applied sciences

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Drosophila Infestations of California Strawberries and Identification of *Drosophila suzukii* Using a TaqMan Assay

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Abstract: In contrast to most other *Drosophila* species that infest processing (overripe) strawberries, the spotted-wing drosophila (SWD) can also infest firm and ripe fruit intended for the fresh market. However, fresh fruit infestations of this invasive species did not become an economic problem for California growers until a decade after SWD's first detection in California strawberries in 2008. This outbreak corresponds to the development of reported insecticide resistance in SWD populations from strawberry and other berry crops following years of incidental exposure of insecticide applications against other key pests. The objective of this study was to determine the current levels of *Drosophila* infestation in fresh market and processing strawberries which would inform the choice of insecticides to use for control. We sampled fresh market and processing strawberries from 17 fields over a two-year period in the three major strawberry production areas of California and determined the numbers of emerged SWD and non-SWD *Drosophila* adult flies. In addition, since holding fruit for adult emergence to determine species composition is impractical for making rapid control decisions and could be inaccurate due to potential interspecific competitions among larvae in the fruit, we developed a TaqMan assay that facilitates larval identification.

Keywords: spotted-wing drosophila; molecular diagnostic assay; larval identification; invasive species

Citation: Ganjisaffar, F.; Abrieux, A.; Gress, B.E.; Chiu, J.C.; Zalom, F.G. *Drosophila* Infestations of California Strawberries and Identification of *Drosophila suzukii* Using a TaqMan Assay. *Appl. Sci.* **2023**, *13*, 8783. https://doi.org/10.3390/app13158783

Academic Editors: Ana Arnaiz and Miguel G. Ximenez-Embun

Received: 30 June 2023
Revised: 26 July 2023
Accepted: 28 July 2023
Published: 29 July 2023

1. Introduction

Many *Drosophila* species are known to oviposit in overripe or damaged fruit. Most species are not considered economic pests of fresh strawberry production since the fruit are harvested prior to becoming sufficiently soft to enable successful oviposition by gravid females. However, strawberries grown for processing are allowed to ripen in the field to facilitate the easy separation of the calyx and core during harvest, thus making those fruit more susceptible to *Drosophila* infestation [1]. Strawberries infested with drosophilid flies also become prone to secondary infections that impair fruit quality and result in yield loss [2]. When larvae are present at detectable levels in strawberries that arrive at processors, entire shipments can be rejected. Therefore, growers must apply insecticides to reduce populations in their fields when *Drosophila* populations become sufficiently high to be detectable in harvested fruit. This situation often occurs late in the growing season when harvest intent changes from delivery to the fresh fruit market to processing [1].

The last study to identify *Drosophila* species present in processing strawberries was performed by Zalom and Toscano in 1994–1995 [3]. This work revealed that *Drosophila simulans* Sturtevant was the predominant species present in fruit. In 2008, the invasive *Drosophila suzukii* (Matsumura), commonly referred to as spotted-wing drosophila (SWD), was discovered in strawberry fields in Santa Cruz County, CA before being detected elsewhere in California and eventually across North America [4,5]. Unlike other

drosophilid flies, this species lays eggs into ripening fruit and therefore poses a unique threat to both fresh and processed berry production. SWD caused substantial yield losses in caneberries, blueberries, and cherries the year following its detection, resulting in growers implementing intensive spray programs aimed at suppressing adult populations to prevent larval fruit infestation. Interestingly, the anticipated impact of this species on fresh strawberry production did not occur in the years following SWD's arrival. We believe that this was due to typical cultural practices such as relatively short harvest intervals and removal of damaged and overripe strawberries in addition to the routine use of insecticides such as spinosad (Entrust and Success, Corteva Inc., Indianapolis IN, USA) and spinetoram (Delegate, Corteva Inc., Indianapolis IN, USA) to control thrips species and light brown apple moth (*Epiphyas postvittana* (Walker), Lepidoptera: Tortricidae), and malathion and pyrethroids targeting other insects especially western tarnished plant bug (*Lygus hesperus* Knight, Hemiptera: Miridae). These insecticides are also effective in controlling SWD, thus providing incidental SWD suppression when they are applied.

