FINAL PROJECT REPORT
WTFRC Project Number: CH-14-106

Project Title: Insecticide Resistance of Spotted Wing Drosophila in Sweet Cherry

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Total Project Request: **Year 1**: $32,058 **Year 2**: $93,397 **Year 3: $83,899**

Other funding sources: None

WTFRC Collaborative Expenses: None

Budget 1
Organization Name: WSU TFREC **Contract Administrator:** Joni Cartwright; Katy Roberts
Telephone: 509-663-8181 x221; 509-335-2885**Email:** joni.cartwright@wsu.edu; arcgrants@wsu.edu

Item	2014	2015	2016 (revised)
Salaries[1]	0	0	10,422
Benefits[2]	0	0	4,022
Wages[3]	7,800	8,112	8,400
Benefits[4]	757	787	843
Equipment	0	0	0
Supplies[5]	1,500	1,500	1,500
Travel[6]	2,966	2,966	4,000
Plot Fees	0	0	0
Miscellaneous	0	0	0
Total	$13,023	$13,365	**$29,187**

Footnotes (year 3 revised budget only):
[1]Salaries: Research Intern, 0.20 FTE
[2]Benefits on salaries: 38.6%
[3]Wages $14/hr, 40 hrs/week, 15 weeks/year;
[4]Benefits on wages: 10%.
[5]Supplies: traps, drosophila rearing supplies, baits and lures, office supplies/electronics
[6]Travel to research sites, motor pool rental, mileage, gas (2 months): $1600; travel to sites in WA and OR (lodging, per diem): $2400.

Budget 2 (Van Steenwyk)
Organization Name: University of California Berkeley Contract Administrator: Lynne Hollyer
Telephone: 510-642-5758 **Email address:** Lhollyer@berkeley.edu

Item	2014	2015	2016
Salaries	0	13,180	13,575
Benefits	0	5,878	6,462
Wages	0	0	0
Benefits	0	0	0
Equipment	0	0	0
Supplies	1,008	388	585
Travel	3,892	6,672	8,340
Miscellaneous	0	0	0
Plot Fees	0	0	0
Total	$4,900	$26,118	$28,962

Footnotes:
Salary: Laboratory Research Assistant II at $2,636 per month for 5 months
Benefits: FY 15 = 44.6% and FY 16 = 47.6%
Supplies: Lab supplies for assay and rearing.
Travel: FY 14 = 35 trip for 200 miles/trip at 0.556/mi, FY 15 = 40 trips for 300 miles/trip at 0.556/mi. and FY 16 = 40 trips for 375 miles/trip at 0.556/mi.

Budget 3 (Zalom/Chiu)
Organization Name: University of California Davis **Contract Administrator:** Guyla Yoak
Telephone: (530) 752-3794 **Email address:** gfyoak@ucdavis.edu

Item	2014	2015	2016
Salaries	0	12,872	13,514
Benefits	0	84	88
Wages	0	0	0
Benefits	0	0	0
Equipment	0	0	0
Supplies	5,000	6,408	6,230
Travel	0	0	0
Plot Fees	0	0	0
Miscellaneous	0	5,636	5,918
Total	$5,000	$25,000	$25,750

Footnotes:
Salary and Benefits: Graduate Student Researcher
Supplies: Lab supplies for molecular assays including DNA/RNA extraction, PCR, and DNA sequencing
Miscellaneous: Fees for Graduate Student Researcher

Budget 4

Organization Name: OSU MCAREC **Contract Administrator:** L.J. Koong
Telephone: 541-737-4066 **Email address:** l.j.koong@oregonstate.edu

Item	2014	2015	2016 (revised)
Salaries	--	10,485	0
Benefits	--	6,763	0
Wages	7,280	7498	0
Benefits	605	623	0
Equipment	--	--	--
Supplies	1,000	1,545	0
Travel	250	2,000	0
Miscellaneous	--	--	--
Plot Fees	--	--	--
Total	$9,135	$28,914	**0**

Footnotes:
Salary: Faculty Research Assistant 3 mo. Yr 2, 3, Benefits 28.24%+$1,267.51/mo. 3% increase/yr.
Wages: Summer assistant, 3 mo, $14/hr. Benefits 8.31%. 3% increase/yr.
Supplies: Lab supplies for assay and rearing. 3% increase/yr.
Travel to field. 0.556/mi. 3% increase/yr.

