

A Filter Method for Improved Monitoring of *Drosophila suzukii* (Diptera: Drosophilidae) Larvae in Fruit

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Abstract

Drosophila suzukii Matsumura (Diptera: Drosophilidae) has become a major economic pest of soft-skinned fruits since it was detected in North America in 2008. Control of this fly is achieved through insecticide sprays applied when ripening or ripe fruit are present. Monitoring to aid informed management decisions is challenging since trapping for adults is not a reliable indicator of potential or existing infestation in the fruit. Moreover, current larval monitoring techniques using brown sugar or salt solutions allow for visual detection of late-instar larvae, but they are time consuming and tend to miss smaller larvae. Here, we describe a method combining a salt solution, coffee filter, and microscope that can reliably and efficiently detect small and large larvae of *D. suzukii* in fruit samples. By sifting the sample liquid through an inexpensive coffee filter, larvae of all instars can be counted quickly and accurately. This method is 1.7 times faster than using a visual tray-based method and can detect more larvae because first instar larvae can be detected. Growers can use this information to target insecticide sprays for curative control of small larvae, identify fields where the presence of larger larvae may indicate an unmarketable crop, or verify that no infestation exists and control programs are working. We provide images to support larval identification of this pest, and we expect this method will become an important component of rebuilding IPM programs in fruit crops affected by *D. suzukii*.

Key words: Spotted wing *Drosophila*, SWD, monitoring, salt flotation, larval sampling

Drosophila suzukii Matsumura (Diptera: Drosophilidae), referred to as the spotted wing *Drosophila*, was first detected in North America in 2008 and has become the primary insect pest of blueberries, caneberries, strawberries, and other soft-skinned fruit (Lee et al. 2011, Asplen et al. 2015, Farnsworth et al. 2017, Mazzi et al. 2017). Females are highly fecund and lay eggs in undamaged ripe and ripening fruit using a serrated ovipositor (Atallah et al. 2014). Its short generation time (10–14 d) can result in large and rapid population increases (Tochen et al. 2014), which is particularly challenging for fruit harvested in the late summer. There is zero tolerance for infestation in fresh marketed or whole frozen fruit, and well-established integrated pest management (IPM) programs have been abandoned to control this invasive pest (Haviland and Beers 2012, Van Timmeren and Isaacs 2013, Diepenbrock et al. 2016, 2017).

Assessing the presence and risk of *D. suzukii* has proved challenging because monitoring for adults using traps deployed with existing baits and lures cannot reliably predict infestation in fruit (Hamby et al. 2014, Burrack et al. 2015). Monitoring for larvae can provide a more reliable indication of fruit infestation, allowing growers to have confidence that their fruit are not infested and can therefore

be harvested without concern of rejection by inspectors. Fruit sampling methods have been reported in which fruit are placed in brown sugar water, salt water, or hot water, followed by counting the larvae that subsequently exit the fruit (Hueppelsheuser 2010, Dreves et al. 2014, Burrack 2014). Similar larval sampling techniques have been used successfully for detection of *Rhagoletis indifferens* Curran (Diptera: Tephritidae) in cherries (Frick 1953, Yee 2014) and *R. mendax* Curran in blueberries (Neilson and Lawrence 1986, Dixon and Knowlton 1994). All of these methods rely on visual assessments of larvae in the liquid, and as a result, only the largest larvae can be seen. For *D. suzukii* management, this often means missing the small (first and second instar) larvae and only detecting third instars that can be most easily seen. As a result, detection occurs only after larvae are mature and these are harder to control than the more immature stages (Wise et al. 2015).

Current methods to detect the smaller larvae are time intensive and cost prohibitive. In addition, current methods involving visual inspection of *D. suzukii* larvae in fruit and/or liquid rely heavily on the skill of each assessor, which could lead to variability in larval detection. This can make it more difficult for growers and scouts to

get an accurate assessment of infestation. Similarly, constraints apply to researchers who may miss the full effects of their treatments on the initial immature instars if only the mature ones can be detected. There is also limited information on the morphology of this economically important life stage of *D. suzukii*, so there is a need for visual tools to assist with identification.

