2016 California Cherry Research Reports

California Cherry Board
University of California
FNIRC
UC Cooperative Extension
Cal Tech
University Notre Dame

HEALTHY FOOD SYSTEMS • HEALTHY ENVIRONMENTS • HEALTHY COMMUNITIES • HEALTHY CALIFORNIANS

University of California
Agriculture and Natural Resources
Making a Difference for California

San Joaquin Pomologist

CALIFORNIA CHERRY RESEARCH REVIEW

CALIFORNIA CHERRIES

January 5, 2017

Evelyn Costa Assembly Room
San Joaquin County Robert J. Cabral Agricultural Center
2101 E. Earhart Avenue, Stockton, California 95206-3949
Sponsored by the University of California Cooperative Extension and California Cherry Board

9:00 am	**Welcome** Joe Grant, UC Cooperative Extension, San Joaquin County
9:05	**Classical biological control of Spotted Wing Drosophila** Dr. Kent Daane, Dept. of ESPM, UC Berkeley and UC Kearney Agricultural Research & Extension Center, Parlier, CA
9:25	**Developing attractive baits for *Drosophila suzukii*** Dr. Zain Syed, University of Notre Dame
9:45	**Management and epidemiology of pre- and post-harvest foliar and fruit diseases of sweet cherry** Dr. Jim Adaskaveg, Dept. of Plant Pathology, UC Riverside
10:05	**Engineered transgenic *Drosophila suzukii* for wild population suppression & eradication: Production, performance assessment, and effective wild releases** Dr. Omar Akbari, Dept. of Entomology, UC Riverside
10:25	**Break**
10:45	**2016 California Cherry Board Update** Chris Zanobini, CCB Executive Director
11:00	**Oviposition deterrents and insecticides for Spotted Wing Drosophila control in cherry** **San Jose Scale control in cherries** Dr. Jhalendra Rijal, UC Area IPM Advisor, Merced, Stanislaus, and San Joaquin Counties
11:30	**Better understanding of sweet cherry post-harvest cracking in California and potential strategies to reduce its incidence** Dr. Irwin Donis-Gonzalez, Dept. of Biological & Agricultural Engineering, UC Davis
11:50	**Update: 2016/2017 chill accumulation** Mike Devencenzi, Devencenzi Ag Pest Management and Research
12:00 pm	**Existing approaches, challenges, and possibilities for mechanical tree fruit and sweet cherry harvesting** Dr. Stavros Vougioukas, Dept. of Biological & Agricultural Engineering, UC Davis
12:30	**Lunch** (courtesy of California Cherry Board)
1:30	**Adjourn**

1.5 hours of PCA Continuing Education Credit pending

CALIFORNIA CHERRY BOARD

2016-2017 RESEARCH COMMITTEE

MEMBER	CCB	PRIMARY REPRESENTATION AREA	INDUSTRY ACTIVITIES
NICK MATTEIS **(Staff)**	S	ALL	CCB (RESEARCH CO-OR)
RICH HANDEL	N	ALL	PACKER, GROWER, SHIPPER
JOE GRANT **(Staff)**	N	SAN JOAQUIN CO.	CCB (RESEARCH CO-OR)
ARNIE TOSO	A	SAN JOAQUIN CO.	CCB, GROWER
ANDREW DASSO, JR.	A	ALL	CCB, PACKER, GROWER, SHIPPER
LAWRENCE SAMBADO	M	ALL	CCB, PACKER, GROWER, SHIPPER
TOM CHINCHIOLO	N	ALL	PACKER, GROWER, SHIPPER
DONALD DRAKE	N	ALL	PACKER, GROWER, SHIPPER
GREG COSTA **(Chair)**	N	ALL	GROWER, PACKER, SHIPPER
ANDY MARIANI	N	SANTA CLARA VALLEY	GROWER, PACKER
STEVE SOUTHWICK	N	ALL	PACKER, GROWER, SHIPPER
MIKE DEVENCENZI	N	SAN JOAQUIN CO.	GROWER, PCA
PAT GOTELLI	N	ALL	GROWER, PACKER, SHIPPER
RYAN LOGOLUSO	N	FRESNO CO.	CCB, GROWER
BRUCE FROST	N	KERN CO.	GROWER
PAUL WOLF	N	SAN JOAQUIN CO.	GROWER
JOHN CALLIS	N	DELTA	GROWER, PACKER
GARY SUTHERS	S	TULARE CO.	CCB, GROWER, PACKER, SHIPPER
BLAKE UEKI	S	TULARE CO.	CCB, GROWER, PACKER, SHIPPER
ROSS VAN VLACK	S	FRESNO CO.	CCB, GROWER, PACKER, SHIPPER
JOE CATALDO	N	SAN JOAQUIN CO.	CCB, GROWER, PACKER, SHIPPER
SCOTT BROWN	N	SAN JOAQUIN CO.	CCB, GROWER, PACKER, SHIPPER
KRISTI FRIIS	N	SAN JOAQUIN CO.	CCB, GROWER, PACKER, SHIPPER

