Optimizing the Salt Filter Test to Monitor Spotted Wing Drosophila Larval Infestation Levels in Blueberries

Amelia R. Davis

RESEARCH SUPERVISOR

Mr. Steven Van Timmeren, M.S.
Research Technician at Trevor Nichols Research Center,
Michigan State University Entomology Department
Fennville, MI

FACULTY SPERVISOR

Dr. Santiago Salinas, Ph.D.
Assistant Professor of Biology,
Kalamazoo College,
Kalamazoo, MI

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Abstract

Spotted wing drosophila (SWD), or *Drosophila suzukii,* is an invasive pest that has cost the United States millions of dollars in crop losses since its first detection in the country in 2008. SWD develops through three larval instar stages between egg and adult and its short generational turnover and unique morphology makes it a serious threat to soft-skinned fruit agriculture. The salt filter test monitoring method described in this study offers accurate quantification of larvae in all instar stages. However, until the present study, variables such as incubation time, fruit crushing, and solution type had not yet been optimized for the salt filter test method. This study found that salt filter testing was significantly more accurate than unfiltered assessment methods for quantifying SWD larvae of all instar stages in blueberries. Additionally, for detecting first instar larvae, allowing at least 30 minutes for incubation is recommended, but for second and third instar larvae, incubation times higher than 15 minutes did not significantly increase larval yield. Similarly, fruit crushing is critical for first instar detection, but not for second and third instar detection. Solution type did not have a significant effect on total larval yield at any instar stage, but did affect activity levels, suggesting that motionless larvae killed by salt water are better for salt filter testing whereas active larvae in distilled water may be better for unfiltered assessment. This optimized technique offers growers accurate information regarding SWD infestation levels in their farms, allowing them to increase the sustainability, effectiveness, efficiency, and affordability of their pest management programs. Future studies should investigate whether larval movement aids in unfiltered assessment. Additionally, researchers should utilize low-level infestation to test for a difference between squished and firm berries for detecting large larvae, as fruit decomposition from heavy infestation in this study may have masked a difference.

Introduction

The total agricultural output of the United States makes up 10.1 percent of global agricultural output, making the United States one of the top agricultural producers in the world, only second to China (Alston & Pardey, 2014). In the United States, agricultural and food industries make up 5.4 percent ($1.053 trillion) of the gross domestic product (GDP), additionally accounting for 11% of employment in the country (USDA, 2019). With so much invested in its agriculture, the United States has strong incentives to manage the organisms that may harm its crops and weaken its global production status. Certain agricultural products, such as soft-skinned fruits, are especially susceptible to bird and insect damage. In fact, most species of finch and sparrow love to eat blackberries, which can result in substantial berry losses for commercial growers that do not use bird netting (Dellamano, 2006). Blueberry maggots attack blueberries, which also reduces crop yields and makes the fruit less marketable (Pelz *et al.,* 2005). However, one of the most harmful pests to soft-skinned fruits is a member of the Drosophila genus: *Drosophila suzukii*.

The appearance of the invasive *Drosophila suzukii,* or spotted wing drosophila (SWD) in Europe and the Americas in 2008 has had severe impacts on the agriculture industry. The pest was first spotted in the contiguous United States in California, but quickly spread to Oregon and Washington within a year (Lee *et al.,* 2011). During the first year of SWD activity in the country, these three states, which account for 76% of total United States commercial soft-skinned fruit production value, saw a 20% yield loss of soft-skinned fruits (Bolda *et al.,* 2010). Of all the fruits evaluated in this study, blueberries suffered the worst value reduction. Over the next three years, SWD reached states in the Midwest, the South, and even the East Coast (Lee *et al.,* 2011). Michigan, as

the leading blueberry-producing state, is one place where the pests have caused severe crop damage (Longstroth & Hanson, 2014). In order to grasp the seriousness of SWD's threat to the American fruit industry, it is important to understand its unique biology.

Unlike other species of vinegar flies, such as *Drosophila melanogaster*, which are drawn to overripe or damaged fruit, *Drosophila suzukii*, or lays eggs in ripening fruit (Asplen *et al.,* 2015). Due to their serrated ovipositors, SWD vinegar flies are capable of penetrating and infesting a wide variety of soft-skinned fruits, including blueberries, raspberries, strawberries, and cherries (Lee *et al.,* 2011). These factors, alongside the flies' high reproductive potential and rapid generational turnover, have put substantial pest pressure on growers. Heavy infestation of fruits by SWD larvae cause the fruits to break down and degrade, making them unmarketable in the worst cases. In addition to crop losses, farmers have suffered the economic burdens of expensive insecticide chemicals, increased production labor, and monitoring tools (Lee *et al.,* 2011).

Drosophila suzukii is native to the Asian continent. Its primary distinguishing traits are the large serrated ovipositor found on females and the dark spots found on the edges of the leading wings on males (Asplen *et al.,* 2015). SWD develop from eggs deposited underneath the skin of soft fruits. According to Atallah *et al.* (2014), flies may stab the fruit skin with their ovipositors several times before successful oviposition (i.e. egg deposition). This activity leaves scars that can be detected under a microscope. If successful, a female may oviposit between one and three eggs per hole (Dreves *et al.,* 2014). After 2 days, the flies emerge from eggs and go through three larval instar stages, distinguishable by size and number of teeth on their mouth hooks (Figure 1; Van Timmeren *et al.,* 2017). Each instar stage lasts about 2 days. The larvae feed on fruit tissues until they pupate and emerge as adult flies about 8 days after being laid as eggs

(Lee *et al.,* 2011). Larvae may either pupate inside the fruit or leave it to pupate. Adult flies are capable of laying eggs 1 to 3 days after emerging and their fecundity decreases slightly with age (Asplen *et al.,* 2015). Fecundity estimates range from about 6 to 25 eggs laid by a mature female per day (Emiljanowicz *et al.,* 2014; Asplen *et al.,* 2015). Given SWD's life history traits, from its rapid generational turnaround to its high fecundity, pest management programs and infestation monitoring are critical to preserving the fruit industry.