Obtaining information on the occurrence of and damage by SWD in California strawberries has become important considering recent grower accounts of drosophilid fly infestations in both fresh and processing fruit. These observations appear to coincide with our findings that some SWD populations have become resistant to spinosad [6,7], malathion [8], and pyrethroids [9]. These sprays would be ineffective if SWD currently represents the major species infesting fruit, providing support for our hypothesis that use of these insecticides for other insect pests in strawberries had been providing incidental protection against SWD. The objective of this study was thus to determine the current levels of *Drosophila* infestation in commercial fresh market and processing strawberries, and the extent to which strawberry production contributes to SWD adult populations.

The lack of current data regarding the species composition of drosophilid flies in strawberries creates challenges for implementing effective management programs. Therefore, our second objective was to develop new molecular tools for identifying SWD among other *Drosophila* species. Species determination using morphological characteristics of adult flies is extremely time-consuming given the large number of larvae typically present in field fruit collections and requires specialist expertise. Moreover, morphological identification cannot be reliably performed on larvae. In order to identify larvae in field-infested fruit, despite the practical problems associated with identifying adults, researchers often rear them from larva-infested fruit to assess species composition. However, previous laboratory studies using drosophilid flies have shown that SWD can be outcompeted by other closely related species when provided a common larval substrate [10], confounding the results obtained when multiple *Drosophila* species are present. A precise and rapid method capable of identifying SWD at all life stages would help to develop targeted management programs for this pest. Molecular diagnostic markers have been developed that enable rapid and unambiguous identification of many *Drosophila* species found in agricultural settings including SWD and *D. simulans* at all life stages [11]. We adapted a TaqMan real-time PCR assay to leverage these molecular markers for species identification. By identifying larvae present in strawberries as well as the adults that emerge from the fruit, we assessed the relative contributions of different *Drosophila* species to the total larval infestation in strawberries.

2. Materials and Methods

2.1. Strawberry Sampling and Fly Collection

Strawberries were collected from commercial fall-planted fields grown on raised beds in California's major production areas near the end of the season when fruit is more susceptible to drosophilid fly infestation. These areas represented 98.6 percent of California production and 86.7 percent of total U.S. production during this period [12]. Four fields (OXN 1, OXN 2, OXN 3, and OXN 4) near Oxnard, Ventura County were sampled on

30 May 2019. A second collection was made from three of these fields (OXN 1′, OXN 2′, and OXN 3′) on 18 June 2020, and a nearby field (OXN 5) was also sampled on that date since the processing strawberry harvest in OXN 4 had already been terminated. Due to COVID-19 and associated travel restrictions, no samples were collected in spring 2020. In the Santa Maria area (Santa Barbara and San Luis Obispo Counties), fruit samples were collected from three fields (SAT 1, SAT 2, and SAT 3) on 12 August 2019, and from two fields (SAT 4 and SAT 5) on 12 August 2020. In the Monterey Bay area (Monterey and Santa Cruz Counties), samples were collected from three fields on 17 October (MON 1, MON 2, and MON 3) and 5 November (MON 1′, MON 2′, and MON 3′) in 2019, and from four fields on 27 October (MON 4, MON 5, MON 6, and MON 7) and 10 November (MON 4′, MON 5′, MON 6′, and MON 7′) in 2020.

On each sampling date, 20 to 40 ripe (suitable for fresh market) and 13 to 28 overripe (suitable for processing) fruit were randomly collected from each site and returned to our laboratory at the University of California, Davis to assess the levels of larval infestation as well as the relative abundance of *Drosophila* species causing infestation. To rear *Drosophila* adults from the samples, strawberries were placed in plastic containers (16 × 28.5 × 9.5 cm) (The Container Store Group, Inc., Coppell, TX, USA) containing a thin layer of cotton topped with sand as a substrate for pupation. The lids of these containers had large openings (10 × 12 cm) covered with fine mesh to ensure ventilation. The containers were then placed in a walk-in growth chamber at 23 ± 1 °C, 55–65% RH and a 14L:10D photoperiod (Percival Scientific Inc., Perry, IA, USA) and checked daily until fly emergence. Emerged flies were aspirated into Fisherbrand drosophila bottles (Fisher Scientific, Inc., Portsmouth, NH, USA) containing Bloomington standard drosophila cornmeal diet, then plugged with cotton. These flies were then anesthetized using CO_2 to facilitate their identification as SWD versus non-SWD *Drosophila* using morphological characteristics.

