 m apart in rows spaced 3 m apart. Green- to blush-colored clusters of 15-20 blueberry fruits were enclosed in organza bags to prevent naturally occurring *D. suzukii* infestation. At fruit ripening, eight 2 wk-old mated female *D. suzukii* were enclosed in each bag for 24 h as described above. Infestations in potted Liberty blueberries were made between 17 and 30 July 2014. Infestations in Elliott blueberries were made between 11 Aug. and 3 Sept. 2015. Predator access and exclusion treatments were created as above. Clusters were distributed randomly among blueberry plants. Dataloggers were attached to one predator exclusion and one predator access cluster for each day of infestation, as described above.

A week after infestation, fruits were collected and taken back to the lab. The number of remaining fruits were counted as some of the experimental fruits were missing (i.e. had fallen from the plant or had been eaten). The number of *Drosophila* larvae in each cluster was assessed via the salt flotation method (Dreves et al., 2014). Briefly, fruits were gently crushed and submerged in a 10 Brix salt water solution. Larvae were collected and counted as they emerged from the fruits. Additionally, natural enemies that emerged from the fruits were identified to family or order.

The natural enemy community was assessed by vacuum sampling with a leaf blower (Echo ES-2000, Echo Inc., Lake Zurich, IL). In strawberries, 10 m long sections of row were vacuumed between 6 and 19 June 2014 and 4 and 10 June 2015. Two to four samples were taken from each row, with an equal number of samples from the two varieties, for a total of 26 samples in 2014 and 14 samples in 2015. In potted Liberty blueberries, each plant was vacuumed on 7 and 13 Aug. 2014. All the plants in a given row were included in a single sample, for a total of 10 samples. Vacuuming of potted blueberry plants was conducted after the biocontrol assessment to avoid reducing the natural enemy community during the assay. In Elliott blueberries, 10 m long sections of row were vacuumed in a sinusoidal pattern along the entire width of the canopy, between 16 June and 14 July 2015, for a total of 12 samples. All samples were taken on sunny days between 8:45 and 13:30. Vacuum samples were frozen and insects and arachnids within each sample were identified to family or order.

2.1.1 Larval Biocontrol Statistical Analysis

All analyses were conducted using SAS software, version 9.4 (SAS Institute Inc., Cary, NC, USA). Because some of the originally infested fruits were not recovered, the total number of larvae collected in each sample was divided by the number of fruits collected for that sample. The number of larvae/ fruit was compared between predator access and predator exclusion treatments using general linear mixed models in PROC GLIMMIX. Infestation date was included as a random block to account for environmental variations during the experiment. Separate analyses were conducted for each year for each variety of strawberry and blueberry. For potted Liberty blueberries in 2014 and Elliotts in 2015, the larvae/fruit were square root transformed to meet the assumptions of normality and homogeneity of variance. Average minimum, maximum, and mean temperature and RH were calculated for predator access and exclusion clusters in both strawberries and blueberries for each date of the larval biocontrol experiment using PROC MEANS. These daily averages were compared between access and exclusion clusters with Wilcoxon Signed Rank Test, a non-parametric test for paired samples that can be used as an alternative to the paired t-test when the

assumption of normality is violated, in PROC UNIVARIATE. Data for strawberries and blueberries were analyzed separately.

2.2 Pupation behavior

Pupation behavior in blueberry fruits was evaluated at a commercial U-Pick orchard in Corvallis, OR (44°35'59.58" N, 123°13'24.32" W). Green- to blush-colored clusters of blueberry fruits were enclosed in organza bags to prevent naturally occurring *D. suzukii* infestation. At fruit ripening, 2 wk-old mated female *D. suzukii* were enclosed in each bag for 24 h. *D. suzukii* were provided with a wet sponge and a 30% sucrose solution wick. After 24 h, *D. suzukii* adults were removed and a 2 L plastic funnel was placed under each infested cluster (Figure 1 A, B) to direct dropping fruit or larvae into a 1.2 L Tupperware (225 cm² square opening, Rubbermaid, Atlanta, GA) filled to a depth of 3 cm with potting soil (Metro-Mix ® 840PC, SunGro Horticulture, Agawam, MA). Four holes were drilled in the bottom of each container and covered with fine-mesh netting to allow water from overhead irrigation to drain from the containers. Each funnel rested on top of a 25 cm tall, 18 cm diameter cylinder made of 0.64 cm hardware cloth secured with zip ties. The entire set-up was enclosed in a 140 cm long, 90 cm diameter sleeve (Figure 1, C) made of no-see-um netting (Skeeta, Bradenton, FL). The sleeve was tied at the bottom of the cylinder and secured to the stem of the fruit cluster with a butterfly hair clip.