Objectives:

1. *Design and test traps to capture live SWD adults for insecticide resistance studies (yr 1)*
   The initial barrier to screening SWD adults was the need to capture them live from the field; monitoring traps, whether they used baits, synthetic lures or sticky panels were designed to kill the flies entering the trap in order to retain them. Despite the technical difficulties, this approach was deemed preferable to collecting infested fruit and rearing out larvae.
2. *Develop discriminating doses of insecticides to test susceptibility of SWD populations (yr 1)*
   The discriminating dose approach (in preference to probit bioassays) was chosen in order to screen more populations and insecticides. The discriminating dose requires only 100 subjects (vs 700) per bioassay. This also allowed us to perform bioassays on F1 progeny from field collections, instead of having to rear through multiple generations to obtain sufficient flies.
3. *Complete development of primers for genetic analyses of SWD alleles that confer resistance (yr 1)*
   The appropriate primers (those encoding for resistance mechanisms) were necessary before genetic analyses could be performed in Obj. 5. Primer design was greatly facilitated by the publication of the entire SWD genome.
4. *Screen SWD from multiple districts in CA, OR and WA for insecticide susceptibility (yr 2-3)*
   In addition to developing the methodology, our goal was to use it to establish the resistance status of SWD populations in sweet cherries at this point in time (ca. 7-8 years post-detection).
5. *Correlate results from discriminating-dose and genetic studies (yr 2-3)*
   Based on the information found in the bioassays, the genetic studies would reveal which alleles would most likely confer resistance in the future.

SIGNIFICANT FINDINGS

- Several styles of traps and techniques are effective for capture of live SWD.
- The numbers of founding females was below optimal in several orchard due to low underlying SWD density.
- In 105 bioassays, there were 10 instances of surviving females in the diagnostic dose screenings, including some in those re-tested due to initial survivorship. This may be indicative of the early stages of resistance, or issues related to the diagnostic dose bioassay (dose selected, evaluation interval, etc).
- Two populations with possible resistance showed ca 10-fold reduction in a cytochrome P450 gene expression. Functional experiments will be necessary to confirm that differential expression of this particular P450 gene confers resistance.

Note: an extension was requested to collect more SWD populations in Washington in the fall of 2017; rearing and bioassays on these populations were completed by February of 2018.

Methods

Obj. 1. Design and test traps to capture live SWD adults for insecticide resistance studies (yr 1)

In the first year of the study, methods will be developed to collect adult SWD populations from orchards. This methodology will be utilized to capture adults for use in discriminating dose and target site and metabolic resistance screening in years 2 and 3. Current traps employ a liquid bait which also served to kill and retain the flies, and is thus not suitable for live capture. Several possible approaches suggest themselves, including 1) using a liquid bait, but utilize a screen to prevent flies from drowning in the fluid; such a trap will include measures to aid fly retention and survival (food, water, and shade); 2) using a dry lure in a similar type of trap.

Obj. 2. Develop discriminating doses of insecticides to test susceptibility of SWD populations (yr 1)

Baseline susceptibility information using a probit bioassay will be generated for candidate insecticides using an SWD population collected in OR in 2009, just after the detection of SWD. Insecticides screened will include Malathion, Sevin, Delegate, Entrust and Warrior. For each insecticide, a minimum of five concentrations will be evaluated which will provide responses between 25 and 95% mortality in addition to two doses that yield 100% mortality. Water was used as a control. For each concentration there was a minimum of 40 adult female SWD. Flies were treated using a Potter Spray Tower, and mortality was assessed 24 h post-treatment. The probit bioassays were analyzed using PoloPlus program, and the diagnostic dose calculated as 2x the LC_{99}.

Obj. 3. Complete development of primers for genetic analyses of SWD alleles that confer resistance (yr 1)

In order to monitor the presence and frequency of mutations that confer target site resistance in *D. suzukii*, adult specimens from the field-collected populations were collected and sent to the Chiu/Zalom lab at UC Davis. PCR-based assays and primers were developed to amplify genomic regions that are associated with development of resistance. Research in this proposal focused on: (i) *ace*, which encodes acetylcholinesterase and is a target for organophosphates and carbamates; (ii) *nAC-hR Dα6*, which encodes a subunit of the nicotinic acetylcholine receptor and is proposed as a target for spinosad/spinosyns; and (iii) *para*, which encodes a voltage-gated sodium channel that is a target for pyrethroids. The *D. suzukii* genome has recently been sequenced and annotated to produce a high quality reference gene set, which will greatly facilitate primer design. Genomic DNA will be isolated from individual flies that are collected from the field populations and stored in 95% EtOH. PCR using primer sets that amplify regions covering potential target site mutations will be performed using Accuprime Taq DNA polymerase (Life Technologies, Grand Island, NY) for high fidelity. Resulting PCR products will be purified using PCR purification kits (Qiagen, Valencia, CA) and subsequently submitted for DNA sequencing at the UC Davis Sequencing Core Facility. Results will be analyzed using sequence alignment packages, e.g., CLC sequence workbench, to determine the presence and allele frequency of nucleotide polymorphisms that might confer insecticide resistance.