For samples collected from the Monterey Bay area in 2020, larvae were extracted from fruits immediately following collection from half of the samples. To extract larvae from the samples, strawberries were gently crushed and floated in a standard salt-water solution (125 g salt in 4 L of lukewarm water) for a minimum of an hour. The solution was then strained through a fine strainer, and the extracted *Drosophila* larvae from each sample were collected with a fine brush and transferred to vials containing 70% ethanol for storage before being processed for DNA extraction and identification as SWD vs non-SWD *Drosophila* using molecular diagnostic markers. The remaining fruit from each sample were used to rear out adults as described above.

2.2. DNA Extraction

Genomic DNA (gDNA) was extracted from *Drosophila* larval samples using the protocol optimized by [13]. For sites where the number of extracted larvae exceeded 30, three replicates of ten first to third instar larvae were used. For sites with ≤10 extracted larvae, all larvae were used as a single replicate. Larvae were homogenized in a 2% CTAB solution (100 mM Tris-HCl (pH 8.0), 10 mM EDTA, 1.4 M NaCl, and 2% CTAB). The sample was incubated at 65 °C for 5 min, and 200 µL of chloroform was added to the tube and then slowly inverted 10 times to mix. To isolate nucleic acids, samples were centrifuged at 13,000 rpm for 10 min at 4 °C. The aqueous layer was transferred to a new tube and mixed with an equal volume of 100% isopropanol and left in −20 °C overnight for gDNA to precipitate. The DNA was then pelleted at 13,000 rpm for 15 min at 4 °C. The DNA pellet was washed with 70% ethanol and spun down at 13,000 rpm for 5 min at 4 °C. After the pellet was air-dried, the gDNA was re-suspended in nuclease-free water. Recovered gDNA was stored at −20 °C and then assessed by the TaqMan PCR.

2.3. TaqMan Assay

TaqMan real-time PCR assay was used to discriminate SWD from other *Drosophila* species (non-SWD) at the larval stage through the detection of single nucleotide polymorphism (SNP). This molecular diagnostic technique relies on the amplification of the polymorphic sequence Sec61 (CG9539) with specific primers (Sec61 Forward primer: TGATGGCCACCAGGAACGAT and Sec61 Reverse primer: GTGTAGAACAGCTTGATGGG) followed by the recognition of the SNP using TaqMan probes labeled with fluorophores. The Sec61 gene from several non-SWD species including *Drosophila melanogaster* Meigen, *Drosophila biarmipes* Malloch, *Drosophila tristis* Fallen, and *D. simulans* was sequenced and aligned to verify that the region of interest is conserved between non-SWD, and that the SNP is only present in SWD (Figure S1). A FAM-labeled probe was used to detect non-SWD, and a Cal Orange 560-labeled probe was used to detect SWD individuals (FAM probe seq: CCAGAACCTGCCCAAT; Cal Orange 560 probe seq: ACAGAACCTTCCCAATC). Dry probes (Biosearch Technologies, Petaluma, CA, USA) were resuspended in nuclease-free water at a 10 µM working concentration. A 2xqPCRbio probe mix (PCR Biosystems, Wayne, PA, USA) was used according to the manufacturer's recommendation using 1 µL of gDNA (at concentration < 1 µg/µL). The TaqMan assay reaction was performed in 96-well plates using a CFX96 thermocycler (Bio-Rad Laboratories, Hercules, CA, USA) with the following settings: 95 °C for 2 min and 40 cycles of 95 °C for 5 s (melting) and 58 °C for 30 s (annealing and extension). Genomic DNA from laboratory-reared samples composed of either six *D. simulans* (all non-SWD), six *D. suzukii* (all SWD), or a mix of *D. simulans* and *D. suzukii* (5:1, 4:2, 3:3, 2:4, and 1:5 SWD: non-SWD) larvae were used as our standard controls to establish a calibration curve. We obtained a linear regression between the relative fluorescence unit (RFU) values for both FAM and Cal orange 560 according to species composition that was used to determine the threshold of detection for experimental samples.