CALIFORNIA CHERRY BOARD -- Research Committee

2016 Research Projects Approved April 7, 2016

	Pre/ Post	Pathogen I.D.	Description	Status	Comments	Project Leader	2016 Projects Requested Funding	2016 Projects Funding
1	Pre	Sp. W. Drosophila	SWD attractants and yeast relationships	On-going	Development of lures, traps	Syed	$ 55,000.00	$ 55,000.00
2	Both	Diseases	Canker & Decay controls	On-going	Continuing annual work; Kasumin	Adaskaveg	$ 52,000.00	$ 52,000.00
5	Post	MRL Trade Barriers	Residue remediation	New	Postharvest residue remediation: Breaking MRL trade barriers for the specialty crop industry TASC Grant WA Tree Fruit	Walse	$5,000	$5,000
8	Pre	Sp. W. Drosophila	Population supression & eradication	On-going	Transgenic, *Medusa* allele	Akbari/Hay	$ 103,150.00	$ 103,150.00
9	Pre	Sp. W. Drosophila	Population control	On-going	SWD natural parasites	Daane	$ 11,685.00	$ 11,685.00
13	Both	San Jose Scale	Management Program	New	Degree day models, phenologies, effectiveness of materials, spray timing	Rijal	$ 10,000.00	$ 10,000.00
14	Pre	Sp. W. Drosophila	Oviposition Deterrents	New	Evaluation of oviposition deterrent compounds	Rijal	$ 10,000.00	$ 10,000.00
16	Both	Sp. W. Drosophila	Regulatory approval	New	A solution to regulatory approval of RNAi SWD for population control/erradication	Turpen	$ 10,000.00	$ 10,000.00
17	Post	Cracking/Splitting	Post harvest fruit condition	New	Cracking/splitting survey of CA packing lines	Gonzales	$ 19,784.00	$ 19,784.00
18	Both	CIMIS Station Error Checking	FNIRC error checking	New	FNIRC error CIMIS chill portion error checking	Crisosto	$ 4,000.00	$ 4,000.00
							$ 280,619.00	$ 280,619.00

CALIFORNIA CHERRY BOARD
2016 FINAL RESEARCH REPORTS

Investigating Biological Controls to Suppress Spotted Wing Drosophila Populations

Xingeng Wang[1], John Jones[1], Alexandra Nance[1], John Hutchins[1], Betsey Miller[2], Vaughn M. Walton[2], Kim A. Hoelmer [3], Mathew Buffington [4], and Kent M. Daane[1]

[1]*Department of Environmental Science, Policy and Management, UC Berkeley*; [2]*Department of Horticulture, Oregon State University*; [3] USDA ARS, Beneficial Insects Introduction Research Unit, Newark, DE; [4] USDA ARS, Systematic Entomology Laboratory, Washington D.C.

Summary. The spotted wing drosophila (SWD) has become a major cherry pest in California. To develop sustainable management options for this highly mobile pest, we worked with cooperators at Oregon State University and the USDA on the importation of novel material from South Korean and China. We found that several larval parasitoids that can readily attack SWD in UC Berkeley quarantine evaluations. We report here on the major results on the parasitoid importation program and our progress towards receiving an approval from USDA APHIS to release material from Quarantine.