Improved monitoring techniques are needed for early detection of *D. suzukii* larval infestation to allow growers to react and take appropriate measures to protect the fruit. Here we describe an improved salt test monitoring technique that uses an inexpensive filter to help detect small and large larvae in fruit samples. We also provide images of the instars, including detailed comparisons of their morphology, to aid growers, fruit inspectors, and researchers in identifying the immature stages of this pest.

Filter Salt Test Methods

The following protocol is recommended for sampling fruit to detect *D. suzukii*. It has been tested in blueberries, blackberries, raspberries, strawberries, and cherries. If sampling other fruit, the methods we describe may need to be modified.

Step 1: Collect Fruit

Collect ripe fruit samples to be assessed. Infestation of fruit by drosophilids varies greatly by fruit maturity level and quality status, so fruit should be sorted according to the needs of the assessor. In commercial fruit fields or orchards where infestation at harvest is a concern, fruit samples should represent the maturity of fruit that will be harvested (Fig. 1A). Sample size should be ~8–16 fl oz (237–473 ml) and this can be scaled up or down based on the amount of fruit available. There is not currently a research-based sampling scheme for *D. suzukii* larvae within fruit, but sampling more fruit will increase the chance of detecting infestation. The fruit sample can be weighed to enable calculation of the number of larvae per pound (kg), or the number of berries in the sample can be counted in order to determine the number of larvae per berry.

Step 2: Lightly Crush Fruit

Place fruit sample in a 1 gallon (3.79 liter) resealable plastic bag and seal it. Set the bag on a hard surface and lightly crush each of the berries to break the skin without releasing the inside pulp (Fig. 1B), to allow the salt solution to enter fruit. Crushing is best accomplished using the thumb or index finger, but a deli cup lid can also be used to increase the surface area for larger samples. For blueberries, this means pushing on them until the skin breaks, whereas for raspberries or blackberries this means lightly depressing the berry to separate the drupelets. Do not mash the berries as this will add pulp to the liquid which makes the larval assessment difficult.

Step 3: Add Salt Solution

Add 1 cup of white table salt (Table 1) to 1 gallon of tap water (312.6 g salt per 3.79 liter water) and shake until the salt is fully dissolved. In practice, make this solution in bulk and have it available before sampling. Pour the solution into the bag, covering the berries with salt water, and seal the bag (Fig. 1C). Remove as much air as possible from the bag to minimize the chances of larvae crawling out of the salt water and towards the top of the bag. Place the bag in a plastic bin to keep the bag propped up and ensure all the berries remain fully covered (Fig. 1C). Let the bag sit for at least 1 h before observing larvae. Shorter intervals may be acceptable if only presence or absence information is required (Dreves et al. 2014), but

waiting the full hour will ensure the maximum number of larvae will have exited the fruit. Blueberry, blackberry, raspberry, and cherry samples can be refrigerated in the salt solution for later assessment, but strawberries should be assessed within an hour of soaking, as fruit will degrade and become difficult to filter.

Step 4: Separate the Fruit and Filter the Larvae

Pour the fruit and salt water out of the bag and through a coarse filter such as a layer of 0.25 in. (6.35 mm) metal hardware mesh (Fig. 1D). Allow the liquid to pass through into a reusable basket style coffee filter designed for an 8–12 cup coffee pot (Table 1, Fig. 1D). Coffee filters are available with stainless steel or polyester mesh, and the stainless steel version is more durable and clogs less frequently than polyester. The coarse filter can be glued inside a funnel as an easy way to separate out the fruit and allow the liquid to drain (Fig. 1D). Rinse the berries with a pressurized spray bottle or sink spray nozzle to remove larvae that may remain stuck on berries. Also rinse out the bag to ensure larvae do not remain stuck to the inside. Once rinsed, discard the berries and bag. Note that heavily infested samples or samples with more pulp may drain more slowly. Filters with slowly draining samples can be placed in a small container to collect draining water.