4. Screen SWD from multiple districts in CA, OR and WA for insecticide susceptibility (yr 2-3)

A target level of 100 adult female SWD (and associated males at a ratio of about 2 males/5 females) were collected from each orchard screened (Table 1) using traps or sweep nets. These females were used to produce cohorts of F_1 progeny for use in the diagnostic dose screening. Only females were used in the diagnostic dose screening. About 100 females (5-12 days old) from each population was exposed to the diagnostic dose of each of the five insecticides from Obj. 2. The females were transferred to Petri dishes, sprayed in groups in a Potter Spray Tower and evaluated for mortality after 24 h. If there are any survivors in the diagnostic dose assay, it was repeated. If there were still survivors in the repeat bioassay, a full probit line was calculated using the methods in Obj. 2. Flies from each population screened will be sent to the Chiu lab for allele frequency tests (see Obj. 3).

Table 1. Cherry production districts within CA, OR and WA where populations of SWD were collected and assayed for susceptibility to various insecticides

CA	OR	WA
N. San Joaquin	The Dalles	Okanogan Cty
Coastal	Hood River	Chelan/Douglas
	Willamette Valley	Col. Basin
		Tri-Cities

5. Correlate results from discriminating-dose and genetic studies (yr 2-3)

Genomic data were correlated with the results of the insecticide bioassays performed on the corresponding fly strains, and compared to the genomic baseline SWD strain.

Results and Discussion.

Obj. 1. Design and test traps to capture live SWD adults for insecticide resistance studies (yr 1) A number of trap designs were tested for live capture of SWD. Trap design focused on 1) attracting flies into the trap body; 2) preventing escape; and 3) keeping flies alive until retrieved. Some of the custom-fabricated prototypes were large, and labor-intensive/expensive to produce. The primary difficulty, however, was simply low SWD densities in the orchard. To overcome this, a larger number of traps (up to 100/block) was deployed, and flies were collected over a 1- to 3-day period. A second technique was found to be a practical means of collecting flies, viz., sweep netting beneath the trees (this technique was used successfully in OR). Where populations were adequate, sufficient flies could be collected in a few hours. The majority of the colonies were started with more than the target number of founding females (100), but a few fell short of this mark (Table 2).

Table 2. SWD population information for diagnostic dose screening

Year	state	Region	Orchard	Mgmt.	Coll. date	No. founding females
2014	WA	Orondo	AU	Conv	10/4/2014	199
2014	WA	Brewster	GL	Conv	11/4/2014	95
2014	WA	Royal City	RZ	Conv	11/10/2014	237
2014	WA	Malaga	SN	Conv	9/16/2014	138
2014	WA	Stemilt Hill	SH	Org	10/27/2014	113
2014	WA	Rock Island	SC	Conv	10/2/2014	106
2015	WA	Orondo	CC	Conv	7/14/2015	164
2015	WA	Orondo	CO	Org	8/17/2015	145
2015	WA	Malaga	SE	Org	7/20/2015	140
2015	WA	Rock Island	SC	Conv	7/28/2015	69
2015	OR	Hood River	MC	Conv	7/31/2015	135
2015	OR	Dallesport	DP	Conv	9/4/2015	125
2015	CA	Brentwood	BW	Org		11
2015	CA	Tracy	TC	Conv		9
2016	OR	Dayton	ST	Org	7/14/2016	94
2016	WA	Brewster	HA	Conv	9/2/2016	68
2016	WA	Prosser	OB	Conv	9/9/2016	561
2016	CA	Brentwood	BW	Org		30
2016	CA	Tracy	TC	Conv		18
2017	WA	Col. Basin	IR	Org	10/10/2017	56
2017	WA	Col. Basin	JM	Conv	10/10/2017	97

Obj. 2. Develop discriminating doses of insecticides to test susceptibility of SWD populations (yr 1)

Discriminating doses (2x the LC_{99}) were developed for five pesticides: (Delegate (94.35 mg AI/liter), Entrust (221.24), Sevin (41,272), Malathion (523.58), and Warrior II (109.18), using a standard probit bioassay (Fig. 1). The reference colony used was named 'OSU', which was collected from a blueberry field in the Willamette valley in 2009, shortly after the first detection of SWD in the Pacific Northwest. This colony has been in continuous culture from 2009 until probit bioassays were conducted in 2014-2015.