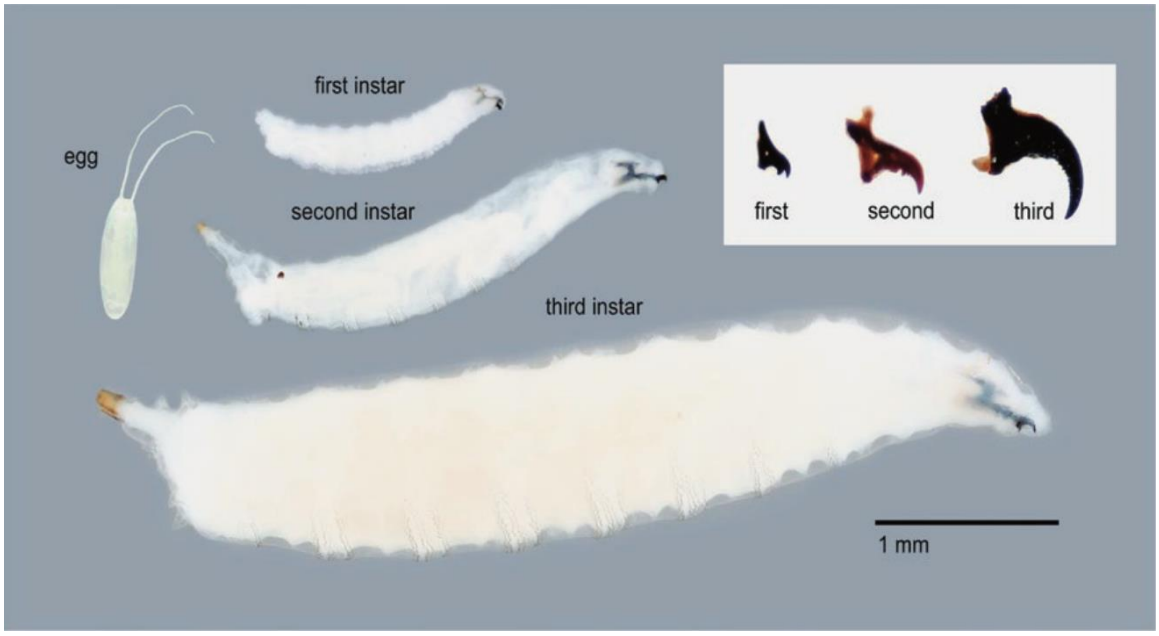

Figure 1. *Drosophila suzukii* egg, instars, and mouth hooks. Egg and first instar were preserved directly in 95% ethanol. Second and third instars were prepared by fixing live larvae in recently boiled water and storing in 95% ethanol. Images were gathered using stacking software. Image and legend from Van Timmeren *et al.* (2017) with permission.

However, these pest management programs must be sustainable. The term "integrated pest management" (IPM) refers to ecosystem-based strategies that emphasize long-term prevention of pest damage. A major goal of integrating a variety of management strategies is to minimize economic damage while also minimizing impacts on non-target organisms (Stenberg, 2017). Optimizing SWD larvae monitoring methods

is in line with goals to decrease insecticide usage both because it can be expensive and because it can have unintended environmental impacts (Ndiath, 2019). For example, excessive insecticide use could lead to pesticide resistance in SWD, be toxic to other insects including critical pollinators, and even result in health concerns for consumers of sprayed fruit, agricultural workers, and people living in the vicinity of regularly sprayed farms (Orozco *et al.,* 2011). If growers have easy access to accurate data on the infestation rates of their crops, they can better regulate their insecticide usage and only spray when there is a serious threat of infestation. They can also evaluate whether biological controls or other IPM strategies are sufficient to manage the problem.

Several methods have been suggested to monitor infestation. Researchers have used baited traps to quickly monitor for the presence of adult flies (Asplen *et al.,* 2015; Lee *et al.,* 2011). However, this method only provides information about the quantity of adults in the area; monitoring larvae quantity instead can provide information about present and future fruit infestation and potentially give growers enough time to take action before their crops are too infested to market. One such method is to collect a sample of berries from the field and rear the larvae inside the fruit to adulthood (Burrack, 2014). This has advantages in that flies can be confirmed to be SWD, but this process can be time-consuming and labor-intensive. For pest management programs requiring quick responses to early infestation, this rearing method would not be a useful tactic. Instead, researchers have adapted several liquid incubation larval extraction methods originally used for other insect species in order to monitor SWD larval infestation.

Some early studies on cherry maggots cooked cherries or froze them for larval analysis, but these processes take time and have been less effective than splitting the cherries open and letting larvae develop to larger, more visible stages (Frick, 1953).

Neilson and Lawrence (1986) and Dixon and Knowlton (1994) studied maggots too, but utilized a liquid density separation method with brown sugar water such that blueberries were crushed and larvae floated to the top of the liquid. This method was faster, but researchers noted that in each sample, some maggots did not float to the top. Hot water, in which larvae sink, has also been tested with Western cherry fruit fly larvae, but has similar drawbacks (Yee 2011, 2014). While assessment methods like these that use the naked eye have been somewhat successful in lab conditions, they may be less effective in the field. Without ideal lighting and magnifying lenses, the ability to see and count larvae varies with light conditions (weather, time of day), stillness of the water, color of the assessment background, and the visual acuity of the assessor.

More recently, another monitoring method known as the salt filter test has been developed to assess SWD larvae quantity in blueberries and other fruits (Van Timmeren *et al.*, 2017). Put most simply, the test involves lightly crushing a fruit sample, incubating the fruit in salt water to extract larvae, filtering the larvae into a coffee filter, and then assessing the larvae under a stereomicroscope. The utilization of a coffee filter allows the SWD larvae to be assessed under a microscope because filtering greatly reduces the depth of field. Microscopic assessment with the filter method helps to control some of the variables that affect unfiltered methods, like light conditions and visual acuity of the assessor. The method is more accurate for assessing smaller larvae and cuts test time almost in half compared to unfiltered methods using lighted magnifiers (Van Timmeren *et al.*, 2017). Because the salt filter test uses a stereomicroscope, it also makes identifying different instar stages of the larvae easier, which may be difficult to assess with the naked eye. Information regarding early infestation levels is key to advising insecticide usage and the success of control programs; if a majority of smaller, first instar larvae are

detected, growers may have enough time to implement an insecticide solution to curb the infestation and save the fruit, whereas if many third instar larvae are detected, the fruit may be unmarketable (Van Timmeren *et al.,* 2017).

Although the salt filter test has many benefits compared to other methods, it is still a relatively novel procedure. The method as outlined in Van Timmeren *et al.* (2017) describes lightly crushing sample blueberries, submerging them in a salt solution for one hour, and then sifting the sample into a coffee filter. However, rigorous testing has not yet been conducted to optimize variables such as incubation time, fruit squishing, and solution type. The purpose of this study was to develop an accurate, efficient, and cost-effective procedure for monitoring SWD larval infestation levels in blueberries. This was achieved by conducting four experiments that sought to address the following: 1) how filtering the larvae from the berry solution and assessing under a stereomicroscope affected counts of larvae extracted from berries compared with unfiltered naked-eye counts, 2) how berry incubation time in salt solution before filtering affected counts of larvae extracted from berries, 3) how lightly crushing berries prior to incubation affected counts of larvae extracted from berries, and 4) how the type of solution used to incubate the berries affected counts of larvae extracted from berries.