Introduction

Spotted wing drosophila, *Drosophila suzukii*, is a pomace fly distributed across eastern China, Japan, the Korean Peninsula, and other regions in Southeastern Asia. In North America, the fly was first detected in 2008 in California (Bolda et al. 2010) and was subsequently reported in most fruit growing regions in the continental United States (US) and Canada (Asplen et al. 2015) and parts of Europe and Brazil. *D. suzukii* is considered a key pest of soft and thin skin fruits such as blueberries, cherries, figs, raspberries, and strawberries in all of these newly invaded regions (Burrack et al. 2013; Lee et al. 2011; Mitsui et al. 2006; Yu et al. 2013).

Control efforts in North America currently rely on the use of insecticides that target adult *D. suzukii*. However, insecticide-based programs can be limited by the fact that many host fruits in non-crop habitats act as reservoirs for *D. suzukii* and support its reinvasion into commercial fields. The lack of effective biological controls in the newly invaded range of *D. suzukii* led to the initiation of a classical biological control program. Our goal is to discover, import and select the most effective but also safest parasitoids for field release to control *D. suzukii* in North America. This will be accomplished through systematic quarantine evaluations of the efficiency, host specificity, and establishment potential (e.g. climatic adaptability) of candidate parasitoid species. Parasitoids were collected from South Korea in 2013 and 2014, and five species have been maintained in quarantine, three larval parasitoids (*A. japonica*, *L. japonica* and *G. brasiliensis*) and two pupal parasitoids (*P. vindemniae* and *T. drosophilae*), and evaluated on various aspects of their biology and efficiency. These evaluations included each parasitoid's egg maturation dynamics, host stage preference and suitability, fecundity, functional response, preference and performance on different host species and olfactory response towards different fruits infested by *D. suzukii*, as well as the outcomes of their potential interaction.

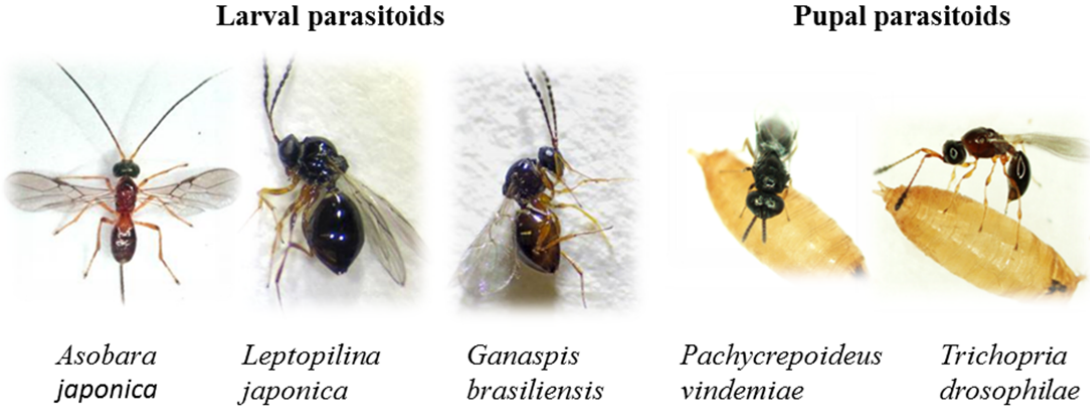
We have also tested these five parasitoid species against a range of 24 non-target Drosophilidae species. Among the larval parasitoid species, *A. japonica* is a more effective SWD parasitoid than the other two parasitoid species, but it is also more of a generalist species whereas the host range of the other two larval parasitoid species is largely limited to the *Melanogaster* group (species that are closely related to *D. suzukii*). The two pupal parasitoids are the most generalist parasitoids and developed from all tested non-target species, and they also occur in North America. Based on these results, a petition to release both *L. japonica* and *G. brasiliensis* in North America has been submitted. In 2016, we conducted more foreign exploration for *D. suzukii* parasitoids in South Korea and China and collected over 1,200

individual parasitoids from *D. suzukii*. These newly collected parasitoids are currently under identification and will also be evaluated in quarantine.

