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# Sieving Fruit Pulp to Detect Immature Tephritid Fruit Flies in the Field

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## Abstract

Fruit flies of the Tephritidae family are among the most destructive and invasive agricultural pests in the world. Many countries undertake expensive eradication programs to eliminate incipient populations. During eradication programs, a concerted effort is made to detect larvae, as this strongly indicates a breeding population and helps establish the spatial extent of the infestation. The detection of immature life stages triggers additional control and regulatory actions to contain and prevent any further spread of the pest. Traditionally, larval detection is accomplished by cutting individual host fruits and examining them visually. This method is labor intensive, as only a limited number of fruit can be processed, and the probability of missing a larva is high. An extraction technique that combines i) mushing host fruit in a plastic bag, ii) straining pulp through a series of sieves, iii) placing retained pulp in a brown sugar water solution, and iv) collecting larvae that float to the surface was tested. The method was evaluated in Florida with field-collected guava naturally infested by *Anastrepha suspensa*. To mimic low populations more representative of a fruit fly eradication program, mangos and papaya in Hawaii were infested with a known, low number of *Bactrocera dorsalis* larvae. The applicability of the method was tested in the field on guava naturally infested by *B. dorsalis* to evaluate the method under conditions experienced by workers during an emergency fruit fly program. In both field and laboratory trials, mushing and sieving the pulp was more efficient (required less time) and more sensitive (more larvae found) than cutting fruit. Floating the pulp in brown sugar water solution helped detect earlier instar larvae. Mushing and sieving fruit pulp of important tephritid hosts may increase the probability of detecting larvae during emergency programs.

## Introduction

Tephritid fruit flies are among the most destructive agricultural pests, with the genera *Anastrepha*, *Bactrocera*, and *Ceratitis* posing the greatest risk[1]. Many areas are at high risk for exotic fruit fly establishment, based on 1) historical incursions and associated delimitation and eradication programs, 2) the high arrival rate of fruit fly host material at ports of entry, and 3) climatic conditions favorable for the establishment of reproducing populations. The state of California experiences multiple incursions and detections of tephritids annually[2]. There have been more than 200 incursions and eradication programs against tephritids globally over the last century, and this has accelerated significantly in recent decades[3]. Though the vast majority of these programs are successful in eradicating the invading fruit fly[3,4], the economic and environmental burden of these invasions remains still high, and the possibility of establishment is always present; a recent catastrophic example is the infection of *Bactrocera dorsalis* in the African continent[5].

During emergency fruit fly programs, a concerted effort is made to detect and control breeding populations of the invading species. For example, the state of Florida responds to tephritid incursions by applying soil drenches (under the dripline of fruit-bearing host plants) and removing host fruit in a 200 m radius around sites where mated females and/or larvae are found[6]. These actions and tactics serve to kill larvae and pupae in the soil and remove any eggs and larvae from fruit within the area. In some eradication programs, a significant amount of host fruit is removed. In 2015, over 100,000 kg of fruit was destroyed during the *B. dorsalis* eradication program in Florida[6]. The economic losses to growers and associated industries in the quarantined area alone were estimated to be over $10.7 million[7].

To find tephritid larvae in the quarantine areas, a small team of entomologists collect host fruits in a 200 m radius around a female fly detection area and cut and visually inspect each fruit for larvae[6]. With limited staff resources and hundreds of possible hosts, the task becomes difficult, particularly in the areas where plant diversity in both commercial production areas and residential yards is high. In addition, larvae may be missed when cutting host fruits. In a study evaluating fruit cutting at the ports of entry, cutting fruit was found not to be as effective in detecting *A. suspensa* when compared to holding the infested fruits for several weeks and counting the larvae and pupae found in the pupation substrate[8].