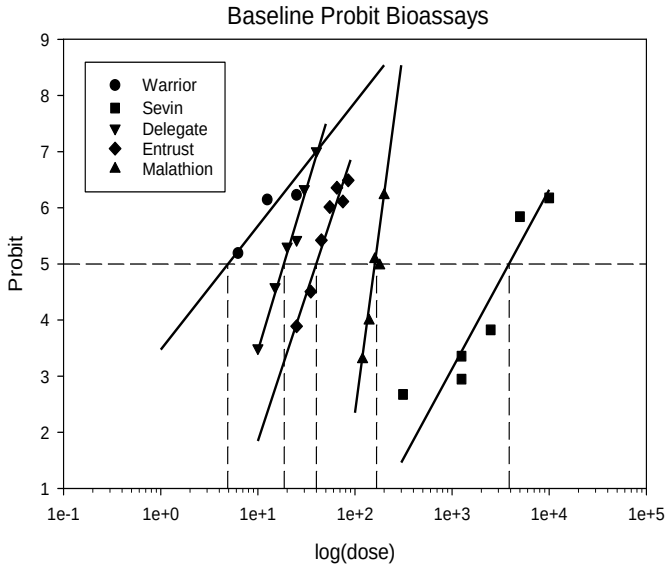

Fig. 1. Baseline probit bioassays for five candidate SWD pesticides.

Obj. 3. Complete development of primers for genetic analyses of SWD alleles that confer resistance (yr 1)

A total of 12 populations of SWD have been sequenced. We focused on the identification of differentially expressed genes (DEG), in particular genes that are involved in conferring metabolic insecticide resistance, e.g., metabolic detoxification (glutathione-S-transferase [GST], cytochrome P450, and esterase) and reduced cuticle penetrance. Results of these analyses are presented in Objective 5. Bioinformatic analysis did not yield single nucleotide variants (SNVs) in protein coding regions that are known to confer target-site resistance. We therefore concluded that any observed resistance in the populations we have examined are likely due to metabolic resistance.

Obj. 4. Screen SWD from multiple districts in CA, OR and WA for insecticide susceptibility (yr 2-3)

To date, 23 populations have been screened against the candidate pesticides (Table 3). For the Washington populations, there were no survivors in the 2014 screenings. Unlike the 2015-16 data, these populations had been in culture 4-6 months versus the 4-7 weeks for the later collections. While the 2014 population screening did not conform to the protocol (collection close to harvest, screening of F_1 females), they represent an initial proof of concept for the diagnostic dose procedure. In 2015, however, there were 3 instances of survivorship in the initial screenings; only one population (CY/Org – Delegate) also had a survivor in the repeat screening. A full probit line was run on this population, and while the LC_{50} was slightly lower than the original OSU line (12.6 vs 18.7), the LC_{99} was slightly higher (59.4 vs 47.2). In 2016, none of the WA populations tested had survivors, and in 2017, one of the population had survivors.. A probit bioassay was conducted for this population (JM), and compared to the original probit used to develop the diagnostic dose, and a contemporaneous bioassays of the OSU colony. The POLO-Plus run of the JM and OSU original probit data indicated rejection of the hypotheses of equality and parallelism. However, the JM LC_{50} was slightly lower (128 ppm AI) than the original Shearer baseline for malathion (167 ppm AI), but due to the difference in slopes, the LC_{99} for JM was much higher (497 ppm AI) than the original Shearer baseline (262 ppm AI). The OSU malathion bioassay was repeated at a lower dosage range to prevent the high mortality levels in C1-C3; the previous bioassays used the diagnostic dose as the

high rate. The repeat bioassay yielded an LC_{50} of 112 ppm AI, and an LC_{99} of 336 ppm. The POLO-Plus comparison of these two bioassays rejected the hypothesis of equality, but did not reject the hypothesis of parallelism. However, only the LC_{10}s were significantly different. The Lethal Concentration Ratio test appears to be overly sensitive to differences, and thus may be too conservative an estimate of shifts in susceptibility.