Note that this report is technical – but this is the detail needed to get material permitted to be released from Quarantine and placed out near the cherry orchards.

1. Evaluated candidate parasitoid species

Fig. 1. Evaluated five major parasitoids from South Korea at the University of California



Berkeley's Quarantine Facility.

In 2016, we continued evaluations of the five parasitoid species collected from South Korea in 2013 and 2014, which are the three larval parasitoids *A. japonica*, *L. japonica* and *G. brasiliensis*, and the two pupal parasitoids *P. vindemniae* and *T. drosophilae* (Fig. 1), at the University of California Berkeley's Quarantine Facility. Previously, we reported some results on the evaluation of *A. japonica*, including its functional response, performance on *D. suzukii* and *D. melanogaster* and olfactory response towards fruits infested by *D. suzukii*. Some results on the two pupal parasitoids have been published (see Stacconi et al. 2015; Wang et al 2016a, 6). Because the two pupal parasitoids also occur in North America, our main interests focused on these larval parasitoids, particularly their biology and efficiency, host specificity, and climatic adaptability. Here we report mainly the results on the evaluations of the three larval parasitoids, especially *L. japonica* and *G. brasiliensis*.

2. Biology and efficiency

2.1. Egg maturation dynamics

It is critical to understand the rate of egg maturation of parasitoids to optimize parasitoid rearing for field release or (in our current work) biological studies. To determine how *A. japonica*, *L. japonica* or *G. brasiliensis* mature eggs over their adult life span, groups of adult female wasps were dissected at different time periods after their emergence. The number of mature eggs was counted while the body size (hind tibia length) of each dissected female was measured with an ocular micrometer.

Both *L. japonica* and *G. brasiliensis* females were reared from *D. suzukii*. The number of mature eggs carried by females was affected by the female age (*L. japonica*: $F_{5,158} = 10.66$, $P < 0.001$; *G. brasiliensis*: $F_{1,141} = 21.79$). Both parasitoid females emerged with approximately 30-60 mature eggs and mature egg-load peaked 3-4 days post-emergence (Fig. 2). Although the mean body size (hind tibia length) of tested females was not significantly different among the different age classes in *L. japonica* ($F_{5,158} = 1.34$, $P = 0.25$) or *G. brasiliensis* ($F_{5,148} = 1.72$, $P = 0.13$), the hind

tibia length of dissected individual females varied, and affected the female's mature egg of *L. japonica* ($F_{1,158}$ = 36.84, P < 0.001) and *G. brasiliensis* ($F_{1,141}$ = 60.93, P < 0.001). There was no interactive effect between the female age and body size on the mature egg load in *L. japonica* ($F_{1,158}$ = 0.72, P = 0.81) or *G. brasiliensis* ($F_{1,141}$ = 0.46, P = 0.61).