2.4. Data Analyses

All data analyses were performed using R version 4.2.1 for Windows [14] and were evaluated for significance at $p < 0.05$. The proportions of SWD in ripe and overripe fruit for each site were compared using a Chi-square test (function 'prop.test').

3. Results

The number of SWD and non-SWD adults that emerged per fruit show variation among sites in both the degree of infestation and the proportion of infestation caused by SWD. In thirteen out of seventeen samples in 2019 and eight out of ten samples in 2020, the total number of *Drosophila* flies was greater in overripe than in ripe fruit. On average, six, three, and fourteen *Drosophila* flies emerged per ripe fruit, and ten, six, and fourteen flies emerged per overripe fruit in 2019 from the Oxnard, Santa Maria, and Monterey Bay areas, respectively. In 2020, the average number of *Drosophila* flies emerging was one and thirteen per ripe fruit, and one and twenty-nine per overripe fruit collected from the Santa Maria and Monterey Bay areas, respectively. The proportions of SWD were significantly greater in ripe than in overripe fruit ($p < 0.05$), except in OXN 2 ($\chi^2 = 0$; $p = 0.9635$), OXN 4 ($\chi^2 = 1.06$; $p = 0.3023$), OXN 1′ ($\chi^2 = 0$; $p = 0.9637$), SAT 3 ($\chi^2 = 0.12$; $p = 0.7341$), and MON 3′ ($\chi^2 = 0$; $p = 1$) in 2019, and SAT 5 ($\chi^2 = 0.62$; $p = 0.4306$), MON 4 ($\chi^2 = 3.61$; $p = 0.0574$), and MON 5′ ($\chi^2 = 1.11$; $p = 0.2931$) in 2020 (Table 1). Compared to the species distribution observed in the Oxnard area, larvae that were extracted from fruits in the Santa Maria and Monterey Bay production areas were primarily SWD (Table 1). In 2019, the percentage of SWD per sample ranged from 0.4 to 73.1%, from 73.5 to 100%, and from 70.5 to 100% in ripe fruit collected from the Oxnard, Santa Maria, and Monterey Bay areas, respectively. For the overripe fruit, the percentage of SWD per sample ranged from 0 to 51.4%, from 18.9 to 98.1%, and from 49 to 100% in the Oxnard, Santa Maria, and Monterey Bay areas, respectively (Figure 1). In 2020, the percentage of SWD per sample ranged from 0 to 50%

and from 22.2 to 100% in ripe fruit and from 14.3 to 40% and from 15.8 to 100% in overripe fruit collected from the Santa Maria and Monterey Bay areas, respectively (Figure 1).

These results were compared to and confirmed by species identification of larvae found in fruits using the real-time PCR TaqMan assay (Figure 2). All samples from the first collection of the MON sites were identified as SWD since the obtained signals for those samples fell below the FAM RFU value for the standard generated from DNA extracted from five *D. suzukii* and one *D. simulans* larvae (702.87) and above the Cal Orange 560 RFU value for the standard with one *D. suzukii* and five *D. simulans* larvae (443.74). For the second collection, all samples showed FAM RFU values lower than the value for the standard with five *D. suzukii* and one *D. simulans* larvae (723.12), and the Cal Orange 560 RFU value was greater than the standard with one *D. suzukii* and five *D. simulans* larvae (422.95) for all samples except for an overripe sample from the MON 6 site (Figure 2).

Table 1. Total number of *Drosophila suzukii* (SWD) and non-SWD *Drosophila* flies emerged per sample collected from the three major strawberry production areas in California in 2019 and 2020.