There are alternatives to fruit cutting for detecting an infestation[9,10,11,12,13]. For example, a brown sugar floatation and a hot water method are both accepted procedures used to detect western cherry fruit flies in harvested cherries[9,10]. The brown sugar method involves placing crushed fruit in sugar water solution and collecting larvae that float to the top. The brown sugar floatation method was developed specifically to meet regulatory rules for exported cherries, which require packing houses to monitor for quarantine fruit fly pests. There is also an approved US-Canada blueberry certification program that includes brown sugar water floatation, salt water floatation, or boiling to support phytosanitation[14]. When testing the accuracy of sugar and hot water floatation, researchers used the sieving method to determine how many larvae are missed[9,10,11,12,13]. A study showed that mixing crushed blueberries in a salt solution and filtering the solution through

a reusable coffee filter was four times better in detecting *Drosophila suzukii* larvae than visually inspecting the surface of salt and sugar solutions[14]. In addition, gas chromatography was used for the detection of *A. suspensa* larvae in citrus[15]. These approaches have not been tested for applicability in field surveys.

Our goal was to develop and test a method to find tephritid larvae in the field using sieving and sugar water floatation. This method allows the more efficient detection of immature fruit flies than the traditional fruit cutting method, supporting the timely control of breeding populations during fruit fly eradication programs.

## Protocol

### 1. Fruit Selection

1. Determine what fruit is available in the area to be surveyed.
2. Select host fruit based on the list of known hosts for the target tephritid species.
3. Choose soft fleshed, ripe fruit, such as mangos, papaya, and guava. Unripe or hard fleshed fruit, such as tropical almonds, should be inspected with a different method, such as fruit cutting.
4. Select fallen, overripe fruit, or ripe fruit on trees that have signs of damage, oviposition scars, and soft spots.
5. Process approximately 2 L of fruits at once (e.g., 5 guavas or 5 medium-sized mangos constitute adequate samples for this method). The number of fruits that can be processed at once depends on the size of the fruits (**Figure 1A**).

### 2. Mushing

1. Cut the fruit into large pieces and place it into a 4 L zip lock storage bag (**Figure 1B**).
2. Add water to the bag until the water covers the chopped fruit by 25-50 mm (**Figure 1C**).
3. Squeeze the fruit gently by hand until all the pulp has dislodged from the peel and has a smooth consistency (i.e., no large chunks) (**Figure 1D**).

### 3. Sieving for late instar collection

1. Stack the sieves. Use large sieves (457 mm diameter) for processing large amounts of fruits (~ 5 fruits at once) and smaller sieves (305 mm diameter) for individual fruit or smaller samples (< 5 fruit).
2. Stack the sieve with a large mesh (No. 8; 2.36 mm) sieve atop a small mesh (No. 20; 0.85 mm) sieve. For the detection of early instars, place a third sieve (No. 45; 0.35 mm) on the bottom of the stack (**Figure 1E**).
3. Pour the pulp into the top sieve (**Figure 1F**).
4. Thoroughly wash the pulp through the stack of sieves using water from a faucet, hose, or a bottle until the fine pulp has passed through the sieves (**Figure 1G**).
5. Visually scan the top sieves for late instar larvae that might have been retained with the peel or any large pieces of fruit (**Figure 1H**).
6. Carefully inspect the second sieve for late instar larvae. With large amounts of fine pulp, additional rinsing may be necessary.
7. Collect larvae from the sieves with larval forceps and place them into vials with 70% EtOH.

### 4. Sugar floatation for early instar collection

1. Premix the sugar solution by dissolving 453 g (1 box) of dark brown sugar in 2 L of tap water, which yields a Brix reading of 19°[10].
2. Wash the pulp from the finer mesh sieves (e.g., No. 20 and No. 45) to the edge of the sieve with tap water, then move the material to a plastic dishpan (11 L).
3. Add the brown sugar solution until it covers the pulp by 25-50 mm and add 2 drops of anti-foamer. Let the pulp sit in the brown sugar solution for about 5 min.
4. Collect larvae that float to the surface of the solution with larval forceps into vials with 70% EtOH.

### 5. Larval curation

1. Label a vial with the collection location, date, type of fruit, and collector for later examination and identification.