In 2014, infestations took place in two late-season varieties, on 12 and 13 Aug. in eight rows of 'Berkeley' blueberries and on 25 Aug. in seven rows of 'Dixie' blueberries. Eight female flies were enclosed with clusters of 15-20 fruits. A total of 47 late-season samples were collected in 2014. In 2015, infestations took place in two mid-season varieties, on 29 June, 7 and 13 July in six rows of mixed 'Bluecrop' and 'Blueray' blueberries. Additionally, infestations were made on 4, 5, and 11 Aug. in six rows of late-season Berkeley blueberries. In all infestations in 2015, 6 female flies were enclosed with clusters of 20-25 fruits, in an effort to decrease infestation rates from the previous year. A total of 21 mid-season and 41 late-season samples were collected in 2015.

The experiment was repeated in raspberries in a seedling field at the USDA-ARS experimental farm in Corvallis, OR, (44°33'14.20"N, 123°13'09.57" W). Green raspberry fruits were enclosed in organza bags to prevent naturally-occurring infestation. Three to five closely spaced raspberry fruits were included in a single bag and treated as a "cluster." At fruit ripening, each cluster was infested with four 2-week old mated female *D. suzukii*. Twenty-six replicates were infested between 2 and 9 Sept. 2014. Thirty-one replicates were infested between 21 July and 10 Aug. 2015. Infested raspberry fruits were enclosed as described above.

After 14 d, samples were collected and returned to the lab for processing. Fruits attached to the plant, fruits that had dropped to the soil, and the soil itself were collected into separate labeled plastic bags. Fruits were dissected and the number of *D. suzukii* larvae and pupae were counted. The soil was sieved through 0.1 and 0.2 cm sieves to remove soil particles substantially larger and smaller than *D. suzukii* pupae. Then remaining soil was examined and *D. suzukii* larvae and pupae were counted.

2.2.1 Pupation Behavior Statistical analysis

The average infestation level of each trial was calculated as the total number of juvenile *D. suzukii* recovered in each sample, divided by the total number of berries in that sample. The number of larvae and pupae in the soil versus in fruit (combined hanging and fallen fruits) were compared with Wilcoxon Signed Rank Test in PROC UNIVARIATE. Data from mid-season blueberries, late-season blueberries, and raspberries were analyzed separately within each year. To assess whether a fruit dropping increased the likelihood of pupation occurring outside the fruit, Pearson correlation coefficients were calculated in PROC CORR for the proportion of fruit that dropped to the ground and the proportion of pupae that were found in the soil, for each of the datasets.

2.3 Pupae biocontrol

Biocontrol on *D. suzukii* pupae was evaluated in 2015 at the USDA-ARS experimental farm in Corvallis, OR, (44°33'14.20"N, 123°13'09.57" W) in Shuksan and Rainier strawberries, a mixed-variety experimental blueberry planting, and a mixed-variety experimental blackberry planting.

Pupae were collected from diet exposed to the *D. suzukii* colony. Large petri dishes containing infested diet were placed on a metal shelving unit such that 3rd instar larvae crawling out of the diet would fall to metal trays placed on the shelf below. The larvae pupated on the metal trays and were collected within 48 h and placed in a -80° C freezer until used in the field.

The ground cover in each crop (soil in strawberries and blackberries, sawdust in blueberries) was used to completely fill 8 cm diameter Petri dishes. Ten pupae were placed on top of each dish, and lightly covered with additional soil or sawdust. In the predator access treatment, dishes were buried flush with the ground surface underneath the crop canopy. In the predator exclusion treatment, dishes were placed within fine-mesh bags to prevent access to arthropod predators. The predator exclusion treatment was used to account for pupae lost in sample processing. Replicates were placed in strawberries on 26 May, 3 and 9 June; in blueberries on 13,

23 and 29 June, and 6 July; and in blackberries on 13 and 20 July. Soil or sawdust containing pupae was collected after 48 h and sieved in the lab. Remaining pupae were counted.