Table 3. Percentage mortality of female SWD in diagnostic dose screening of five candidate insecticides

State	Year	Orchard	Regime	Delegate 5.04 oz	Entrust 11.82 fl oz	Malathion 6.99 fl oz	Sevin 34.4 qt	Warrior 5.61 fl oz
WA	2014	AU	Conv	100	100	100	100	100
		GL	Conv	100	100	100	100	100
		RZ	Conv	100	100	100	100	100
		SN	Conv	100	100	100	100	100
		SH	Org	100	100	100	100	100
		WB	Conv	100	100	100	100	100
	2015	CY	Conv	100	100	100	100	100
		SC	Conv	100	100	97	100	96
		SC rep	Conv			100		100
		CY	Org	89	100	100	100	100
		CY rep	Org	99				
		SN	Org	100	100	100	100	100
		DP	Conv	100	100	100	100	100
	2016	HA	Conv	100	100			
		OB	Conv	100	100	100	100	100
	2017	IR	Org	100	100	100	100	100
		JM	Conv	100	100	97	100	100
		JM rep	Conv	100	100	98	100	100
CA	2015	BW	Org	100	100	100	100	100
		TC	Conv	91	97	90	100	100
	2016	BW	Org	100	98			
		TC	Conv					
		GL	Conv	100	100	100		
		GN			100			
OR	2015	HR	Conv	100	100	100	100	100
	2016	ST	Org	100	100	100	100	100

Cells highlighted in yellow had 1 or more survivors. The designation ‘rep’ indicated a screening that was repeated due to survivors.

Obj. 5. Correlate results from discriminating-dose and genetic studies (yr 2-3)

The goal of this objective is to correlate our genomic data with insecticide bioassays performed on the corresponding fly strains in comparison to the genomic baseline SWD strain, Specifically, we will focus on (1) gene expression changes indicative of metabolic upregulation of detoxification enzymes or genes known to be involved in reducing cuticle penetrance of insecticides; as well as (2) single

nucleotide variants (SNVs) in protein coding regions that can potentially confer target-site resistance. Whereas the bioinformatic analysis for SNVs did not yield known mutations that confer target-site resistance, differential gene expression analysis identified a large number of genes that are up- and down-regulated in the various populations of SWD as compared to the SWD genome strain (Tables 4, 5). Although some of these differentially expressed genes could be the result of local adaptations and genetic variations, it is likely differentially expressed metabolic detoxification genes might have contributed to the observed changes in insecticide response as shown in Table 3.

Table 4. Number of Up- and Down-regulated genes in SWD populations as compared to the genome strain.

Strain	Collection Location	Collection Date	Up-regulated	Down-regulated
BT	Brentwood, CA	7/1/2015	867	1374
TC	Tracy, CA	9/10/2015	690	1696
CY	Bray's Landing, WA	7/14/2015	743	1331
CYO	Bray's Landing, WA	8/17/2015	767	1754
SN	Malaga, WA	7/20/2015	776	1726
SC	Rock Island, WA	7/28/2015	544	1552
DPt	Dallesport, WA	9/3/2015	1392	2264
HR	Hood River, OR	7/30/2015	1063	2008

Table 5. Differential expressions of selected metabolic detoxification genes. Values are $\log_2$(fold_change) compared to the SWD Genome Strain, and only shown if they are significant, i.e., value of +1 = 2-fold increase.

Strain	Cyp12a4	Cyp12b2	Cyp12c1	Cyp12d1-d	Cyp18a1	Cyp28c1	Cyp28d1	Cyp301a1	Cyp304a1
BT								0.54	
CY	-0.94		-0.60	-0.74			-0.78		2.76
CYO	-0.55	-0.96	-0.57			-1.03	-0.79		2.25
DP	-0.65	-1.12	-0.77	-0.89	-0.60	-1.38	-1.21		
HR	-0.45	-0.91	-0.58	-0.51		-1.11	-0.68		
SN						-1.59	-0.76		2.37
SC		-0.94						1.08	2.00
TC	-0.46	-0.85	-0.73			-1.28	-0.55		2.78