Step 5: Count and Identify Larvae

The contents of the coffee filter can be observed using a stereomicroscope set to $\times 5$ – 10 magnification (Fig. 1E and F), and the number of *D. suzukii* larvae can be counted using their size to distinguish among instars (Fig. 2). Counts of each can then be recorded for each sample. The number of *Drosophila* eggs can also be counted if present (Fig. 2), but eggs are not as reliably dislodged as larvae are from fruit by the salt test. While assessing fruit samples for *D. suzukii*, larvae of tephritid flies such as *Rhagoletis* species can also be counted. Early instar larvae of the tephritids may be similar in size to late instar *Drosophila* larvae but they can be identified using diagnostic characteristics (Fig. 3) (Hauser 2016). Larger tephritid larvae and other potential fruit contaminant insects can be easily differentiated from *Drosophila* larvae by their size and shape.

If greater precision is needed in identifying the instars of *D. suzukii* then the mouthparts and spiracles can be examined (Figs. 4 and 5). We analyzed the number of teeth on the mouth hooks (mandibles) of 15–21 larvae of each instar, and found that they have an average (\pm SD) of 1.0 ± 0.0 tooth in first instars, 4.2 ± 0.7 teeth in second instars, and 13.1 ± 2.2 teeth in third instars (Fig. 4). Under the magnification used for fruit sampling (Fig. 2), the mouth hooks can be distinguished between instars. Those in first instars are short, each having a single large tooth that is about the size of the tip of the hook; second instars have a large basal tooth on each hook with a number of smaller teeth separating it from the tip of the hook; and third instar hooks appear long, curved, and somewhat smooth, their teeth being numerous but small and blunt (Fig. 4). The anterior spiracles can also be used to differentiate among the instars as first instars have no visible spiracles or spiracle openings, second instars have spiracles ending in trunks but no apparent spiracle openings (appear as small club-like organs), and third instars have spiracles ending in finger- or pencil-like papillae and have several visible spiracle openings (Fig. 5) (Bodenstein 1950). Determining the different stages of larval development can be time consuming, and for most pest management decision making it will be sufficient to classify larvae by size class (Fig. 2).

After counting and classifying larvae, coffee filters should be rinsed and washed thoroughly before reuse.