## Representative Results

**Early and late instar *Anastrepha suspensa* extraction from field collected fruit**

In this experiment, we have compared the fruit cutting and the mushing, sieving, and floating (MSF) methods in respect to the proportion of larvae detected and the mean time required to detect them. Guava, highly infested with the larvae of *Anastrepha suspensa*, were collected from a plant located at the University of Florida, Institute of Food and Agricultural Sciences, Tropical Research and Education Center, Homestead, FL. The fruit were randomly sorted into groups of 5 and assigned to 1 of 2 larval extraction methods: 1) hand cutting or 2) the MSF method. The time to collect all the larvae visible to the naked eye using each extraction method was recorded.

The hand cutting method followed the method currently being used in an eradication program. Each of the 5 workers (n=5) was assigned 5 fruits to search for all stages of larvae by cutting the fruits into smaller pieces and visually inspecting the pulp. To determine whether larvae were missed in the visual inspection, the hand-cut fruit pieces were re-inspected using a dissecting microscope (10x).

For the MSF method, 5 fruits were cut into large pieces (50-80 cm), placed in zip lock bags, and squeezed gently by hand until all the pulp was dislodged from the peel and the pulp had a smooth consistency (i.e., no large chunks). The mushed fruit was strained through a series of large (45.7 cm) brass sieves. The largest mesh (No. 8) was stacked on the top, followed by a number No. 20 and a No. 45 mesh sieve. The staff assigned to this treatment washed the pulp through the mesh using water from a hose connected to a sink faucet. The late instar larvae were apparent in the sieves. The smaller instars were mixed with pulp, making them difficult to see and remove. Therefore, the pulp/larvae mixture from the sieves was put into buckets with 1 L of brown sugar water solution. The larvae immediately floated to the surface. The solution was gently stirred, and after 5 min, larvae were removed from the buckets and counted. The time to process the fruit was a combination of mushing, sieving, and removing the larvae from the sugar water solution. Data for the number of larvae found through the hand cutting or sieving and floatation methods were analyzed using the Kruskal-Wallis non-parametric test ($p = 0.05$)[16].

The MSF method yielded greater numbers of larvae (**Figure 2A**) and more larvae per min (**Figure 2B**) than hand cutting. Although detection of the different instars was not quantified in this study, we observed that all instars (first, second and third) were found using sieves, whereas only later instars (second

and third) were seen using hand cutting. When the previously cut and visually inspected samples were re-inspected with a dissecting microscope scope, 40% of the late instar larvae infesting the fruits were missed. However earlier instars were primarily found with the re-inspection.

This experiment showed that using the MSF method is more effective and efficient for finding larvae in highly infested fruit. However, fruit infested with lower numbers of larvae are more likely encountered in an eradication program, where the invading species would be very rare. Therefore, we conducted a laboratory study in which the host fruit was infested with a known, low number of larvae.

**Manual infestation of mango and papaya to simulate low *Bactrocera dorsalis* infestation**

This experiment compared the fruit cutting and MSF methods with respect to the proportion of larvae detected and the time required to detect them when infestation was relatively low. Manual infestation was used as an experimental tool to evaluate the efficacy of each method, as the number of larvae present was known with certainty.

A cork borer (1.0 cm diameter) was used to make 5 holes in individual mango and papaya fruits that were free of fruit fly larvae. A single late second to early third instar *B. dorsalis* larva was placed into each of the 5 holes of a subset of the fruit. The holes were capped using the piece bored from the fruit and the remaining fruit were capped without inserting larva to visually simulate manual infestation. The fruits were held at 27 °C for 48 h to allow for larval development. The experiment was conducted at the ARS laboratory in Hilo, Hawaii Island (n = 5 workers) and the APHIS-PPQ laboratory on Oahu Island, Hawaii (n = 4 workers).

For fruit cutting, each worker was given 5 mangos (1 infested with 1 larvae and 4 not infested) and 4 papayas (one infested and 3 not infested). A worker cut each fruit individually into smaller and smaller pieces and continuously inspected the pulp for any immature fruit flies. Searching was stopped when the pulp was thoroughly inspected. The total number of larvae found and the time spent by each worker to process all the fruits by cutting were recorded (**Figure 3**) and (**Figure 4**).