Materials and Methods

Study Site and Organisms Used

This study was conducted between June and August 2019 at the Trevor Nichols Research Center (TNRC) in Fennville, MI in Allegan County. The vinegar flies used for infestation in these experiments were SWD from a laboratory colony established from flies reared out of wild fruit samples collected from an unsprayed site in Van Buren county in 2018. The flies were 6 days old upon time of fly loading (adding flies to the infestation cups) and were from generations F9-F15.

General Experimental Procedures

The general procedure for all experiments involved berry infestation, oviposition hole counts, salt filter testing, and filter assessment. A complete "run" of a given experiment spanned one week. Three "runs" were conducted for all four experiments.

Berry Infestation

Store-bought blueberries (Rainier Fruit Company organic blueberries from Washington state) were used in all experiments. The berries were first rinsed in tap water and allowed to air dry. All soft berries were discarded in case of pre-existing infestation. Seventeen berries were added to a 473 ml clear plastic deli cup such that the berries fit snugly in a circle around the bottom ring of the cup without rolling around. Each cup was fitted with a lid with a cloth mesh hole in the top to allow air inside. Five male and ten female SWD were knocked out with carbon dioxide (Figure 2A) and transferred to a small plastic condiment lid which was carefully placed in the center of the berry circle in each cup (Figure 2B). After fly loading, the mesh lids were placed back on the deli cups and the cups were left for 24 hours in environmental chambers set to 23.4°C.

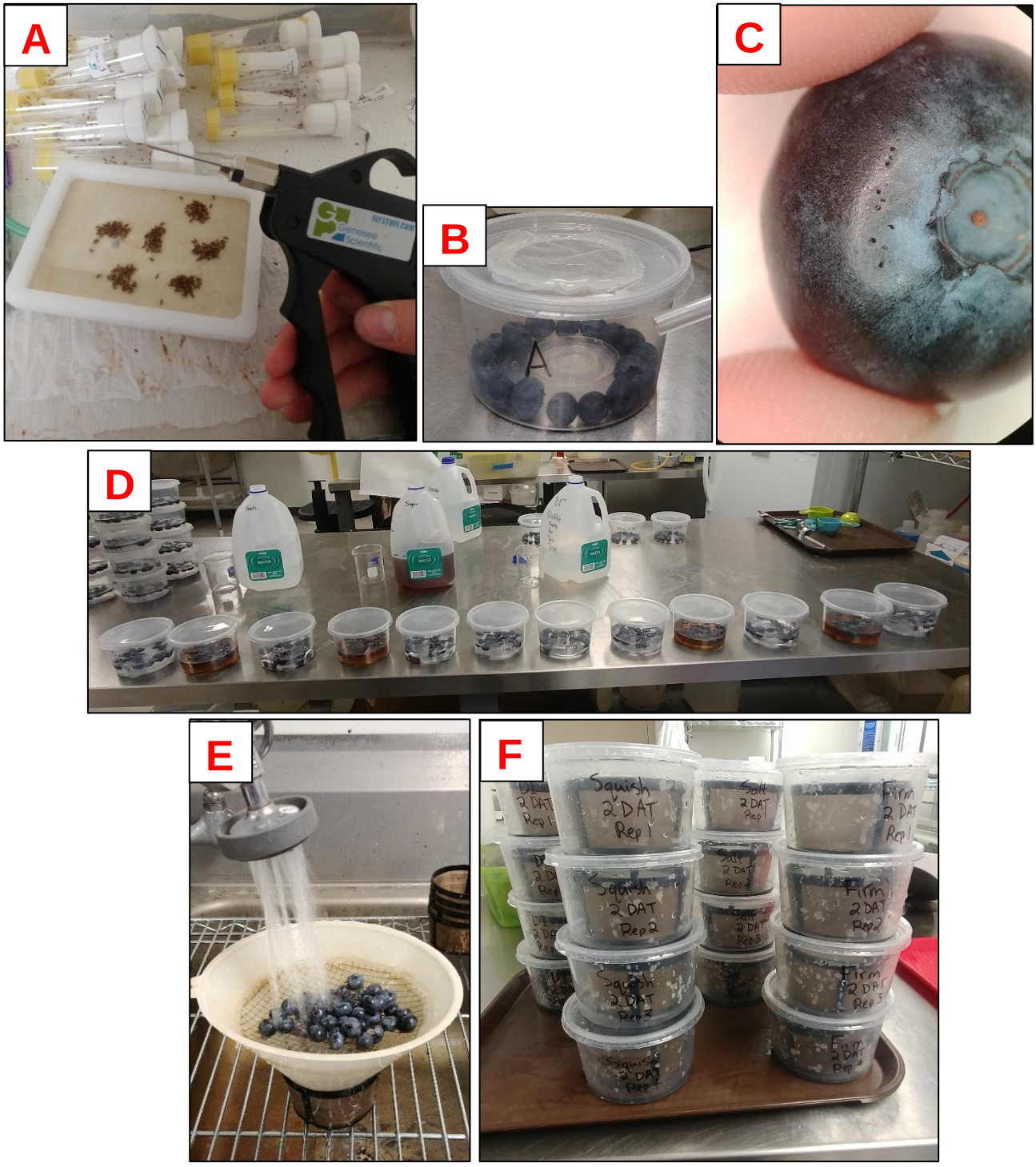

Figure 2. Visual overview of materials and methods. Image A depicts a CO_2 gun and knocked-out SWD flies on the fly pad. Image B depicts an infestation cup with a mesh lid and 17 berries arranged in a circle around the edges of the cup. Image C depicts oviposition holes on a blueberry under a light microscope. Image D depicts a line of cups incubating in liquid. Image E depicts the rinsing process with the industrial sink sprayer, mesh-lined funnel, and coffee filter underneath. Image F depicts finished salt test cups ready for assessment.

After 24 hours, the flies were extracted one by one with an insect aspirator and killed in a collection cup partially filled with 95% ethanol. Fly extraction was conducted on the cups in the same order as fly loading to improve the equalization of fly egg-laying time. In order to ensure that there would be a relatively similar number of eggs per cup, two things were done. First, the berries were redistributed into new cups, ensuring that each of the new cups received some berries from cups that had been loaded earlier and some from cups that had been loaded later. This controlled against slight variations in infestation (egg-laying) time as well as possible cases of high fly mortality or abnormal fecundity in original infestation cups. Second, the mass of berries in each cup was recorded to ensure relatively similar masses between cups, as flies may prefer to lay eggs in larger berries. After this, each of the new seventeen-berry cups was fitted with a solid (hole-less) lid and stored in the environmental chambers at 23.4°C.