2.3.1 Pupae Biocontrol Statistical Analysis

The number of pupae recovered per sample was compared between predator access and predator exclusion treatments using general linear mixed models (strawberries and blackberries) or generalized linear mixed models (pupae recovery data for blueberries were fit to a negative binomial distribution), in PROC GLIMMIX. Sample deployment date was included as a random block to account for environmental variations during the experiment. Separate analyses were conducted for sentinel pupae in strawberries, blueberries, and blackberries.

2.3.2 Video recording

In a subset of predator access treatments, digital surveillance equipment was used to record predators foraging on *D. suzukii* pupae. Adapting the methods of Grieshop et al. (2012), three water-proof surveillance cameras (Alibi 600 TVL IR, Supercircuits, Austin TX, USA) were deployed for 48 h on 26 May and 3 June in strawberries and 13 and 23 June in blueberries. Cameras were suspended 11 cm above the ground surface via conduit clamps attached to metal conduit pounded vertically 30 cm into the soil. The cameras were connected by coaxial cables to a one-channel MPEG-4 Mobile DVR (MDVR25HR, Supercircuits, Austin, TX, USA), where video was recorded onto a 32 GB SD card. The cameras and DVR were powered by a pair of 12 V marine batteries (ML22-12, Maxlife Battery, Kumeu, New Zealand) linked in parallel. The DVR and batteries were housed in a 28 L (28 x 40 x 25 cm) plastic storage tub with lid to keep the equipment dry. Holes were cut in the sides of the tub to feed power and coaxial cables through and to allow for ventilation to prevent the DVR from over-heating. Video could be viewed as it was being recorded on a portable LCD TV (SC-195, SuperSonic Inc, Commerce CA, USA) connected to the output channel of the DVR, to confirm proper camera placement.

2.3.3 Video processing

After 48 h of recording, SD cards were taken to the lab for video viewing. Four video recordings were watched simultaneously at 4x speed to identify frames with arthropod activity. Relevant frames were then viewed one-at-a-time at normal speed. Arthropods entering the petri dishes were identified to Family or Order. Arrival and departure times and behaviors of each arthropod were recorded. Arthropods of the same taxonomic group that left and re-entered the screen within 5 min were considered a single individual. An arthropod of the same taxonomic

group that re-entered the screen after a period of >5 min was recorded as a separate individual.

3. Results

3.1 Larval biocontrol

Significantly fewer *D. suzukii* larvae were extracted from fruits in predator access treatments than from fruits in predator exclusion controls, for all fruit varieties in both years (Table 1). Infestation rates were high in strawberry fruits, > 18 larvae per fruit, based on the average number of larvae per fruit recovered in the predator exclusion controls (Figure 2). In the presence of predators, infestations were reduced by an average of 3.6 to 6.6 larvae per fruit. Initial infestation rates were much lower in blueberry fruits, <1.5 larvae per fruit, with correspondingly smaller numerical decreases in larvae in the presence of predators (Figure 3). Across experiments, natural enemies reduced larval survival by between 19 and 49%, based on estimates of least square means for the treatments.

Average daily minimum, maximum, and mean relative humidity and temperature were often similar between predator exclusion and predator exclusion treatments, for both the strawberry and the blueberry larval biocontrol experiments (Table 2). In the strawberry experiment, the average daily and average maximum daily temperatures were higher in the predator exclusion, while the average minimum daily temperature was higher in the predator access treatment. In the blueberry experiment, only the average daily RH was higher in the predator access clusters than in the predator exclusion clusters. Although in each case, the actual difference represents a few degrees or one percent of RH.

The most common natural enemies collected in vacuum samples were anthocorids, ants, parasitoid wasps, and spiders (Table 3). The most common natural enemies found inside infested fruits when samples were processed were ants, parasitoid wasps, staphylinids and spiders (Table 4).

3.2 Pupation behavior

The average infestation level in the pupation experiments was low in blueberries (less than 1.3 juveniles/berry) and slightly higher in raspberries (Table 5). During the course of the pupation experiments, 60-80% of the infested fruit dropped to the soil surface. Between 87 to 98% of the immature *D. suzukii* had reached the pupal stage at the time that the samples were processed. The vast majority of those pupae, 82-93%, were found in soil (Table 5). No significant correlations were

found between the proportion of fruits in a sample that dropped to the ground and the proportion of pupae in that sample found in the soil.