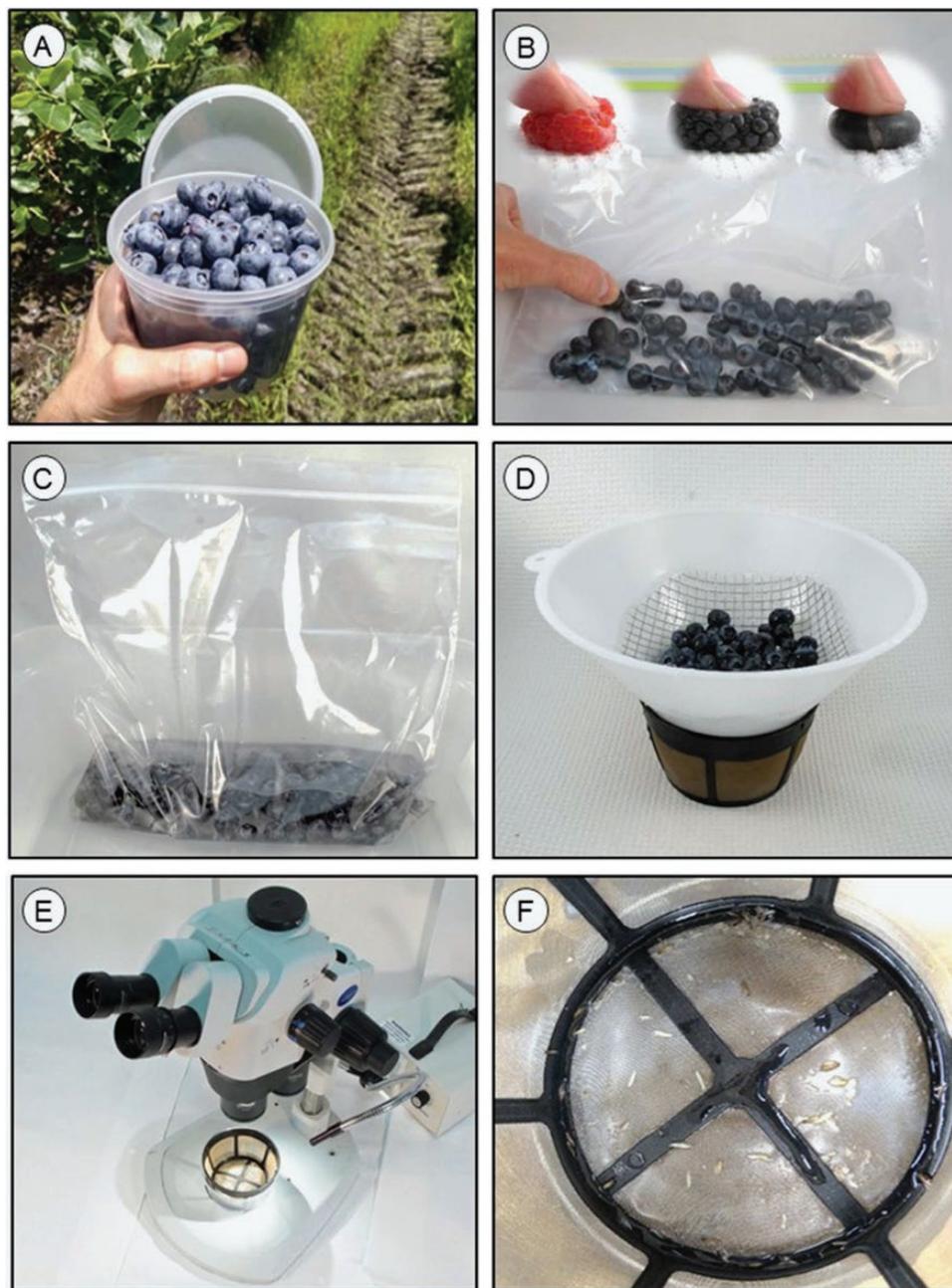


Fig. 1. Follow these steps to sample fruit for *D. suzukii* larvae: (A) Collect 8–16 oz (237–473 ml) of ripe berries; (B) place berries in a 1 gallon resealable plastic bag and lightly crush berries; (C) add salt water to berries, place bag upright in a plastic bin, and incubate for 1 h; (D) pour salt water and berries into course filter funnel and rinse berries off to wash larvae into reusable coffee filter; (E) use microscope or other magnifier to view larvae in the coffee filter; and (F) count the number of larvae in the bottom of the filter.

Table 1. Supplies list for conducting the filter salt test method to detect *Drosophila* larvae in fruit samples

Product	Product details	Company	City
White table salt	Cargill brand Top-Flo granulated salt	Cargill Salt	Minneapolis, MN
Hardware cloth	Mat Midwest 23-gauge hardware cloth	Mat Holdings, Inc.	Long Grove, IL
Plastic funnel	Plews 48 oz plastic utility funnel, Model #75-064	Plews & Edelmann	Dixon, IL
Pressurized spray bottle	Solo One-Hand Pressure Sprayer, Model #418	Solo Inc.	Newport News, VA
Reusable coffee filter	Medelco 12 cup basket coffee filter, Model #BF215	Medelco, Inc.	Bridgeport, CT

Testing Detection of *D. suzukii* Larvae

To test this method, an experiment was conducted where ripe store-bought blueberries were exposed to adult *D. suzukii* for 6 d and

the resulting infested fruit were placed in plastic resealable bags, lightly crushed, and placed in a liquid solution. A salt solution and brown sugar solution were compared as these are two commonly