Oviposition Hole Counts

Between 24 and 48 hours after treatment (before the developing SWD emerged from their eggs) 3-6 cups were selected for oviposition hole counts. Each berry in each cup was examined under a light microscope and oviposition holes were counted (Figure 2C). The counting of oviposition holes helped to determine whether infestation was successful and that relatively similar numbers of eggs were present between cups. In all cases, they were.

Salt Filter Tests

Salt testing took place at three different times: 2 days after treatment (DAT), 4 DAT, and 6 DAT, with “treatment” being the time when the berries were first exposed to the flies (fly loading). These DAT times targeted the different growth stages of SWD larvae; two days after treatment, most larvae were at the first instar stage, while four days

and six days after, most larvae were at the second instar and third instar stages respectively.

Using nitrile gloves, all seventeen berries were lightly crushed in the 473 ml deli cup. On ripe berries, the “light crushing” pressure just breaks the skin. Then about 125 ml of 1.3 M salt water (Cargill brand Top-Flo granulated salt, Cargill Salt, Minneapolis, MN) were poured over the gloves and into the cup to ensure that any larvae on the gloves were washed into the cup. A lid was placed on the cup and the berries were left to incubate with the liquid at room temperature for one hour (Figure 2D).

At the expiration of the incubation time, the lid of each deli cup was removed and the berries and liquid were poured through a funnel (Plews 48 oz plastic utility funnel, Model #75-064, Plews & Edelmann, Dixon, IL) with a wire mesh berry filter (Mat Midwest 23-gauge hardware cloth, Mat Holdings, Inc., Long Grove, IL) with a reusable coffee filter placed underneath to catch the flowthrough (Medelco 12 cup basket coffee filter, Model #BF215, Medelco, Inc., Bridgeport, CT). Using an industrial sink sprayer with tap water at a flowrate of about 225mls/sec, the inside of the deli cup was rinsed into the filter and the berries were gently disturbed and rinsed for about 10 seconds (Figure 2E). The berries were then discarded, the funnel filter was rinsed, and the coffee filter was put in the deli cup and lidded (Figure 2F). Cups were assessed immediately for larva contents under a microscope (see Filter Assessment below) or stored in the fridge at around 1.7°C until assessment. If assessment was delayed, some liquids as follows were added to the cup to preserve the sample. For delayed assessment of more than 6 hours, about 20 ml of tap water was added, for more than 24 hours, about 75 ml of tap water was added, and for over 72 hours, about 75 ml of 5% ethanol solution was added to the cup.

Filter Assessment

Using a stereomicroscope set to 5-10x magnification, the coffee filter was examined. Eggs, pupae, and first, second, and third instar larvae were classified by size and the number of each recorded. Classification helped to confirm that 2 DAT assessment targeted first instar larvae, 4 DAT assessment targeted second instar larvae, and 6 DAT targeted third instar larvae. After data collection, the filters were rinsed and washed in a dishwasher for reuse.

Statistical Analysis

Systat 13 software was used to conduct statistical analyses (Systat Software, Inc., Chicago, IL). Total egg, pupa, and larva counts for each DAT within each treatment were averaged and compiled among all three runs of each experiment. Egg and pupa counts were included in total larval yield averages. Data were tested for normality using a Shapiro-Wilk test and tested for homogeneity of variances using a Levene's test. Non-normal data were log(X+1) transformed to achieve normality. The analysis types utilized varied by experiment and will be specified in the following sections.

Specific Experimental Procedures

The four experiments conducted utilized the procedures described above, but each with slight variations, described below. The experimental design is summarized in Table I.

Table I. Summary of experimental design.

Experiment	Treatments	Number Replicates per Treatment	Statistical Test(s) Used	Predicted Relative Larval Yield Between Treatments
Unfiltered Vs Filtered	Unfiltered with naked eye, Filtered with microscope	11	t-test	Filtered>Unfiltered
Incubation Time	15, 30, 45, and 60 minutes	12	ANOVA, Tukey's HSD	60>45>30>15
Squish Vs Firm	Squished, Firm berries	12	t-test	Squished>Firm
Solution Type	Salt water (1.3 M), Distilled water, Sugar water (0.64 M)	12	ANOVA	Salt>Sugar>Distilled water

Unfiltered Vs Filter Experiment

This experiment aimed to simulate the conditions of unfiltered assessment methods without magnification like those described in Yee (2012) and Dreves *et al.* (2014) and compare the accuracy to that of the salt filter test described in Van Timmeren (2017). For the Unfiltered Vs Filter Experiment, an unfiltered assessment was conducted just prior to berry rinsing. After one hour in salt water, the cups were taken to a garage with the overhead garage door open to allow sunlight to enter. The cups were placed on a dark brown background and the total number of larvae seen with the naked eye within approximately 45 seconds was recorded for each cup. Following this, each cup was rinsed in accordance with the normal salt test procedure. The Student's two-tailed t-test was used to compare mean larvae totals between unfiltered assessment and filtered assessment. It was predicted that filter assessment would be more accurate (yield more larvae) than unfiltered assessment because it is easier to detect small larvae under a microscope and because filter assessment eliminates some uncontrolled visual condition variables like lighting and visual acuity.

Incubation Time Experiment

This experiment aimed to increase the time efficiency of the salt test by testing different liquid incubation times. While Dreves *et al.* (2014) suggest that 15 minutes is sufficient if scouts are only interested in the presence or absence of larvae, Van Timmeren *et al.* (2017) recommends one hour to ensure that the maximum number of larvae have left the fruit interior. For the Incubation Time Experiment, incubation time in salt water varied among the four time treatments of 15 minutes, 30 minutes, 45 minutes, and 60 minutes. A one-way analysis of variance (ANOVA) was used to compare the mean larvae totals between groups. Post-hoc means separation in the ANOVA test was conducted using Tukey's honest significant difference (HSD) test. It was hypothesized that the 60-minute incubation condition would yield the most larvae and that accuracy would decrease as incubation time decreases.

Squish Vs Firm Experiment

This experiment also aimed to increase the time efficiency of the salt test by testing whether or not berry squishing is necessary for test accuracy. Although some researchers argue that squishing the fruit helps the salt solution permeate the fruit pulp and reach larvae, other researchers found no difference in larval yield between squished and not squished berries (Dreves *et al.,* 2014; Hueppelsheuser, 2010). For the Squish Vs Firm Experiment, berries in the "squish" treatment were crushed in accordance with the general experimental procedure, while berries in the "firm" treatment were not crushed. The Student's two-tailed t-test was used to compare mean larvae totals between the two treatments. It was predicted that squishing is necessary and that the squish condition would have higher larval yields than the firm condition.