In blueberries and raspberries in both 2014 and 2015, significantly more pupae were found in soil than in fruits (Table 5; Figure 4). In all crops except 2015 raspberries, significantly more of the larvae that had not yet pupated at the time that samples were collected were found in fruit than in soil (Table 5).

3.3 *Pupae biocontrol*

Significantly fewer *D. suzukii* pupae were recovered from predator access treatments than from predator exclusion treatments, in strawberries, blueberries, and blackberries, representing an approximate 61 – 91% decrease in pupae (Table 6), based on the estimates of least square means for each treatment.

A total of 481 hours of video was recorded of pupae biocontrol samples, during which 674 observations of arthropod activity were documented. The most common natural enemies observed were ants (356 observations), spiders (106 obs.), and opiliones (59 obs.). Centipedes (9 obs.) and earwigs (14 obs.) were also observed. Additionally, ants were the only natural enemy observed to dig up *D. suzukii* pupae and carry them away, which was recorded on 39 independent occasions.

4. Discussion

This study provides important information about the pupation behavior of *D. suzukii* and the subsequent consumption of pupae on the ground by natural enemies. Although *D. suzukii* have been observed to sometimes emerge from fruit to pupate, and have been collected from the soil underneath strawberry plants (Gabarra et al., 2015), it was unknown how frequently pupation occurred on the ground. In the pupation behavior study, the majority of pupae were found in soil, while larvae that had not yet pupated at the time of collection were predominantly found in fruit, suggesting that larvae typically emerged from fruit and dropped to the ground to pupate. When more than one *D. suzukii* larvae is developing in a fruit, one might expect fruit to decay faster, increasing the chances of a larva emerging from fruit to pupate. However, in this study, *D. suzukii* were observed to pupate in the soil at both relatively low infestation levels in blueberries and somewhat higher infestation levels in raspberries. Infestation levels in blueberries were estimated to be less than 1 or 2 individuals per fruit. Similarly, between 0.76 to 2.7 *D. suzukii* emerged per blueberry from fruit collected from an unsprayed field (from Fig. 3. in Lee et al., 2015) suggesting that our infestation levels were within the range of what has been observed in the field.

Furthermore, there was a lack of correlation between the proportion of fruits in a sample that dropped to the ground and the proportion of pupae in that sample found in the soil. This suggests that whether a fruit remained hanging on the plant or dropped to the ground had no effect on the behavior of the pupae. In other words, the majority of pupae were found in the soil, even for samples with few or no dropped fruits. Limiting fruit on the ground through in-field sanitation is already considered a critical part of *D. suzukii* management (Goodhue et al., 2011; Lee et al., 2011b). However, these data suggest that pupae may be dropping onto the ground from hanging fruits, necessitating additional management to control pupae in the soil. Knowing this, cultural management tactics that disrupt the pupal stage warrant investigation. Currently, weed mats are being examined as a way to suppress immature *D. suzukii* (V. Walton et al. *pers. comm.*). Dark weed mats are expected to become hotter throughout the day than the soil underneath, exposing infested fruit or larvae that drop to the ground to higher temperatures. Additionally, the weed mat may interfere with the ability of pupating larvae to bury themselves, increasing the time larvae are exposed to high temperatures and predation. Both high temperature and predation are sources of mortality for wandering *Anastrepha* spp. larvae (Diptera: Tephritidae) between the time they emerge from fruit and the time they bury themselves (Aluja et al., 2005).

Wiman et al. (2016) demonstrated that the majority of individuals in a *D. suzukii* population are in the juvenile stage once oviposition begins. For this reason, controlling adults with chemical sprays has limited effect on the overall population size and must be repeated throughout the growing season, whereas controlling larvae or pupae has more promise for reducing overall population sizes. Natural enemies significantly reduced *D. suzukii* larvae in strawberry and blueberry fruits, as well as pupae in strawberry, blueberry, and blackberry plantings. There are a few important caveats to address for these experiments. For the pupal biocontrol study, the risk of predation for pupae in the soil varies with pupation depth (Hodgson et al., 1998). All pupae in this study were placed near the soil surface, where they would have had the highest risk of predation. However, Kanzawa et al. (1939) demonstrated that *D. suzukii* typically pupate at a depth of no more than 4 mm, which would make them fairly accessible to natural enemies and is shallower than pupation depths for which predation differences were seen in *Anastrepha* spp. (Hodgson et al., 1998).