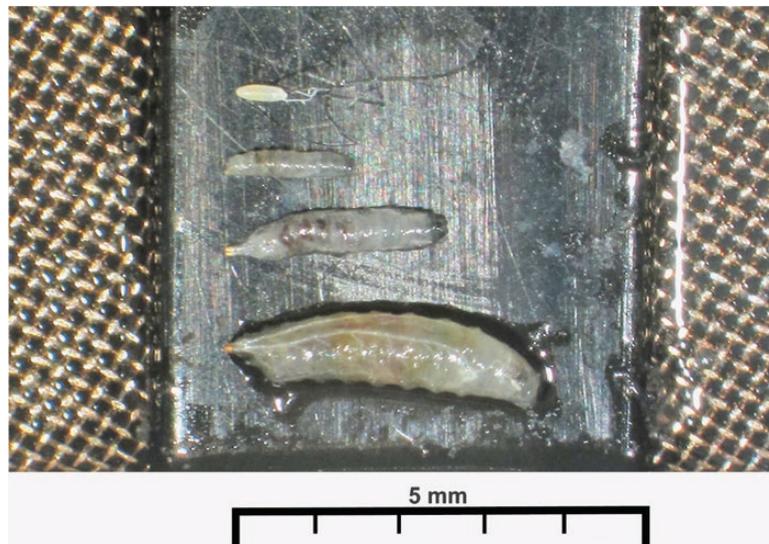


Fig. 2. *Drosophila suzukii* egg and small, medium, and large larvae as seen under a microscope after sifting using a reusable coffee filter. Scale is in millimeters.

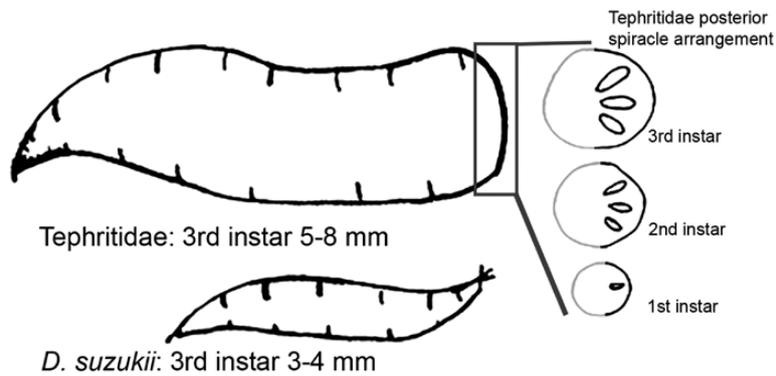


Fig. 3. Tephritid (top) and drosophilid (bottom) larval shapes and approximate third instar sizes and posterior spiracle slit arrangement in Tephritidae (right). Tephritid larvae have a flattened posterior, and first instar larvae have one pair of posterior spiracle slits, while second and third instars have three pairs of posterior spiracle slits.