Solution Type Experiment

This last experiment targeted both the accuracy and the cost-effectiveness of the test. Salt water, sugar water, and distilled water have all been used to draw larvae out of berries (Dreves *et al.*, 2014; Hueppelsheuser 2010; Yee 2011, 2014). However, sugar water is roughly twice as expensive as salt water; for example, Meijer, Inc. (Grand Rapids, MI) sells Meijer-brand light brown sugar at 75 cents per pound and Meijer-brand table salt at 42 cents per pound (Meijer Grocery). The cost of the salt or sugar is added to the cost of distilled water to make salt or sugar solutions, thus plain distilled water is the most cost-effective between the three liquids. For the Solution Type Experiment, salt water was used in accordance with the general procedure for the salt treatment. However, distilled water was used for the distilled water treatment and 0.64 M brown sugar water (Meijer brand light brown sugar, Meijer, Inc., Grand Rapids, MI) was used for the sugar treatment. Even though tap water is arguably the most cost-effective liquid, it was not used in this experiment because of the mineral level variability of tap water from taps in different locations, which could impair repeatability of the experiment (Ander *et al.*, 2016). A one-way analysis of variance (ANOVA) was used to compare the mean larvae totals between treatments. It was hypothesized that salt water would have the highest larval yields, followed by sugar water and distilled water.

Results

Oviposition hole counts of representative cups were similar, indicating that infestation occurred and was relatively equal between all cups in all runs. A maximum of four eggs were found per filter assessment (almost all egg counts were zero) and a maximum of four pupae were found per filter assessment (almost all pupae counts were also zero) between all cup assessments. Their effect on total SWD averages is thus virtually negligible and these averages are referred to as total larval yield or average larval yield.

Unfiltered Vs Filter Experiment

Across all runs of the experiment and at DAT, the filter assessment was more effective than the unfiltered assessment at detecting larvae (Figure 3). Two days after infestation, filter assessment yielded significantly more larvae than unfiltered assessment ($t_{10} = -10.01$, $P < 0.001$). A similar result was also observed at four days after treatment ($t_{10} = -7.81$, $P < 0.001$) and six days after treatment ($t_{10} = -4.71$, $P = 0.001$).

Two days after treatment, filter assessment in this study yielded 2.69 times as many larvae as unfiltered assessment. The difference in effectiveness between the methods decreased however at four days and six days after treatment, where filter assessment in this study yielded 1.58 times and 1.40 times as many larvae as unfiltered assessment (Figure 3).

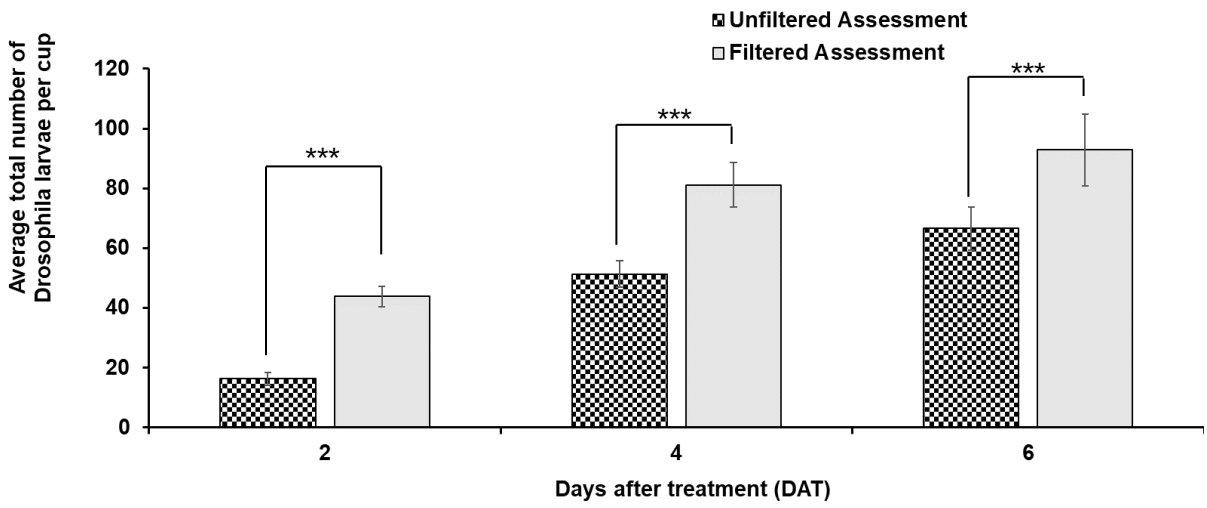

Figure 3. Mean total SWD larval yields by assessment type for the Unfiltered Vs Filter Experiment. Error bars are displayed as standard error. Larval yields by filtered assessment were significantly higher than by unfiltered assessment for all three DAT. Three asterisks (***) indicate significance at the 0.001 level. DAT is the number of days after berries were first exposed to flies.

Incubation Time Experiment

Incubation time influenced the total larvae yield per cup for the 2 DAT group, but not for 4 DAT or 6 DAT (Figure 4). At two days after treatment, there was a significant effect of incubation time on total larval yield ($F_{3, 44} = 8.15$, $P < 0.001$). Post hoc comparisons using the Tukey HSD test indicated that the mean total larvae detected in the 15-minute incubation time treatment was significantly lower than the mean total larvae detected in the 30-minute ($P = 0.02$), 45-minute ($P = 0.001$), and 60-minute ($P = 0.001$) incubation times. However, the 30-minute, 45-minute, and 60-minute incubation times did not significantly differ from each other at 2 DAT.

At four ($F_{3, 44} = 1.5$, $P = 0.23$) and six ($F_{3, 44} = 0.79$, $P = 0.51$) days after treatment, there was no significant effect of incubation time on total larval yield. However, the average larval yields for 15-minute incubation times tended to be lower than those of the other incubation time treatments (Figure 4).