Area	Date	Site	Fruit Ripeness	Sample Size (Fruit)	SWD	Non-SWD	*Drosophila* per Fruit	χ^2	*p*
Oxnard	May 2019	OXN 1	Ripe	31	114	42	5.0	136.27	<0.0001
			Overripe	23	36	209	10.7		
		OXN 2	Ripe	22	3	39	1.9	0.00	0.9635
			Overripe	23	3	58	2.7		
		OXN 3	Ripe	20	27	20	2.4	14.37	0.0002
			Overripe	28	60	158	7.8		
		OXN 4	Ripe	35	33	117	4.3	1.06	0.3023
			Overripe	22	37	95	6.0		
	June 2019	OXN 1′	Ripe	26	2	452	17.5	0.00	0.9637
			Overripe	13	0	163	12.5		
		OXN 2′	Ripe	28	87	70	5.6	31.03	<0.0001
			Overripe	15	39	122	10.7		
		OXN 3′	Ripe	28	147	82	8.2	7.68	0.0056
			Overripe	15	133	126	17.3		
		OXN 5	Ripe	24	19	9	1.2	31.00	<0.0001
			Overripe	16	22	116	8.6		
Santa Maria	August 2019	SAT 1	Ripe	38	193	4	5.2	108.22	<0.0001
			Overripe	17	115	103	12.8		
		SAT 2	Ripe	39	50	18	1.7	33.45	<0.0001
			Overripe	19	10	43	2.8		
		SAT 3	Ripe	40	105	0	2.6	0.12	0.7341
			Overripe	20	53	1	2.7		
	August 2020	SAT 4	Ripe	40	0	49	1.2	20.73	<0.0001
			Overripe	20	14	21	1.8		
		SAT 5	Ripe	40	3	3	0.2	0.62	0.4306
			Overripe	20	1	6	0.4		

Table 1. *Cont.*

Area	Date	Site	Fruit Ripeness	Sample Size (Fruit)	SWD	Non-SWD	*Drosophila* per Fruit	χ^2	*p*
Monterey Bay	October 2019	MON 1	Ripe	20	1070	50	56.0	147.76	<0.0001
			Overripe	10	418	135	55.3		
		MON 2	Ripe	20	103	43	7.3	10.67	0.0011
			Overripe	10	48	50	9.8		
		MON 3	Ripe	20	191	1	9.6	26.69	<0.0001
			Overripe	10	52	11	6.3		
	November 2019	MON 1′	Ripe	40	18	0	0.5	N/A	N/A
			Overripe	20	6	0	0.3		
		MON 2′	Ripe	40	425	0	10.6	N/A	N/A
			Overripe	20	222	0	11.1		
		MON 3′	Ripe	40	6	0	0.2	0.00	1.0000
			Overripe	20	11	1	0.6		
	October 2020	MON 4	Ripe	40	5	0	0.1	3.61	0.0574
			Overripe	20	17	22	2.0		
		MON 5	Ripe	40	76	0	1.9	152.18	<0.0001
			Overripe	20	28	149	8.9		
		MON 6	Ripe	40	476	2	12.0	11.60	0.0007
			Overripe	20	399	16	20.8		
		MON 7	Ripe	40	858	0	21.5	N/A	N/A
			Overripe	20	369	0	18.5		
	November 2020	MON 4′	Ripe	40	6	21	0.7	3.89	0.0485
			Overripe	20	14	13	1.4		
		MON 5′	Ripe	40	3	0	0.1	1.11	0.2931
			Overripe	20	24	23	2.4		
		MON 6′	Ripe	40	955	49	25.1	55.87	<0.0001
			Overripe	20	1166	197	68.2		
		MON 7′	Ripe	40	1523	107	40.8	13.29	<0.0001
			Overripe	20	586	15	30.1		