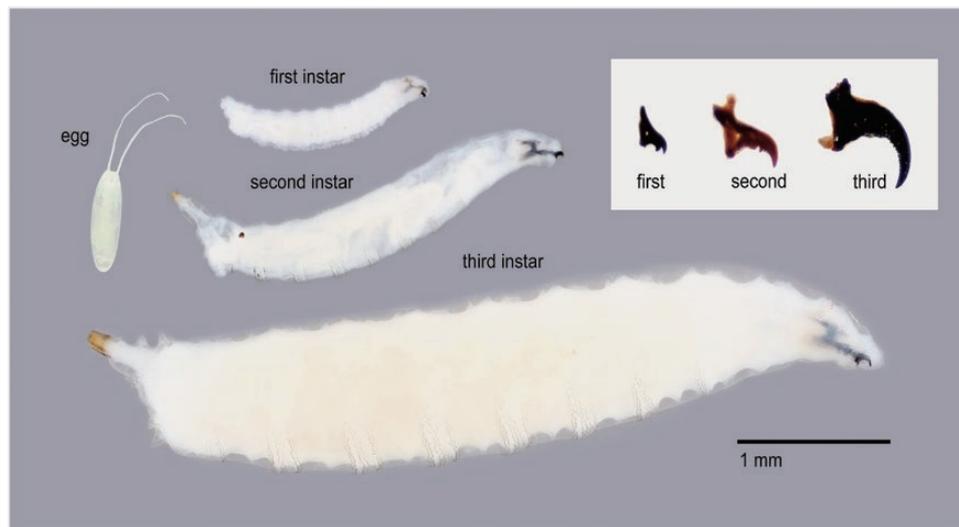


Fig. 4. *D. suzukii* egg, instars and mouth hooks. Egg and first instar were preserved directly in 95% ethanol. Second and third instars were prepared by fixing live larvae in recently boiled water and storing in 95% ethanol. Images were gathered using stacking software.