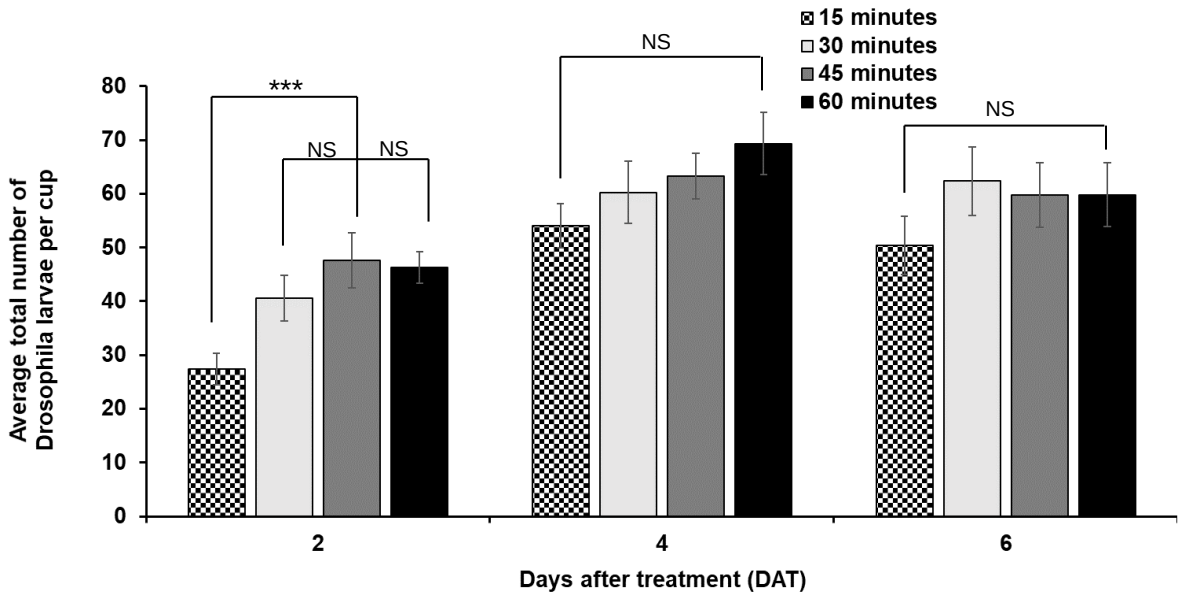

Figure 4. Mean total SWD larval yields by incubation time at different days after treatment for the Incubation Time Experiment. Error bars are displayed as standard error. Larval yields for the 15-minute incubation time treatment were significantly lower than yields for the 30-minute, 45-minute, and 60-minute incubation times two days after treatment, but not four or six days after treatment. Three asterisks (***) indicate significance at the 0.001 level and NS indicates no significance. DAT is the number of days after berries were first exposed to flies.

Squish Vs Firm Experiment

For all three DAT times, the average total larval yield for the squish condition tended to be higher than for the firm condition (Figure 5). Two days after treatment, the squish condition yielded significantly more total larvae on average than the firm condition did (t_{22} = -3.93, P = 0.001). While this trend was also present four days after treatment, the difference was only marginally significant (t_{22} = -1.89, P = 0.07). Furthermore, at six days after treatment the squish condition tended toward higher larvae totals than the firm condition, but the Student's t-test revealed no significant difference at the six DAT time (t_{22} = -0.48, P = 0.64).

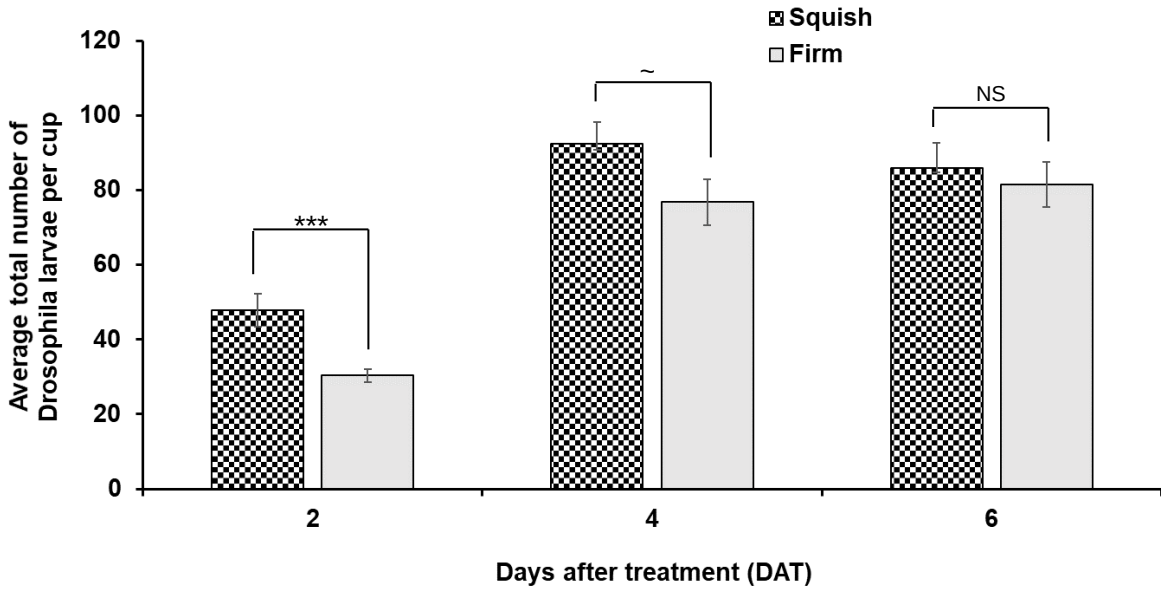

Figure 5. Mean total SWD larval yields by squish condition at different days after treatment for the Squish Vs Firm Experiment. Error bars are displayed as standard error. Larval yields for the squish condition were significantly lower than yields for the firm condition two days after treatment, but not four or six days after treatment. Three asterisks (***) indicate significance at the 0.001 level, a tilde (~) indicates marginal significance, and NS indicates no significance. DAT is the number of days after berries were first exposed to flies.

Solution Type Experiment

The sugar condition tended toward lower average larvae totals than the salt and distilled water conditions at four and six days after treatment, but there was no significant effect of solution type on total larval yield at any DAT time (Figure 6; 2 DAT: $F_{2, 33} = 2.51$, $P = 0.10$; 4 DAT: $F_{2, 33} = 2.25$, $P = 0.12$; 6 DAT: $F_{2, 33} = 2.39$, $P = 0.11$). Nevertheless, Larvae that incubated in distilled water were still mobile during filter assessment and some of the smallest larvae from the distilled and sugar water conditions crawled through the filter into the cup. Larvae that incubated in salt water were immobile and presumably dead.