For the larval biocontrol study, which included treatments with and without mesh bags, the potential for between-treatment differences in temperature and humidity to affect *D. suzukii* survival and development should be considered (Kinjo et al., 2014; Tochen et al., 2016, 2014). There were slight differences in temperature between the treatments in the strawberry portion of

the larval biocontrol experiment. However, the direction of the differences was not consistent: the minimum temperature was 0.7 °C higher in the predator access (unbagged) and the overall average daily temperature was 1 °C higher in the predator exclusion (bagged). The biggest difference was ~4 °C higher average maximum temperature in the predator exclusion. It is unlikely that the nearly 39 °C daily maximum temperature could explain the higher survival of larvae in the predator exclusion than predator access treatment. Based on studies, 30 °C is near the limit of tolerance for *D. suzukii* development; oviposition, egg-hatching, and both larval and adult survival are low at this temperature (Kinjo et al., 2014; Tochen et al., 2014). If anything, the higher maximum temperatures in predator exclusion treatments in strawberries might have reduced larval survival. In the blueberry larval biocontrol experiment, all environmental measures were the same between treatments except for a ~1% difference in average RH. Similar developmental and reproductive parameters for *D. suzukii* were found at 71 and 82% RH (Tochen et al. 2016), a much larger difference in RH than found between the predator access and exclusion treatments in this study. In general, survivorship would be expected to be high at the average daily RH and temperature found in both treatments of both experiments (Tochen et al., 2016, 2014).

This study provides important information about the role of natural enemies in consuming *D. suzukii* juveniles in small fruit crops. Five groups of natural enemies were observed or collected repeatedly from a variety of different sampling methods. Ants and spiders were the most commonly observed natural enemies on video, were among the most abundant natural enemies in vacuum samples, and were additionally observed inside infested fruit. Parasitoid wasps and anthocorids were common in vacuum samples, and staphylinids were observed inside infested fruits. Spiders, staphylinids, and anthocorids have been directly observed eating *D. suzukii* individuals in the field (AJ Dreves *pers. obs*), and furthermore, the natural enemy taxa collected and observed in this study are known to prey on juveniles of other similar orchard pests. Ground predators such as ants, spiders, staphylinids and carabids have been reported to prey on wandering third instar larvae, pupae, and teneral adults of fruit flies (Diptera: Tephritidae) in orchards (Aluja et al., 2005; Hennessey, 1997; Hodgson et al., 1998; Monzó et al., 2011, 2009; Thomas, 1995; Urbaneja et al., 2006; Wong and Wong, 1988).

Given that ants were observed to dig up and carry off pupae in the pupae biocontrol experiment, they seem a promising target for conservation biological control. Ants are often credited as a major source of mortality for juveniles of orchard pests. Ants have been credited with consuming the majority of pupae of Caribbean fruit fly *Anastrepha suspense* (Loew) in soils in a carambola grove (Hennessey, 1997) and the majority of Mexican fruit fly *Anastrepha ludens* (Loew)

third instar larvae and pupae from the soil under citrus plants in Texas (Thomas, 1995). Ants were also responsible for the majority of mortality of *Anastrepha* spp. larvae emerging from fruit to pupate in soil in an experiment in Veracruz, Mexico (Aluja et al., 2005). Although *D. suzukii* is a drosophilid and not a tephritid, we have demonstrated that it participates in the same behavior of emerging from fruit to pupate, and therefore may be susceptible to similar types of predation as wandering third instar larvae or pupae in the soil.