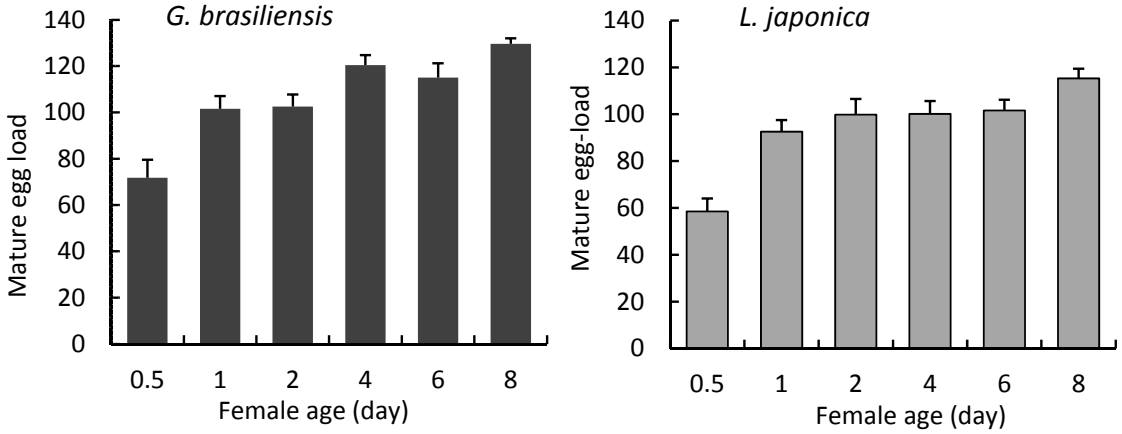


Fig. 2. Egg maturation dynamics of female *L. japonica* and *G. brasiliensis* when deprived of host but provided with food. Values are mean ± SE (n = 20 to 29 for each age group).

A. japonica females were reared from both *D. suzukii* and *D. melanogaster*. The number of mature eggs of *A. japonica* females was affected by the rearing host ($F_{1,486}$ = 93.52, P < 0.001) and female age ($F_{10,486}$ = 30.44, P < 0.0001), as well as the interactive effect of both factors ($F_{10,486}$ = 2.04, P = 0.03). The parasitoid females matured eggs rapidly and the mature egg load reached a peak within 2-3 days post-emergence and then decreased when derived of hosts (Fig. 3A). In general, females reared from the larger host of *D. suzukii* contained more mature eggs than those reared from the smaller host of *D. melanogaster* (Fig. 3A) and female wasps reared from the larger host also had a large body (hind tibia length: $F_{1,506}$ = 216.3, P < 0.001; ovipositor length: $F_{1,}$ 506 = 224.0, P < 0.001) (Fig. 3B). We will further determine how female wasp's body size would affect their parasitization efficiency.

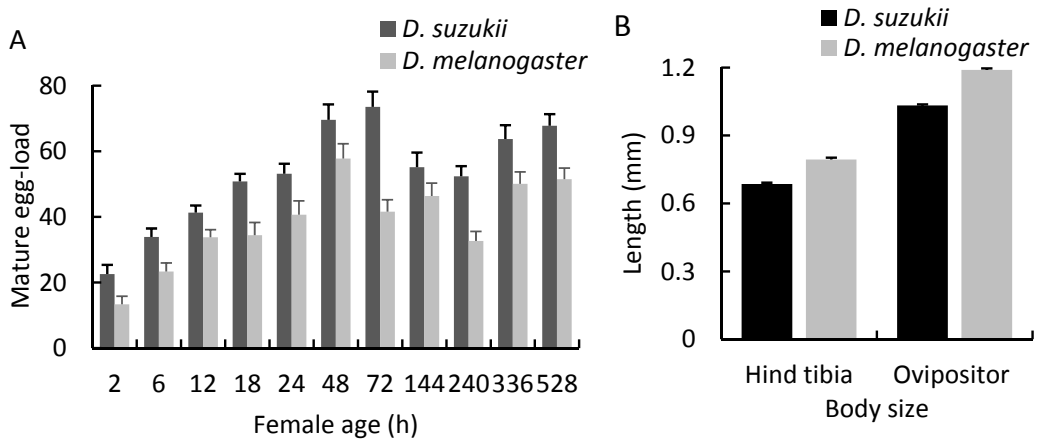


Fig. 3. (A) Egg maturation dynamics of female *A. japonica* reared from *D. suzukii* and *D. melanogaster* and (B) the effect of rearing host species on the body size (hind tibia and ovipositor length) of the female parasitoid. Values are mean ± SE (n = 19 to 25 for each age group).

2.2. Host stage preference and suitability

It is important to determine host stage preference and suitability by a parasitoid to optimize its effective rearing and for future modeling of field efficacy. Possible difference among different species in host stage selection could avoid or reduce interspecific competition and increase their chance of coexistence and synergistically affect the pest population. We conducted both choice and non-choice tests to determine the host stage preference and suitability by each of the three larval parasitoids. In no choice test, 10 hosts of each stage (1, 2, 3, 4 days old) were exposed to one female for 6 h, respectively, and half of the exposed hosts were dissected while the other half were reared to determine the parasitism, developmental time and body size of emerged female wasps. In choice test, 5 larvae of two distinct ages (1-2 or 3-4 days old) were exposed to one female wasp for 6 h and all exposed hosts were dissected to determine the parasitism of each host age class.