Each worker received another similar set of fruits (5 mangos and 4 papayas) for mushing or sieving (with no fruit cutting involved), with 2 pieces infested as previously described. Pulp was poured into the top sieve and washed through the stack of sieves using water from a faucet and larvae removed, as described in the protocol. The experiment was conducted twice, with sugar floatation and without sugar floatation, to determine whether removing the floatation step would increase the speed of the process without losing sensitivity (i.e., all or most larvae were found) (**Figure 3**). The number of larvae found and the time spent by each worker to process the fruit through the cutting, MSF, or MS method were recorded.

For both mangos and papayas, the full MSF method (floatation included) resulted in higher numbers of larval detections and was faster than fruit cutting (**Table 1**). Workers using the traditional fruit cutting method missed 32% and 35% of the larvae placed in mangos and papaya, respectively (**Table 1**). Processing fruits in bulk using the MSF technique required 30% less time than cutting individual mangos and 35% less time than cutting individual papayas (**Figure 3**). More larvae were found per minute using the MSF method for papaya (**Figure 3C**) and mango (**Figure 3D**) when compared to the fruit cutting method. All larvae found were alive.

Larval morphological identification is only possible for late instars. We repeated the above experiment but omitted the

floatation procedure to determine whether the recovery of larvae remained high and the speed of fruit processing increased. The MS method (with floatation omitted) resulted in more larval detections for papaya (**Figure 4A**) and mango (**Figure 4B**) compared to cutting and visual inspection. Additionally, the technique was faster than cutting and visually inspecting papaya (**Figure 4C**) and mango (**Figure 4D**). Removing the floatation step from the MSF method reduced the time to find late instar larvae by 90% for papaya and by 48% for mangos (**Table 2**). The percentage of larvae found was high for both methods and was consistently higher for MS (floatation omitted). For papaya, 80% and 85% of the larvae were recovered from the MSF and MS methods, respectively (**Table 1** and **Table 2**). For mango, 88% and 95% were recovered from the MSF and MS methods, respectively (**Table 1** and **Table 2**).

**Field comparison of the fruit cutting and MSF methods**

The goal of this experiment was to compare the fruit cutting and MSF methods under field conditions, mimicking an emergency fruit fly program. Fruit processing was conducted without the convenience and infrastructure of the laboratory to test the field readiness of the two larval extraction methods. Work was conducted in a guava orchard located at the USDA-ARS Tropical Plant Genetic Resources and Disease Research Unit Germplasm near Hilo. A total of 40 guavas showing signs of infestation were collected and divided into 2 groups. A total of 20 guavas were subjected to cutting/visual inspection followed by MSF (floatation included), which allowed for assessment of the sensitivity of the cutting method compared to the MSF method. Dissection proceeded as described above. When detected, larvae were removed and counted. Four workers dissected 5 guavas each, and the time required for cutting and inspecting was recorded for each worker. Post-cutting MSF was conducted as above, except that a third smaller-mesh sieve (No. 40, 0.420 mm) was used in addition to the No. 8 and No. 20 sieves to collect smaller larvae. The second set of 20 guavas were placed in 2 zip lock bags (10 fruits per bag) and were subjected to MSF only (i.e., no cutting), which allowed a comparison of the time needed for fruit cutting versus MSF. As above, three sieves were used in this procedure. The number of larvae found and the total time to process fruit (mushing and holding the fruit for 5 min in the bag/sieving/floating in sugar solution) were recorded.

As found in the laboratory, fruit cutting underestimated fruit infestation and was highly variable, detecting 25%-83% fewer larvae than what could be recovered using MSF methods (**Table 3**). Moreover, in the sample with low numbers of larvae, MSF recovered 500% more larvae, providing higher assay sensitivity and a greater chance to identify the infesting organism. Fruits were processed much faster using the MSF method compared to cutting; cutting and inspecting 5 fruits required about the same amount of time as processing 10 fruits *via* MSF.