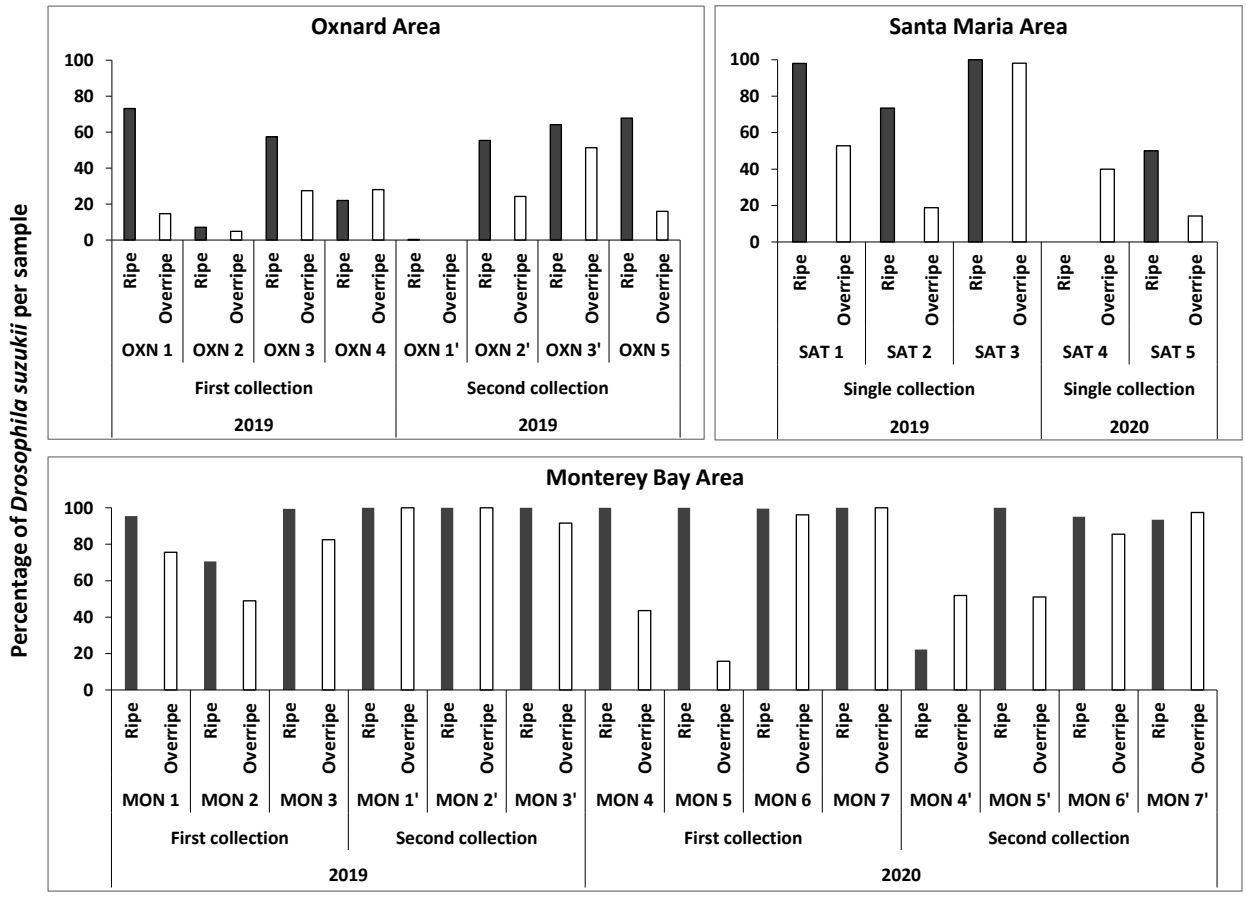

Figure 1. Percentage of *Drosophila suzukii* (SWD) flies emerged per sample collected from the three major strawberry production areas in California in 2019 and 2020. The symbol ′ for a site name indicates a second, later collection from the same site.