In no choice tests, host stage affected the parasitization efficiency by *A. japonica* ($F_{3,96}$ = 5.74, P = 0.81), *L. japonica* ($F_{3,96}$ = 11.74, P = 0.81) and *G. brasiliensis* ($F_{3,96}$ = 17.70, P = 0.61). There was no difference in the number of hosts parasitized by *A. japonica* for 1- to 3-days old larvae, but the parasitoid attacked fewer 4-day old host (Fig. 4). Both *L. japonica* and *G. brasiliensis* were more successfully in parasitizing young (1-2 days old) than old (3-4 days old) host larvae. The results on the effects of host stage on the parasitoid's fitness (body size, developmental time etc.) have not been analyzed yet and the choice tests are still in progress. Also, we will conduct further studies to determine the distribution of different host stages inside the fruit and how this would affect the host location by each parasitoid species.

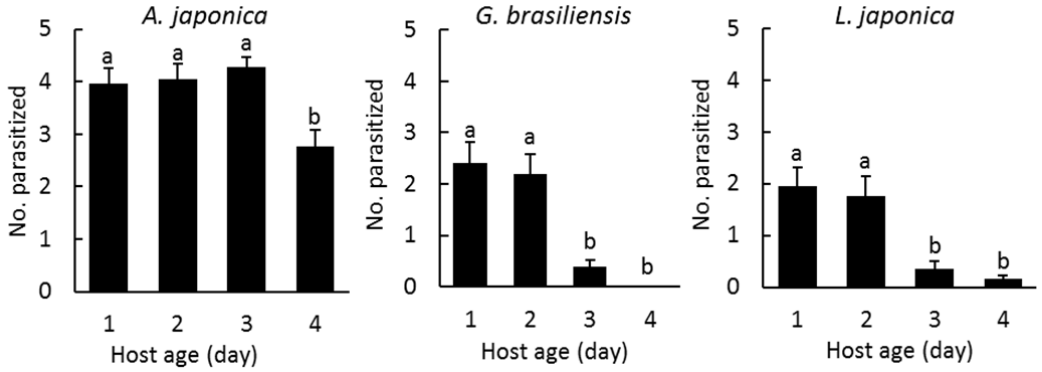


Fig. 4. Host stage effect on the parasitization efficiency by *A. japonica*, *L. japonica* or *G. brasiliensis*) when attacking *D. suzukii* in a no-choice test. Values are mean ± SE (n = 25) and bars bearing different letters are significantly different (Tukey's HSD, $P < 0.05$).

2.3. Life-time fecundity

We compared the reproductive potential of the three larval parasitoids by giving them unlimited access to *D. suzukii* larvae. A pair of newly emerged (<12h old) female and male were provided 20 larvae in a diet vail daily, until the female was dead. Female longevity, numbers of offspring developed, offspring sex ratio and survival rate, and juvenile developmental time were calculated. From these data, life table fertility parameters were estimated for each parasitoid including *net reproductive rate* (R_0), *intrinsic rate of natural increase* (r), *mean generation time* (T), and *doubling time* (DT). We have reported the results for *A. japonica* (as reported previously), but the data for the life-time fecundity of *L. japonica* and *G. brasiliensis* have not been analyzed yet and will be present in the next report.

2.4. Foraging efficiency and host species preference

To be effective these parasitoids must be able to locate host in more realistic setting, i.e. on host fruit. We are comparing the foraging efficiency of the three larval parasitoids when parasitizing *D. suzukii* on host fruit as well as their preference between *D. suzukii* and *D. melanogaster* in different combinations of host density (i.e. 20 *D. suzukii* only, 15 *D. suzukii* + 5 *D. melanogaster*, 10 *D. suzukii* + 10 *D. melanogaster*, 5 *D. suzukii* + 15 *D. melanogaster* and 20 *D. melanogaster* only). The data have not been analyzed yet and will be provided in the next report.