**Figure 1: Steps of the fruit fly larvae extraction protocol.** (**A**) Process approximately 2 L by volume of fruit at once (e.g., 5 guavas or 5 medium mangos constitute adequate samples for this method). (**B**) Cut the fruit into large pieces and place it into a 4 L zip lock storage bag. (**C**) Add water to the bag until the water covers the chopped fruit by 25-50 mm. (**D**) Squeeze the fruit gently by hand until all the pulp has dislodged from the peel and has a smooth consistency (i.e., no large chunks). (**E**) Stack the sieve with the large mesh (No. 8; 2.36 mm) sieve atop followed by the small mesh (No. 20; 0.85 mm) sieve. For early instars, place a third sieve (No. 45; 0.35 mm) on the bottom of the stack. (**F**) Pour the pulp into the top sieve. (**G**) Thoroughly wash the pulp through the stack of sieves using water from a faucet, hose, or a bottle until the fine pulp has passed through the first sieve. (**H**) Visually scan the top sieves for late instar larvae that might have been retained with the peel or any large pieces of fruit. Please click here to view a larger version of this figure.

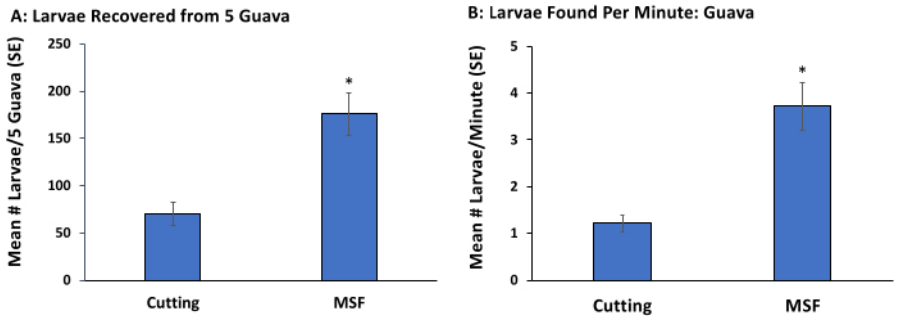


**Figure 2: Early and late instar *Anastrepha suspensa* extraction from field collected fruit.** The mean number (± standard error of the mean [SE]) of *Anastrepha suspensa* larvae from five guava fruit collected by cutting and visually inspecting (cutting: 70.4 ± 11.9) or washing the pulp through a series of three sieves followed by soaking the pulp in a sugar water solution (MSF: 175.6 ± 21.91) (**A**). The mean number of larvae (±SE) collected per minute from 5 guavas processed by cutting (1.21 ± 0.16) and by MSF (3.71 ± 0.50) (**B**). Each method was replicated 5 times, and asterisks above the bars indicate significant differences for the number of larvae ($\chi^2$ = 6.81, $p$ < 0.01) and the time to process ($\chi^2$ = 6.80, $p$ < 0.01) based on a Kruskal-Wallis test. Please click here to view a larger version of this figure.