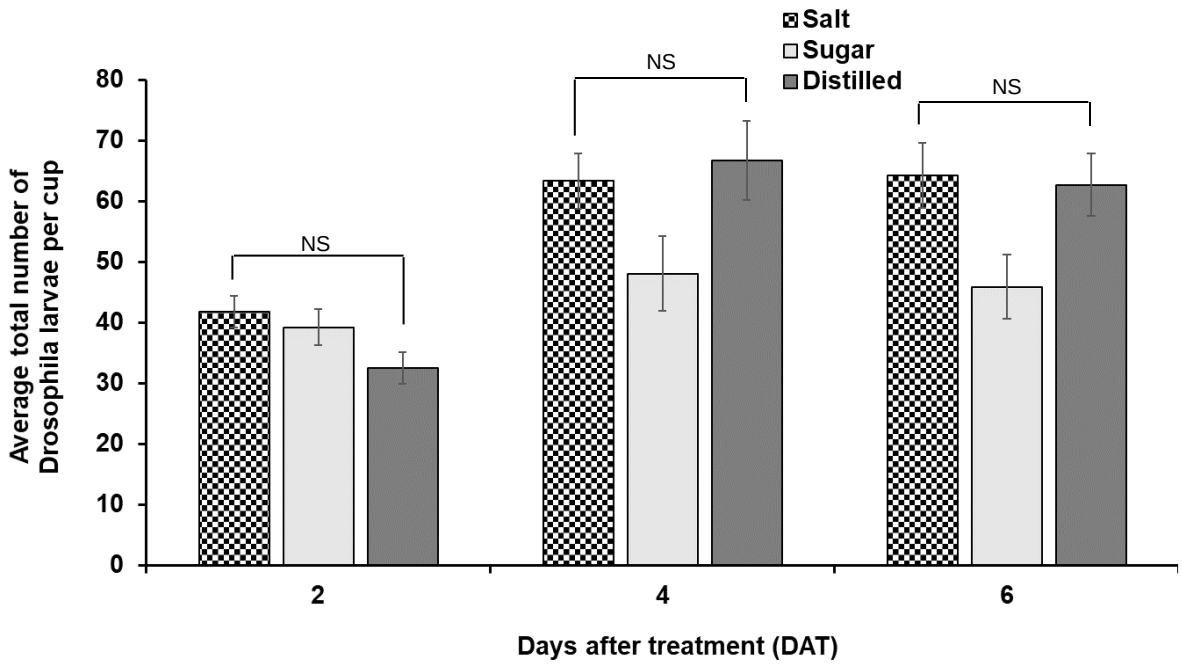

Figure 6. Mean total SWD larval yields by solution type at different days after treatment for the Solution Type Experiment. Error bars are displayed as standard error. Larval yields for the sugar condition appear to be lower than for the other conditions at four and six days after treatment, but no statistically significant differences were found between the treatments. NS indicates no significance. DAT is the number of days after berries were first exposed to flies.

Discussion

The purpose of this study was to determine what conditions optimize the efficiency and accuracy of the salt filter test to detect SWD larvae in blueberries. In order to do so, four experiments were designed to investigate different aspects of the test. This study found significant differences in average larval yield in all experiments except for the Solution Type Experiment. These results will be reviewed and discussed in the following sections.

Unfiltered Vs Filter Experiment

The primary finding of this experiment was that for all DAT times, filter assessment detected more larvae than unfiltered assessment. This supports the hypothesis that salt filter testing with microscopic assessment is more accurate than visually assessing berries in liquid using the naked eye without filtering. Because this difference was found across all DAT, this suggests that regardless of what growth stage the larvae are at (first, second, or third instar), they are more likely to be observed through salt filter testing than unfiltered testing. Nevertheless, the advantage to using a salt filter test instead of unfiltered assessment seems to be most pronounced when detecting the smallest first instar larvae, as the greatest difference between the methods was identified at two days after treatment. This is likely because larger larvae are easier to spot visually, whereas smaller larvae may be more difficult to distinguish; studies such as Yee (2011) and Yee (2014) identified higher detection of larger third instar larvae than first instar larvae for a variety of unfiltered naked eye methods. Microscope usage is not practical with the large amounts of water associated with unfiltered methods because of the depth of field and the many planes that larvae may be on. Additionally, larvae may be mobile, moving between planes in the three-dimensional space of the water and making unfiltered

microscopic quantification exceptionally difficult. The use of a filter reduces the depth of field, allowing efficient microscopic assessment and permitting detection of the smallest first instar larvae.

While this assessment method seems to be more accurate, there are contextual factors that may come into play regarding a scout's choice of assessment type. Salt filter testing requires access to a light microscope and a sink sprayer which may be unavailable to a scout in the field. However, even if the scout decides to use an unfiltered assessment method, the data collected in this study provide information that they could use to arrive at more accurate larval estimations. For example, given that filter assessment detected about 2.69 times more larvae than unfiltered assessment two days after treatment, scouts could potentially multiply their first instar unfiltered larvae counts by 2.69 to better estimate first instar totals. Based on the data collected at four and six DAT in this study, they could follow a similar procedure to estimate second and third instar larvae totals by multiplying their unfiltered larvae counts by 1.58 and 1.40 respectively. Additionally, research by Dreves *et al.* (2014) suggests that if an unfiltered method is used, a magnifying lens may improve larval count accuracy compared with naked-eye assessment.

However, when considering the applications of this study, it is important to note that unfiltered assessments were conducted within deli cups instead of in gallon-sized plastic bags which are used more frequently by scouts; the shape of the container could influence how easy the larvae are to spot, which in turn could affect the multiplication value needed. Additionally, the multiplication values generated in this experiment are best calibrated for the researcher's visual acuity level. Because visual acuity varies from person to person, this factor must also be taken into account.

Incubation Time Experiment

It was hypothesized that shorter incubation times would yield fewer larvae than longer incubation times. This was partially supported by the data in this experiment because two days after treatment, the 15-minute incubation time yielded fewer larvae than the 30-minute, 45-minute, and 60-minute incubation times. This suggests that when it comes to smaller first instar larvae, more time than 15 minutes is required to force them out of berries. Because the larval yield between 30 minutes, 45 minutes, and 60 minutes was not significantly different, 30 minutes may be enough time to effectively quantify first instar larvae in blueberries. This result has major implications for scouts using the salt filter test method. Scouts can cut salt test time in half from the 60-minute standard and not worry about compromising the accuracy of the test. This could save scout employers money as well as potentially allow scouts to reach more sample sites in a work day.

The data collected at four and six days after treatment, which targeted larger second and third instar larvae, also have implications for scouts. Given that there were no significant differences between incubation times, 15 minutes may be sufficient for extracting second and third instar larvae from berries. This parallels strategies used by Dreves *et al.* (2014) to extract SWD larvae from fruit using just 15 minutes for liquid incubation. Although scouts may miss some first instar larvae with this method, it could still be useful in some cases. For example, if the growers suspect that their field is infested, this shorter test could either deny or confirm that the fruit is too infested to harvest and sell; if lots of larvae in later growth stages are present, the fruit could be unmarketable, but if few are present, then there could be a basis for more tests. Future

experiments could investigate the effectiveness of even shorter incubation times, such as 5 and 10 minutes, for detecting second and third instar larvae.