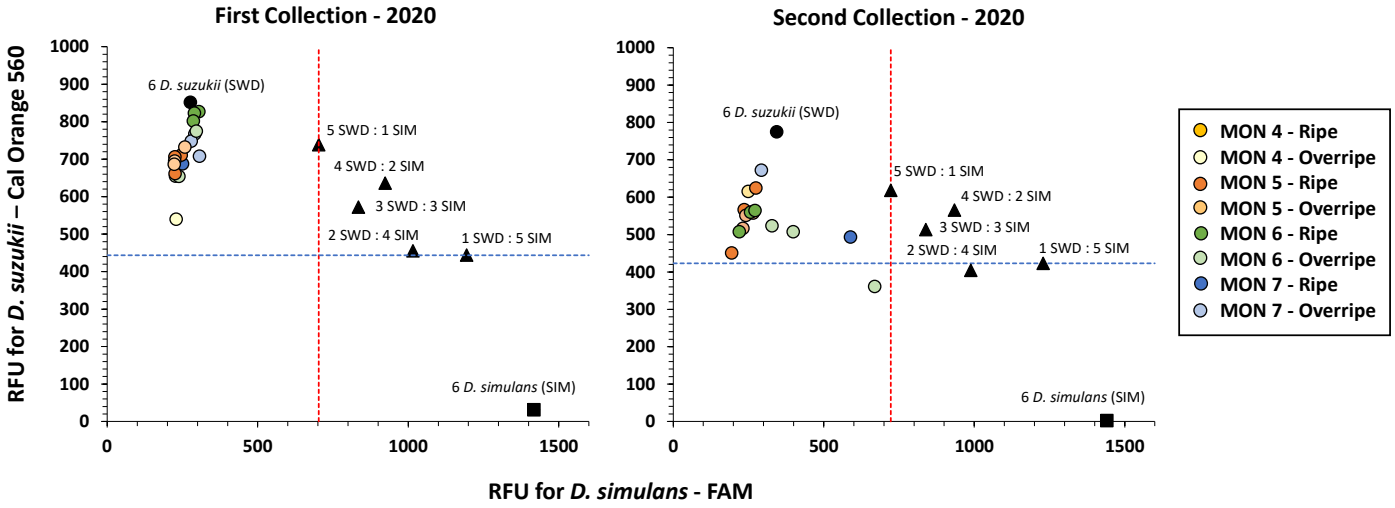

Figure 2. Allelic discrimination results. The PCR reactions included fluorogenic probes to detect SNP associated with *Drosophila simulans* (FAM-labeled probe) or *Drosophila suzukii* (Cal Orange 560-labeled probe). Larval gDNA from *D. suzukii* only (circle), *D. simulans* only (square) or mix of both species (triangle) used as a template for internal controls are represented in black. The red and blue dashed lines are the RFU values for the standards with five *D. suzukii* and one *D. simulans* larvae and five *D. simulans* and one *D. suzukii* larvae at each collection, respectively, serving as a threshold for identification of *D. suzukii* in the samples.

4. Discussion

Our results confirmed recent grower accounts of *D. suzukii* outbreaks in California strawberry fields harvested for both the fresh and processing markets. In their analysis of economic losses attributable to SWD infestation, Goodhue et al. [15] excluded California fresh strawberries from their projected revenue loss estimates because significant yield loss had not been observed. They attributed the lack of reported infestation in fresh strawberries to management practices (especially insecticides) used for other pests, shorter harvest intervals for fresh relative to processed fruit, and earlier harvest timing for fresh fruit. It seems likely that insecticide resistance to multiple classes reported in California berry crops documented since 2017 could be an important factor in the recent outbreaks. This serves as a caution for growers elsewhere to implement research focusing on SWD resistance management and integrated pest management [16].

In general, our hypothesis that ripe fruit will have higher proportions of SWD was supported in both years, which agreed with findings from previous research. For instance, Karageorgi et al. [17] investigated the oviposition behavior of *D. suzukii* and five closely-related *Drosophila* species on ripe and rotten fruit using a two-choice laboratory assay and found that *D. suzukii* females laid almost all their eggs on ripe fruit, whereas *Drosophila ananassae* Doleschall, *D. melanogaster*, *Drosophila eugracilis* Bock and Wheeler, and *Drosophila takahashii* Sturtevant only infested rotten fruit, while *D. biarmipes*, a close relative of *D. suzukii*, showed a mild but not statistically significant preference for rotten fruit. When the choice assay was repeated with sliced ripe fruits with exposed flesh and rotten fruits, they found that *D. biarmipes* laid approximately equal numbers of eggs on both substrates, while *D. melanogaster* maintained a strong preference for the rotten fruit. These results suggest that the preference for oviposition on ripe fruit has evolved in the lineage leading to *D. suzukii* [17]. In addition, these authors indicated that *D. suzukii* is attracted to overripe rotten fruit mostly for feeding, and ripe fruit are mostly selected for oviposition [17], which is also in agreement with findings of Mori et al. and Littler et al. [18,19].