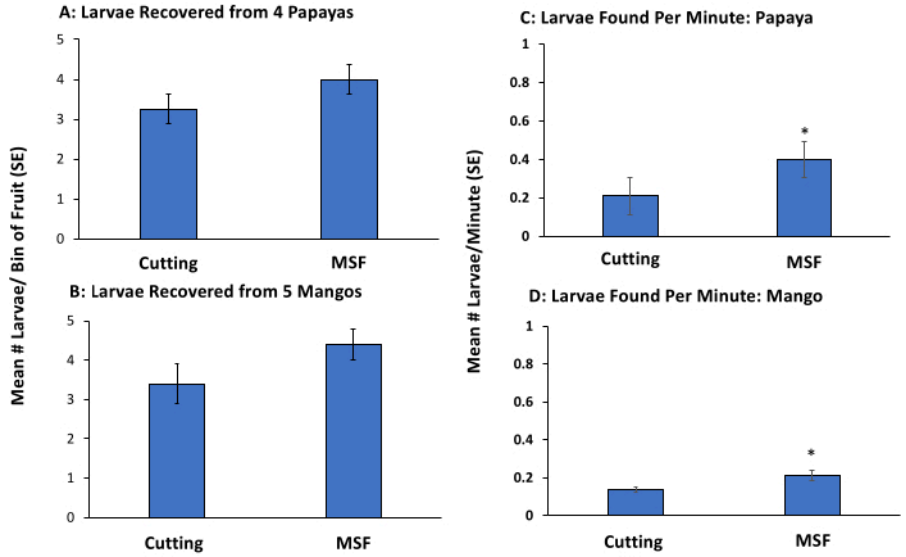


**Figure 3: Validation of the full mushing-sieving-floatation method using manual infestation of mango and papaya to simulate low *Bactrocera dorsalis* infestation.** The mean number of *Bactrocera dorsalis* larvae (±SE) found in papaya (cutting: 3.25 ± 0.51, MSF: 4.0 ± 0.4) (**A**) and mango (cutting: 3.4 ± 0.51, MSF: 4.4 ± 0.4) (**B**) fruits and the mean number of larvae (±SE) collected per minute from papaya (cutting: 0.21 ± 0.1, MSF: 0.4 ± 0.15) (**C**) and mango (cutting: 0.14 ± 0.01, MSF: 0.21 ± 0.03) (**D**). Fruits that were processed using the cutting or the MSF methods (floatation included, n = 5) manually infested with 5 third instar larvae. Asterisks above the bars indicate significant differences for the number of larvae found in papaya ($\chi^2$ = 5.39, $p$ = 0.02) and mango ($\chi^2$ = 3.94, $p$ = 0.05) when compared to fruit cutting based on Kruskal-Wallis tests.

Please click here to view a larger version of this figure.