Squish Vs Firm Experiment

It was predicted that squishing berries would increase larvae detection by perforating the berry skin and allowing salt water to better permeate the berry and force out larvae (Van Timmeren *et al.*, 2017). This hypothesis was partially supported by the results of this experiment. Two days after treatment, squishing the berries was significantly more effective than not squishing them. However, four and six days after treatment, squishing did not seem to have an effect on total larval detection. One study found that in the Caribbean fruit fly, pupae can withstand brief periods of anoxia (complete lack of oxygen), whereas larvae and adults are less tolerant of anoxia (Visser *et al.*, 2018). It is thus possible that squishing and the concomitant influx of salt water causes panic in the younger first instar SWD larvae, whereas older second and third instar larvae which are closer to the pupal stage are less bothered by the disruption. Contact between the larvae and the water may be delayed or more gradual (less cause for alarm) in firm berries because of fewer water entry points in the berry.

Another factor that could be related to the difference in behavior between first instar larvae and second and third instar larvae in response to berry squishing is size. The larger size of second and third instar larvae decreases their surface-area-to-volume ratio. According to a study on caddisfly larvae, larvae's ability to regulate their internal salt concentration breaks down when the external environment reaches a particular salinity threshold of 170 mM concentration (Sutcliffe, 1961). The concentration of salt water in this experiment was 1.3 M, thus the ability of larvae to maintain homeostasis likely began to fail shortly after the larvae were exposed to the liquid. This is supported by the

observed immobility (and presumable death) of larvae incubated in salt water compared to the mobility of larvae incubated in distilled water. Diffusion of water out of skin when exposed to a hypertonic solution (salt water) may be slightly slower for second and third instar larvae with lower surface-area-to-volume ratios than first instar larvae. Slower diffusion may allow them to survive in salt water slightly longer and might decrease alarm and berry-exiting behavior.

Given the results of the experiment, the squish method would be recommended for estimating total larval infestations because it best quantifies larvae at all growth stages. However, if scouts are only interested in determining heavy infestation and the marketability of certain suspect crops, then they may be able to save time by not squishing the berries without compromising the accuracy of the quantification of second and third instar larvae.

Still, one way this experiment's applicability could be limited is in the decomposition of the berries over the course of the experiment in lab conditions. Because the berries were heavily infested at the beginning of the experiment, they started breaking down quickly, appearing mushy at the four DAT and six DAT marks. As Dreves *et al.* (2014) explain, developing larvae cause fruit to soften and collapse around their feeding site. This may have reduced the effect of squishing the berries because even berries in the "firm" condition were mushy by these DAT. Berries in the field would still be provided with nutrients by the branch and thus may be more likely to stay firm despite heavy infestation. Future studies should decrease infestation levels and thus reduce berry decomposition by adding fewer flies to larger quantities of berries and examining the squishing vs. not squishing methods. Infesting berries still on the branch and testing them may add to the real-world applicability of the study as well. Additional research should

aim to determine the mechanism by which squishing increases larval yields, and subsequently develop ways to better standardize the squishing method. For example, if the mechanism at play is the creation of an entry point in the berries for salt water, then pricking each of the berries with a large needle could standardize the size of the entry point.

Solution Type Experiment

It was hypothesized that salt water would be the most effective liquid for the salt filter test, followed by sugar water and distilled water. The data collected did not support this prediction because no significant differences of solution type were found on total larval detection. It is important to note that the original purpose of using salt water or sugar water was to cause the larvae to float to the surface, making them easier to detect with the naked eye (Neilson & Lawrence, 1986; Dixon & Knowlton, 1994). Because the salt filter test sifts the liquid through a coffee filter, this floatation purpose is irrelevant for the filter method. Qualitative observations regarding larval mobility in the distilled water condition also suggest that different solutions may be better suited for different detection methods: for filter assessment, salt water is best and for unfiltered assessment, water is best. For filter assessment, sugar water and distilled water are not recommended because they do not kill the larvae. It is more difficult to assess the filters when the larvae are moving and some wiggle their way through the coffee filter. However, if funds are an issue, distilled water could be a cheaper liquid alternative for the salt filter test that would not compromise its accuracy. Tap water may also be used if necessary, though the results from this study do not account for varying mineral levels in tap water compared with distilled water. Future studies should compare water with varying mineral levels to determine whether this influences filter assessment accuracy.

In terms of unfiltered assessment, larval movement could aid identification of larvae instead of hinder it, therefore distilled water would be a better choice for that method. Sugar water would not be recommended for either method because it is more expensive, yet no more effective than salt water or distilled water at quantifying larvae according to this experiment (Meijer Grocery). As Dreves *et al.* (2014) point out, brown sugar can also be sticky and messy, while its darker hue can make it more difficult to spot larvae visually. Future studies should instead compare salt water and distilled water using the unfiltered assessment method to determine whether larval movement aids quantification. Another potential benefit of using distilled water and not killing the larvae is that they may be reared to adulthood if identification of species is important. While this study specifically infested berries with SWD larvae, a field sample of berries could contain several different species, which are more easily identifiable to species as adults than as larvae.

Conclusions

In conclusion, this study has been successful in optimizing the salt filter test method in several ways. Moving forward, scouts that monitor SWD can save time by incubating samples for only 30 minutes instead of 60 minutes. If they are only interested in gaging severe late-stage infestation, they can save even more time by incubating for only 15 minutes and by not squishing the fruit. Scouts can also save money by using distilled water to incubate berries instead of more expensive solutions. Even if scouts do not have access to the equipment necessary to perform salt filter tests, they can utilize data from this study to arrive at more accurate larval estimates from their unfiltered assessments. Incorporating salt filter tests as another tool to inform IPM programs offers many benefits too. Growers can quickly and accurately assess larval infestation levels

and infer whether or not their strategies—from mass SWD traps, to biological controls, to insecticides—are working. From there, major fruit-producing states in the United States like Washington, Oregon, and California may recover from SWD-related crop losses and revitalize their fruit industries.

Even beyond the direct outcomes of monitoring *Drosophila suzukii*, studies are showing that effective IPM programs for a variety of pests, from flea beetles to green peach aphids, can reduce insecticide applications (Slone & Burrack, 2016). This is critical because commercial insecticides seem to play a role in the decline of pollinator populations (Miranda & Nazzi, 2017). Since 1947, there has been a 59% loss of honey bee colonies in the United States, likely associated with pesticide usage (Potts *et al.*, 2010). Given that 75% of human food crops depend on insect pollination, primarily by bees, it is imperative that growers seek to limit their insecticide usage to protect these insects and preserve the future of worldwide agriculture (Potts *et al.*, 2010). Movement in this direction all begins with effective IPM programs developed through accurate pest monitoring.

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