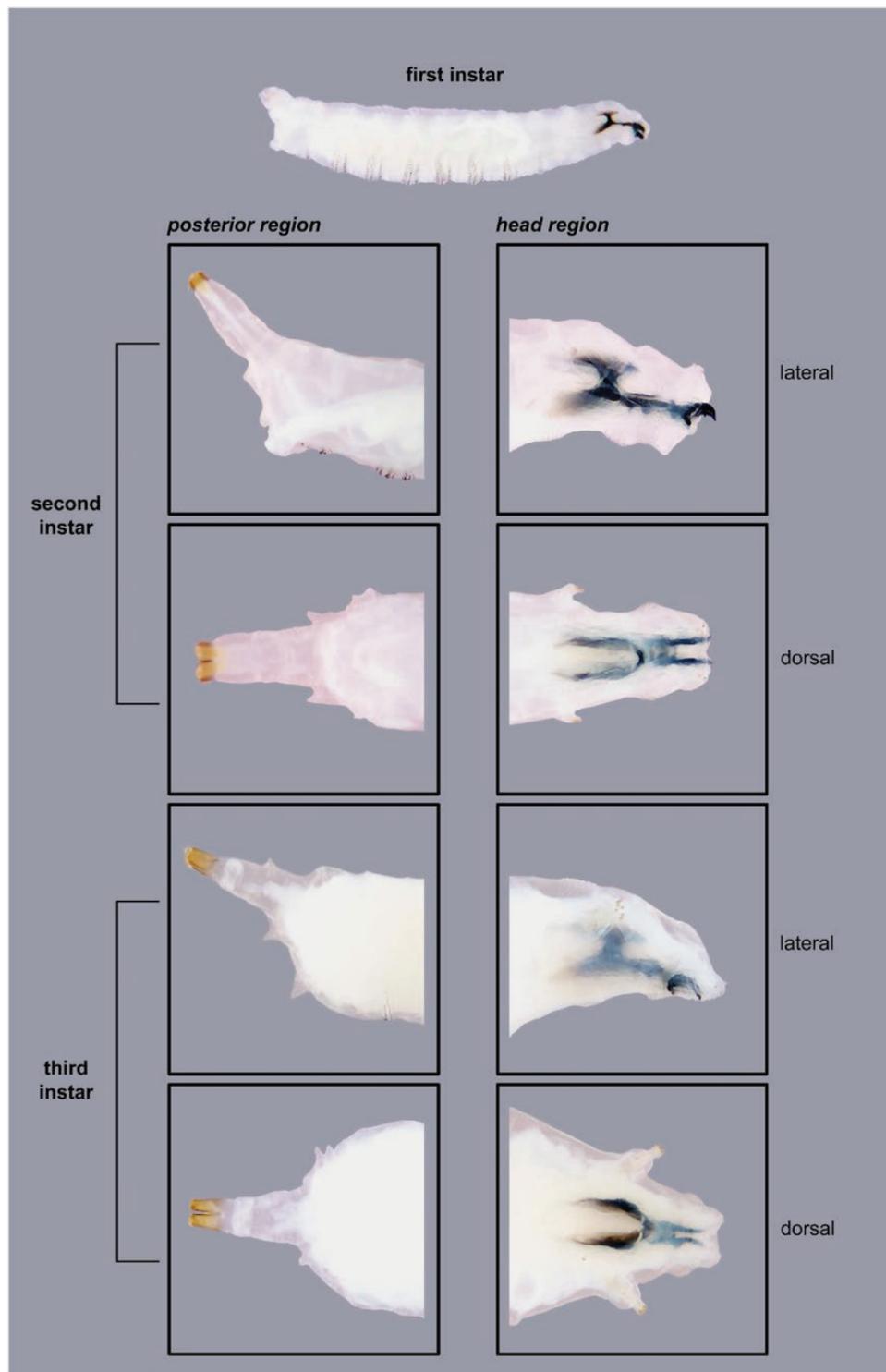


Fig. 5. *Drosophila suzukii* instars and showing posterior (left) and anterior (right) spiracles. First instar was preserved directly in 95% ethanol. Second and third instars were prepared by fixing live larvae in recently boiled water and storing in 95% ethanol.

used sampling solutions (Yee 2014), with room temperature tap water used as a control. There were five samples of each liquid, to compare the number of eggs and larvae detected using each method. Samples were first assessed visually in the bags (standard method) by trained observers and were subsequently assessed using the methods described here using a filter and microscope. We also compared the time it took to assess a 4 oz (118.3 ml) sample of infested blueberries using the filter salt test described here versus the tray-based method

outlined by Dreves et al. (2014) using a lighted magnifier (Global Industrial 3 Diopter Magnifying Desk Lamp, Model #WB695231, Global Equipment Company, Port Washington, New York, $\times 1.75$ magnification) and a digital timer. Four replicates of each treatment were set up and assessed on 5 April 2017. Assessment times were recorded as the total time required for an experienced assessor to prepare and sift salt test samples and subsequently count all larvae that were present in the filter or tray.

All statistical analyses were performed using Systat 13 (Systat Software, Inc., Chicago, IL). To compare sampling methods, the total number of eggs and larvae detected visually were analyzed using one-way analysis of variance (ANOVA) and Tukey's honest significant difference (HSD) test for means separation. The total number of eggs and larvae detected visually or using the filter salt test method were compared using a two-sample *t*-test. Total assessment times for the filter or tray-based sampling methods were compared using a two-sample *t*-test.

Results and Discussion: Integrating Larval Sampling into IPM for *D. suzukii*

The larval sampling method outlined here can improve the accuracy and efficiency of existing larval sampling methods for *D. suzukii*. It enables assessment of small larvae in a way that is not possible with the typical visual assessment methods used by many growers, crop consultants, and fruit processors. More larvae were detected by visual observation (without filter) using the brown sugar and the salt solutions when compared with a tap water control ($F = 16.48$; $df = 2, 12$; $P < 0.0001$), and there was no significant difference between the salt and brown sugar solutions ($P < 0.84$). However, four times as many larvae were detected using the filter method we describe here (82.1 ± 9.5 larvae per sample) compared with larvae found by visual observation (24.5 ± 3.7 larvae per sample) ($t = 7.67$; $df = 1, 6$; $P = 0.002$). Importantly, first and second instar larvae were detectable using the filter method, while few could be detected using visual observation alone.

The filter method described here also decreases the chance that external factors will affect the number of larvae detected in a sample. Factors such as lighting, sampling tray color, and visual acuity of the assessor may affect larval detection frequency in a standard visual assessment, but will not affect detection frequency in the filter sampling method that employs a microscope to improve the detection of larvae. It is important to note that methods for sampling smaller larvae using magnification already exist (Yee 2012). However, the time-consuming nature of using these types of magnified assessments make them less practical for use in an IPM program and more cumbersome for use by researchers assessing experiments. The filter method described here is a fast, efficient, and cost-effective way to assess fruit samples for smaller larvae. Fruit sample assessments were almost twice as fast using the filter method (3.8 ± 0.1 min per sample, including preparation time) when compared with the tray-based method (6.5 ± 0.4 min per sample) ($t = -6.6$; $df = 1, 6$; $P = 0.004$).