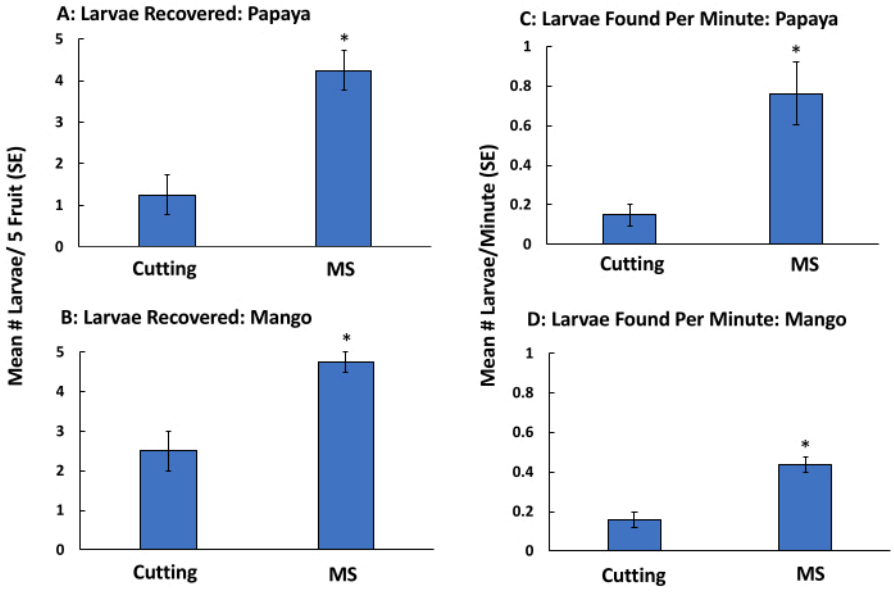


**Figure 4: Validation of the mushing-sieving method (floatation removed) using manual infestation of mango and papaya to simulate low *Bactrocera dorsalis* infestation.** The mean number of larvae (±SE) found in papaya (cutting: 1.25 ± 0.48, MS: 4.25 ± 0.48) (**A**) and mango (cutting: 2.5 ± 0.5, MS: 4.75 ± 0.25) (**B**) fruits and the mean number of larvae collected per minute (±SE) in papaya (cutting: 0.15 ± 0.05, MS: 0.76 ± 0.15) (**C**) and mango (cutting: 0.16 ± 0.04, MS: 0.44 ± 0.04) (**D**). Fruits were manually infested with 5 third instar *Bactrocera dorsalis* larvae and processed by cutting and visually inspecting (cutting) or mushed in a bag and washed through sieves (only mushing and sieving, without floatation, n = 4). Asterisks above the bars indicate significant differences for the number of larvae found in papaya ($\chi^2$ = 5.46, $p$ = 0.02) and mango ($\chi^2$ = 5.25, $p$ = 0.02) and the time to process papaya ($\chi^2$ = 5.39, $p$ = 0.02) and mango ($\chi^2$ = 5.39, $p$ = 0.02) compared to fruit cutting, based on Kruskal-Wallis tests. Please click here to view a larger version of this figure.

| Fruit | # Fruit processed | #Larvae added | Processing method | #Larvae found | Processing time (min)* | % Recovery |
|---|---|---|---|---|---|---|
| Mango | 25 | 25 | Cutting | 17 | 158 | 68% |
| Mango | 25 | 25 | MSF | 22 | 113 | 88% |
| Papaya | 16 | 20 | Cutting | 13 | 62 | 65% |
| Papaya | 16 | 20 | MSF | 16 | 40 | 80% |
| *Total time summed over 5 workers. | | | | | | |

**Table 1: The number of larvae recovered and the time to process fruit by the cutting and visually inspecting (cutting) or the full mushing, sieving, and floating (MSF) method.** The test fruit was manually infested with 5 third instar larvae mixed with bored and capped only fruit (1 of the 5 mangos, 1 of the 4 papayas).

| Fruit | # Fruit processed | #Larvae added | Processing method | #Larvae found | Processing time (min)* | % Recovery |
|---|---|---|---|---|---|---|
| Mango | 20 | 20 | Cutting | 10 | 66 | 50% |
| Mango | 20 | 20 | MS | 19 | 44 | 95% |
| Papaya | 16 | 20 | Cutting | 5 | 38 | 25% |
| Papaya | 16 | 20 | MS | 17 | 25 | 85% |
| *Total time summed over 4 workers. | | | | | | |

**Table 2: The number of larvae recovered and the time to process fruit by cutting or mushing and sieving only, floatation omitted (MS).** The test fruits were manually infested with five third instar larvae mixed with bored and capped only fruit (1 in 5 mangos, 1 in 4 papaya).

| Worker/method | #Fruit processed | Time to process (min) | #Larvae found cutting | #Larvae found MSF* | % of overall count larvae found via cutting |
|---|---|---|---|---|---|
| Worker 1: cutting | 5 | 18 | 33 | 14 | 70% |
| Worker 2: cutting | 5 | 18 | 1 | 5 | 17% |
| Worker 3: cutting | 5 | 26 | 9 | 11** | 75% |
| Worker 4: cutting | 5 | 20 | 24 | | |
| Worker 5: MSF | 10 | 22 | NA | 22 | NA |
| Worker 6: MSF | 10 | 18 | NA | 37 | NA |
| * Pulp from the cutting and visual inspection processed again using the MSF method to determine the number of late 2nd-3rd instar larvae missed | | | | | |
| ** Pulp of workers 2 and 3 fruit pooled prior to processing using the MSF method | | | | | |

**Table 3: The number of larvae found in field-collected guava by cutting and visually inspecting the fruit (cutting) or by mushing, sieving, and floating (MSF) the fruit.**

## Discussion

Our goal was to develop an efficient and effective way to find tephritid larvae in the field. The motivation of launching an eradication program or establishing a quarantine area is the detection of mated female(s) or larvae[6], which indicates a breeding population. The current method of cutting and visually searching fruit is inefficient in finding larvae as there are usually many more host fruits present than can be individually inspected. In addition, the populations of the tephritids are likely low in an area of new invasion, making the chances of finding larvae in a large amount of fruit incredibly difficult. For example, in the 2015 *Bactrocera dorsalis* eradication program in Florida, 54 different host species were identified, and more than 4,000 fruits were cut. In this eradication program, only a few larvae were found in mango, and no other hosts were found to be infested[6]. We found that the MSF/MS method was both more sensitive and faster in detecting *A. suspensa* and *B. dorsalis* larvae when processing fruits that had a large amount of pulp (mangos, guava, and papaya) in bulk compared to fruit cutting. The larger amount of host fruits that it is possible to inspect using the mushing and sieving method, combined with the increase of detection of a rare larva, could increase the probability that an infestation would be found early. The early detection of a breeding population could increase the likelihood of eradication and reduce the costs of the program.