Lower proportions of SWD emerging from overripe fruit could also be due to competition among larvae of *D. suzukii* and other *Drosophila* species, mostly *D. simulans* in California strawberries, that prefer the overripe fruit for oviposition. There have been several studies of interspecific competition between *Drosophila* species [20–22]. One of a few studies that show competition among *Drosophila* species in the field was conducted by [23], who reported that the Sauternes vineyards in France captured *D. suzukii* in traps, but they were not present in collected grape samples. Although *D. suzukii* was known to be the first species to attack ripe grapes in the field, 96.7% and 3.3% of the *Drosophila* infestation in grapes were identified to be caused by *D. melanogaster* and *D. simulans*, respectively [23]. One explanation for this could be the interspecific competition between larvae. *Drosophila* larvae within the same food source compete, leading to increased mortality, decreased growth, and reduced fecundity as density increases [24–26]. Several studies have shown that interference competition between *D. melanogaster* and *D. suzukii* may occur since *D. melanogaster* larvae have a faster feeding rate compared to *D. suzukii* larvae and develop faster than *D. suzukii* [20,22,27]. Shaw et al. [28] also indicated that the presence of *D. melanogaster* significantly reduced *D. suzukii* emergence and the potential oviposition of the females in a substrate pre-inoculated with the eggs of the same or the other species in a laboratory choice assay. Another study showed that the number of *D. suzukii* offspring in both pairwise and cage experiments was dramatically reduced when in competition with *D. melanogaster* [11]. Despite other *Drosophila* species that are not able to compensate developmentally from feeding on low-protein hosts [29], *D. suzukii* can develop in nutrient deficient hosts but may avoid interspecific competition by ovipositing in intact, carbohydrate-rich, and protein-poor fruit [29,30].

Validation of the TaqMan assay confirmed that it is accurate and can differentiate SWD and non-SWD in a mixed population of flies. This molecular diagnostic assay provides an alternative to performing labor-intensive and time-consuming SWD species identification by the traditional approach of rearing adults from *Drosophila*-infested fruit and using

morphological identification. In addition, it is a more flexible and convenient technique since the samples can be stored in ethanol for shipment or later evaluation, and it also provides diagnostic results within a day, allowing for a faster response to mitigate pest infestations.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/app13158783/s1, Figure S1: Alignment of Sec61 sequence from five *Drosophila* species (*D. simulans*, *D. melanogaster*, *D. biarmipes*, *D. suzukii*, and *D. tristis*). The arrow indicates the presence of a single nucleotide polymorphism (SNP) detected in *D. suzukii* ([T/G] in position 38) and used for the TaqMan assay to discriminate SWD from non-SWD species.

Author Contributions: Conceptualization, J.C.C., F.G.Z, F.G., A.A. and B.E.G.; methodology, F.G., A.A., B.E.G., J.C.C. and F.G.Z; software, F.G. and A.A.; validation, F.G., A.A. and B.E.G.; formal analysis, F.G. and A.A.; investigation, F.G., A.A. and B.E.G.; resources, J.C.C. and F.G.Z; data curation, F.G., A.A. and B.E.G.; writing—original draft preparation, F.G. and A.A.; writing—review and editing, F.G., A.A., B.E.G, J.C.C. and F.G.Z.; visualization, F.G.; supervision, J.C.C. and F.G.Z.; project administration, J.C.C. and F.G.Z.; funding acquisition, J.C.C. and F.G.Z. All authors have read and agreed to the published version of the manuscript.

Funding: This research was funded by grant 2020-51181-32140 from the USDA-NIFA, Specialty Crops Research Initiative, grant 21-0001-048-SF from the CDFA Specialty Crops Block Grant Program, and grant ST21-4 from the California Strawberry Commission.

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Data Availability Statement: The data presented in this study are available on request from the corresponding author.

Acknowledgments: We thank Peter Shearer, Oleg Daugovish, Mark Bolda, and Hillary Thomas for their assistance in arranging access to the strawberry field locations for fruit sampling. We also thank Chase Matterson, Marisol Magaña, and Nicole Nicola for their assistance in fruit sampling.

Conflicts of Interest: The authors declare no conflict of interest.

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