Natural enemies are actively consuming *D. suzukii* juveniles in fruit crops, and their efficacy may be enhanced through conservation biological control. Wandering larvae and pupae on the ground may be a better target for conservation biological control than larvae inside fruit. Larvae inside fruit are somewhat protected from predators, especially immediately after infestation when the fruits are still firm. Natural enemies are rarely found inside infested fruit until the fruit decomposition is fairly advanced (JMW *pers. obs.*). In contrast, the shallow pupation depth of *D. suzukii* make them more accessible to predators. These observations are supported by the higher removal rates of pupae than of larvae in the respective biocontrol experiments. Populations of ground predators have been increased in fruit crops using habitat management practices like aisle ground cover plantings (O'Neal et al., 2005) and surface mulches (Mathews et al., 2004; Thomson and Hoffmann, 2007). Research on the potential for such practices to increase predation on *D. suzukii* pupae would also have to assess whether *D. suzukii* are independently affected by changes to the microclimate these practices may cause. Habitat management practices could be used simultaneously with weed matting to make the soil surface directly under plants more hostile for *D. suzukii* juveniles. Conservation biological control techniques may provide growers with much needed tools for combatting *D. suzukii*. Furthermore, the ubiquitous nature of the natural enemies observed in our study suggest that biocontrol could play a substantial role in suppressing pest populations in surrounding unmanaged areas. Ambient biological control in unmanaged areas near farms should be further examined, as *D. suzukii* uses many wild hosts and may build up populations in such areas.

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Table Captions

Table 1. Comparison of *D. suzukii* larval abundance between treatments where predators have access and no access to infested fruit. N indicates the number of replicate clusters for each treatment of a given variety. Significantly fewer larvae were recovered from predator access than predator exclusion treatments for all varieties and crops.

Table 2. Minimum, maximum, and average daily temperature and % relative humidity values recorded in the strawberry and blueberry larval biocontrol experiment, for the predator access and predator exclusion treatments. Values in bold were found to be significantly different between treatments ($p < 0.05$).

Table 3. Species composition of natural enemies collected in vacuum samples in larval biocontrol experiment.

Table 4. Species composition of natural enemies collected from inside infested fruit in larval biocontrol experiment.

Table 5. *D. suzukii* pupal location during fruit ripening and larval-pupal interface. Significantly more pupae were found in the soil in each experiment.

Table 6. *D. suzukii* pupae reduction due to predation. Significantly fewer pupae were recovered from predator access than predator exclusion treatments for all fruits. The numerical and percent decrease in number of recovered pupae indicate how many fewer *D. suzukii* pupae were found, on average, when the samples were exposed to predators.

Figure Legends

Figure 1. *D. suzukii* pupation experiment. Clusters of blueberries were infested with *D. suzukii* larvae and monitored for approximately 14 d to assess whether larvae remained in fruit or dropped to the soil to pupate. A & B) Dropping fruit and larvae were directed into a container of soil by a funnel supported on a metal cylindrical frame. C) The entire set-up was enclosed in mesh to prevent predation on the *D. suzukii* during the experiment.

Figure 2. Mean ($\pm$ S.E.) number of *D. suzukii* larvae per strawberry, by variety and year, in strawberries that predators have access to, or from which predators have been excluded. The percentages above each set of bars indicate the decrease in larvae in the predator access treatments. The asterisks (*) indicate whether the treatments were significantly different (** $p < 0.01$, * $p < 0.05$, * $p < 0.1$).

Figure 3. Mean ($\pm$ S.E.) number of *D. suzukii* larvae per blueberry, by variety and year, in blueberries that predators have access to, or from which predators have been excluded. The percentages above each set of bars indicate the decrease in larvae in the predator access treatments. The asterisks (*) indicate whether the treatments were significantly different (** $p < 0.01$).

Figure 4. Mean ($\pm$ S.E.) number of pupae from the pupation experiment found in soil or in fruits, by variety and year. The asterisks (*) indicate that the number of pupae found in soil and fruit were significantly different (** $p < 0.01$).

Figure1

Figure2

Figure3

Figure4

Table 1. Comparison of *D. suzukii* larval abundance between treatments where predators have access and no access to infested fruit. N indicates the number of replicate clusters for each treatment of a given variety. Significantly fewer larvae were recovered from predator access than predator exclusion treatments for all varieties and crops.

Crop	Year	Variety	N predator exclusion, predator access	F, p
Strawberry	2014	Shuksan	64, 60	$F_{1,89}=12.35$; $p=0.0007$
		Rainier	65, 57	$F_{1,85}=3.67$; $p=0.059$
	2015	Shuksan	69, 47	$F_{1,107}=4.44$; $p=0.0374$
		Rainier	59, 43	$F_{1,91}=8.21$; $p=0.0052$
Blueberry	2014	Liberty	63, 61	$F_{1,102}=4.35$; $p=0.0395$
	2015	Elliott	112, 104	$F_{1,205}=7.33$; $p<0.0001$

Table 2. Minimum, maximum, and average daily temperature and % relative humidity values recorded in the strawberry and blueberry larval biocontrol experiment, for the predator access and predator exclusion treatments. Values in **bold** were found to be significantly different between treatments ($p<0.05$).