Having a fast and accurate assessment method for larval detection is valuable for growers, crop scouts, and consultants for whom sample assessment time is an important consideration. Fruit for salt test samples can be prepared and set in the salt solution immediately after collection during visits to fields during the day and allowed to sit while conducting other scouting tasks, and then the samples can be filtered as described above and larvae counted. The speed at which samples can be assessed using this method is advantageous for researchers as well, and will allow for larger numbers of samples from replicated experiments to be assessed accurately. An additional advantage for researchers is that the filtered samples could be sealed in plastic deli containers along with a small amount of water or ethanol and refrigerated for later assessment in genetic, morphological, or other studies. This method may also be useful for fruit processors who are testing fruit samples for infestation. Samples can be set up rapidly and assessed in a quick, yet accurate manner. Identification of tephritid larvae will also be useful for fruit destined for certain export markets. Many processors and crop consultants

use microscopes in their businesses already, and those that do not could use a magnifier placed over the filter to conduct the assessments. Microscopes fitted with a camera system or cellphones with inexpensive macro attachment could also be used with this approach to take photos of the sample to provide verification that samples are clean or that larvae were detected. In addition, all of the items used for the filter method (Table 1) are available for purchase online or from local food and hardware stores.

Being able to detect smaller *D. suzukii* larvae efficiently will improve IPM programs. Wise et al. (2015) found that some insecticides can provide post-infestation curative activity against *D. suzukii* larvae in blueberries, and post-harvest cold storage can significantly reduce survival of immature *D. suzukii* within fruit (Aly et al. 2016). This easier method for detecting smaller larvae may enable the use of larval sampling to inform the use of insecticide applications in fields where only eggs and young larvae are detected and where curative control may provide sufficient control for the fruit to be harvested. Efficient larval detection can allow growers to identify at-risk fields and use appropriate management actions (e.g., insecticide application, fruit removal, or cold storage) to minimize the impacts of infestation. Likewise, growers can use this sampling method as verification that control programs are working.

An important component of successfully integrating this filter sampling method into an IPM program is assessing enough samples to accurately detect *D. suzukii* infestation in a field. Samples should be collected and tested from as many locations within the field as time permits, making sure that each sample is collected from multiple bushes. The more samples tested the greater the confidence that larvae detected (or not detected) are accurately representing infestation levels in the field. Sampling should also take microclimatic factors into account. Recent research has shown that berries on the interior of blackberry canes have greater *D. suzukii* infestation than berries on the exterior of the bush, a result that is likely related to higher relative humidity in the interior location (Diepenbrock and Burrack 2016). Separate samples could be collected from the interior and outer portions of the bush and tested for *Drosophila* larvae, to provide an indication of whether insecticide coverage is adequate. Fields that receive insecticide applications via airplane could have tests conducted on fruit collected at the top of the bush and closer to the bottom of the bush, to determine whether vertical stratification of infestation is present. Additional research on sampling design is needed to optimize the use of larval sampling for IPM programs.

One disadvantage of this filter sampling method is that fruit pulp can potentially clog the filter. This concern can be reduced by only lightly crushing fruit. However, for larger fruit such as cherries this can be a challenge. Another disadvantage is that this modified method cannot be used to reliably detect eggs because it relies on larvae actively moving out of the fruit. In addition, care must be taken not to count eggs from other species such as *Zaprionus indianus* Gupta or *D. melanogaster* Meigen. Another aspect of this and many other fruit sampling methods is that while larvae can be identified to family, drosophilid larvae cannot easily be identified to species. If determining species is important, then a sugar solution may be substituted for salt water and collected larvae placed on diet and reared to adulthood.

Overall, the filter assessment method for *D. suzukii* larvae presented here is a useful monitoring tool for supporting informed management decisions. Unlike adult trapping of flies, this method provides a real-time measure of in-field infestation for this pest. The presence of large larvae can provide warning of fruit that cannot be marketed, while detection of small larvae can provide an opportunity to apply control measures or to manipulate post-harvest handling practices. Having this reliable method for monitoring infestation will

be an important component in the process of rebuilding IPM programs for the fruit crops affected by *D. suzukii*.

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