Strain	Cyp305a1	Cyp308a1	Cyp309a2	Cyp311a1	Cyp312a1	Cyp4ac1	Cyp4ad1	Cyp4d1	Cyp4d14
BT	-0.60		-0.80	-0.64				-0.75	2.10
CY			-0.89	-1.50		-0.73		-0.70	1.45
CYO	-0.63		-0.95	-1.50		-0.91		-0.59	1.74
DP	-1.08		-1.48	-1.65		-1.21		-0.85	1.38
HR	-0.96	2.17	-0.94	-1.47		-0.93		-0.57	1.94
SN		1.64					1.16	-0.75	1.65
SC			-0.45	-1.26	-3.33	-0.75		-0.96	1.61
TC			-0.43	-1.09	-3.34	-0.56			1.81

Strain	Cyp4d20	Cyp4d8	Cyp4g15	Cyp4p1	Cyp4p2	Cyp4s3	Cyp6a13	Cyp6a14	Cyp6a20
BT	-0.66	-1.82	0.77			-0.50		0.58	
CY	-1.39	-2.05		-0.56					
CYO	-0.97	-1.28		-0.49		-0.95	-0.68		
DP	-0.88	-2.21		-0.85	-0.57	-1.54	-0.80		-0.62
HR	-0.66	-2.04	0.72	-0.64		-1.71			
SN	-1.33	-1.17	0.64			-0.71			
SC	-0.81		1.01			-0.75			
TC	-1.23	-1.95	0.61			-0.53			

Strain	Cyp6a22	Cyp6a23	Cyp6d4	Cyp6d5	Cyp6w1	Cyp9b2	Cyp9c1	Cyp9h1	Est-6
BT	-1.12		1.12		0.72	-0.61			-0.55
CY	-0.94		1.09				-1.17		
CYO			0.75	0.67					-0.79
DP	-0.55	-0.57	0.69			-0.77	-1.27		-1.00
HR			0.76			-0.87		-1.09	-0.80
SN	-0.64		0.73	0.72					-0.54
SC	-0.64		0.78		0.59	-0.65			
TC	-0.74		0.83						-0.92

Strain	Est-Q	GstZ2	α-Est1	α-Est2	α-Est3	α-Est8
BT	-0.93					
CY	-1.57					
CYO	-1.44					
DP	-2.13	-0.78	-0.85	-1.44	-0.71	-0.77
HR	-1.33	-0.82			-0.43	
SN	-2.45					
SC	-1.00					
TC	-1.46					

Among all the metabolic detoxification genes, there is only one that shows changes in gene expression that occur in slightly resistant/tolerant populations, as shown in our bioassays. Cyp312a1 is a cytochrome P450 gene whose expression level is reduced by roughly 10-fold in the Spanish Castle and Tracy populations. It seems counter-intuitive that a reduction in a cytochrome P450 gene can promote insecticide resistance, but since the exact molecular substrate of Cyp312a1 is known, this remains a possibility and will have to be tested through functional experiments in the future. We performed the same analysis for genes that are involved in regulating cuticle penetrance of insecticides to identify any correlation between SWD populations that are more tolerant to insecticides, and identified 3 genes that are differentially expressed (CCAP-R, Cam, l(3)mbn). Elevated expression of cuticular proteins is a widespread mechanism that confers insecticide resistance in addition to metabolic and target-site resistance.

Executive Summary

This project provided a blueprint for determining resistance in SWD populations in the future, as well as a snapshot of the current status. The methodology developed can be easily used by different research groups with commonly available equipment (synthetic lure traps, Potter Spray Tower). The bioassays of the OSU (reference) colony serve as a type of baseline; the insects (and their recent ancestors) were not likely unexposed to insecticides, but this colony represents the status of susceptibility prior to intense selection in western US specialty crops.

The assumption of a diagnostic dose bioassay is that there will be 100% mortality. This did not occur in all cases (10 of 105 had <100%). However, the levels of mortality and the proscribed follow-up probit bioassays do not present a compelling case for resistance at this point. Some of the sub-100% bioassays deviated from the protocol in terms of the numbers of founder females and the numbers dosed, but a few others had persistent survivorship or difference in lethal concentration ratios of the follow-up bioassays.

Resistance development in SWD continues to be a concern given the lack of alternative IPM tactics in use in sweet cherry. There is a reluctance to use thresholds for determining the need of spray applications because of the potentially severe negative consequences. This has promoted an over-reliance on insecticidal control, much of which is prophylactic. The economics of this course (high value of cherry crops relative to the cost of insecticides) will continue to favor preventive pre-harvest sprays to protect fruit from infestation. Although several different modes of action are in common use, rotation of materials may not provide insurance against resistance.