		Minimum	Maximum	Average
Strawberry Temp (°C)	Access	10.66 ± 0.54	34.82 ± 1.68	19.85 ± 0.76
	Exclusion	9.94 ± 0.47	38.65 ± 1.99	20.83 ± 0.83
% RH	Access	48.07 ± 4.23	99.96 ± 0.04	85.33 ± 1.73
	Exclusion	46.77 ± 6.60	99.93 ± 0.07	83.62 ± 2.45
Blueberry Temp (C)	Access	11.02 ± 0.70	29.61 ± 1.07	19.38 ± 0.58
	Exclusion	11.04 ± 0.67	29.05 ± 1.12	19.35 ± 0.57
% RH	Access	39.44 ± 3.02	93.88 ± 1.24	67.84 ± 2.28
	Exclusion	38.48 ± 3.01	93.73 ± 1.27	66.47 ± 2.55

Table 3. Species composition of natural enemies collected in vacuum samples in larval biocontrol experiment.

	Strawberries 2014	Strawberries 2015	Blueberries 2014	Blueberries 2015
# of samples	26	14	10	12
Dermaptera	1	0	0	2
Anthocoridae	82	18	0	0
Nabidae	7	0	0	0
Reduviidae	2	1	0	0
Formicidae	144	35	4	0
Parasitoid	694	303	12	39
Coccinellidae	12	2	1	0
Cantharidae	1	1	0	0
Staphylinidae	2	0	0	0
Aranaea	219	128	50	9
Opiliones	3	8	0	0

Table 4. Species composition of natural enemies collected from inside infested fruit in larval biocontrol experiment.

	Strawberries 2014	Strawberries 2015	Blueberries 2014	Blueberries 2015
# of samples	246	218	124	216
Dermaptera	0	0	1	0
Formicidae	1	3	0	1
Parasitoid	3	2	1	0
Staphylinidae	18	3	0	0
Aranaea	1	1	18	2

Table 5. *D. suzukii* pupal location during fruit ripening and larval-pupal interface. Significantly more pupae were found in the soil in each experiment.

Fruit	Year	Mean juveniles/berry ¹	% pupae in found soil	More pupae in soil than fruit	More larvae in fruit than soil
Blueberry late-season	2014	1.30 ± 0.19	92.77 ± 0.02	$S = 110.5; p < 0.0001$	$S = -56.5; p = 0.0004$
Raspberry		4.46 ± 0.78	89.76 ± 0.04	$S = 448.5; p < 0.0001$	$S = -88.5; p < 0.0001$
Blueberry mid-season	2015	0.21 ± 0.04	82.53 ± 0.06	$S = 69.5; p = 0.001$	$S = -15.5; p = 0.04$
Blueberry late-season		0.70 ± 0.08	81.99 ± 0.03	$S = 423.5; p < 0.001$	$S = -59.5; p = 0.01$
Raspberry		2.28 ± 0.59	83.01 ± 0.06	$S = 171.5; p < 0.0001$	$S = 2.5; p = 0.75$

1. Mean juveniles/ berry indicates combined number of larvae and pupae recovered in each sample, divided by the number of berries in that sample, and serves as an approximation for the infestation rate.

Table 6. *D. suzukii* pupae reduction due to predation. Significantly fewer pupae were recovered from predator access than predator exclusion treatments for all fruits. The numerical and percent decrease in number of recovered pupae indicate how many fewer *D. suzukii* pupae were found, on average, when the samples were exposed to predators.

Crop	N	F, p	Numerical decrease in pupae	% decrease
Strawberry	soil = 68; excl = 15	$F_{1,81}=11.61$; $p=0.001$	5.95 ± 0.94	61%
Blueberry	Sawdust = 96; Excl =	$F_{1,128}=128.09$;	8.40 ± 0.38	91%
Blackberry	Soil = 35; Excl = 35	$F_{1,66}=104.01$; $p<0.0001$	6.44 ± 0.63	67%