Our experiments showed that the number of larvae detected by workers cutting and visually inspecting fruits varied considerably. Workers cutting fruit missed 50% and 75% of the *B. dorsalis* larvae placed in mangos and papaya, respectively. In contrast, only 5% and 15% of the larvae were missed using the MS method for processing mango

and papaya fruit, respectively. Similarly, a study evaluating fruit cutting at ports of entry showed there was considerable variation in the number of infested fruits and larvae found by the inspectors[8]. The study showed that experienced port inspectors missed 64%-99% of *A. suspensa* larvae and 16%-82% of the infested fruit when fruit was cut and visually inspected[8]. Our results suggest that the mushing and sieving method could decrease the likelihood that a worker would miss detecting an infested fruit.

Sugar and hot water floatation are accepted protocols in a systems approach method for ensuring cherries and blueberries are free of fruit flies[14]. A subset of a shipment is crushed into the solution, whereupon an inspector visually screens the surface of the sugar solution for the presence of eggs and larvae. Although a larger number of fruits can be processed compared to cutting individual fruit, the probability of finding larvae using these techniques is still affected by the ability of the inspector, the stage and number of larvae present, and the type of fruit[8]. We found that, like other tephritids, *B. dorsalis* and *A. suspensa* become dislodged from the fruit pulp and float to the surface. Interestingly, we found that with larger late instar larvae, which are the target in emergency and eradication programs as they can be identified morphologically, including sugar floatation did not increase the accuracy of the method. In fact, adding the floatation method increased the processing time by 90% for papaya and by 48% for mango. Increased processing time plus the additional materials (i.e., water, bins, sugar, etc.) do not operationally support adding this step when searching for large instars in the field. The sugar floatation method may be appropriate when the goal is to detect all stages including early instars, such as at ports of entry and packing houses. Filtering the sugar solution with a fine mesh sieve would most likely provide the most accurate detection of eggs and early larval instars[11,12].

The MS and MSF techniques work well with fruit that can easily be mushed and have a large volume of pulp. Tephritid larvae tend to burrow into fruit pulp, which makes visual detection difficult. A critical aspect of the MS and MSF methods is separating the larvae from the pulp. The sieving process removes the pulp, thus exposing the larvae on sieve screens. Similarly, the sugar water method separates the larvae from the pulp by making the larvae float, while the pulp sinks to the bottom of the pan. Larvae separated from the pulp by the MS or MSF methods are readily observed moving on the sieve screen or water surface. Although the mushing, sieving, and optionally floating method greatly improved the speed and accuracy of detecting tephritid larvae in important host fruit, the process may not be appropriate for all fruits. For example, host fruit with hard pulp, such as green avocados or fruit with a large seed/pit and relatively small amount of pulp, such as tropical almonds, may be easier to process by hand cutting and visual inspection.

We found that the MS and MSF methods were faster when a relatively small number of fruit (5-10) were processed. The difference would likely be greater if larger amounts of fruits were processed, which might be necessary and typical of emergency fruit fly programs. Removing the floatation step further increased the detection speed without compromising the accuracy of finding large tephritid larvae (>3 mm). We showed that these techniques could be taken to the field, which simulated the conditions experienced by workers during an emergency fruit fly program. Our studies indicate that the MS methods may allow for a timelier detection of late instar larvae and subsequent eradication of tephritid breeding

populations. MSF could be used to detect eggs and early instars currently not targeted by eradication programs.

## Disclosures

The authors declare that they have no conflicts of interests.

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