2.5. Interspecific interaction

It is important to understand and predict potential interactions, such as competitive outcomes, among natural enemies when designing biological control programs that employ multiple natural enemy species. We are conducting a series of experiments to (1) determine which candidate parasitoid species is more superior and what are the mechanisms of the competitive outcomes (physical combats, physiological suppression, or both); (2) can one parasitoid species discriminate against the hosts previously attacked by another parasitoid species? What are the discrimination mechanism (odor avoidance, antennal or oviposition examination, external or internal marks etc.), and (3) effect of interspecific interaction on host suppression. Studies on the intrinsic competition and host discrimination mechanisms are still in progress, here we report partial results on the outcomes of interspecific interactions between any two of three larval parasitoids using additive-series design, i.e., the total number of each parasitoid species in the two-species release treatment is same as in the single species treatment. The observed levels of host mortality (parasitism) in the two species release treatment was compared to the expected levels of host mortality calculated using data from the single species release treatments only. The observed and expected levels of host mortality were compared across replicates, using t-test.

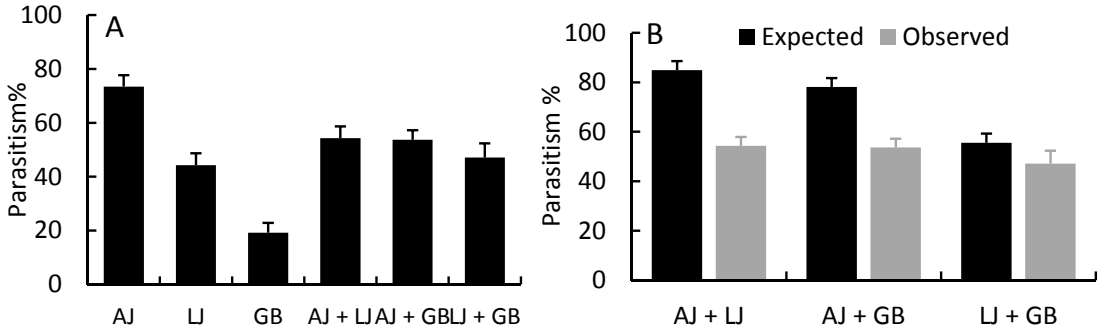


Fig. 5. (A) Numbers of *A. japonica* (AJ), *L. japonica* (LJ) and *G. brasiliensis* (GB) adults emerged from *D. suzukii* and (B) the observed and expected parasitism of *D. suzukii* by the parasitoids when they were present singly or simultaneously in an additive design experiment. Values are mean ± SE (n = 25).

In single species release, *A. japonica* attacked more host larvae than *L. japonica* or *G. brasiliensis* ($F_{2,72}$ = 27.72, P < 0.001) while in the two species release the number of total hosts attacked was no significant difference between any of two species combination ($F_{2,72}$ = 1.00, P = 0.372) (Fig. 5A). When *A. japonica* and *L. japonica* ($F_{2,72}$ = 35.53, P < 0.001) or *G. brasiliensis* ($F_{2,72}$ = 23.42, P < 0.001) were released together, the observed parasitism was lower than the expected one, indicating interspecific competition. However, the expected parasitism was not different from the observed parasitism when *L. japonica* or *G. brasiliensis* were released together ($F_{2,72}$ = 1.72, P = 0.195), suggesting the two parasitoids acted independently (Fig. 5B). The competition outcomes may depend on host density; we are continuing the tests of the outcomes of interspecific competition using different host densities.

2.6. Functional response

To predict the host suppression potential by these larval parasitoids, the functional responses of each parasitoid to nine different densities of *D. suzukii* larvae (3-42 larvae per female per 24 h) was examined. The functional of *A. japonica* has been reported previously. Here we report the results on the functional responses of *L. japonica* and *G. brasiliensis*. Both parasitoid species displayed a Type I functional response when parasitizing *D. suzukii* larvae as the number of host attacked increased linearly over the tested host density range (Fig. 6). This kind of behavior clearly indicates that the biocontrol activity of these parasitoids is host density dependent.

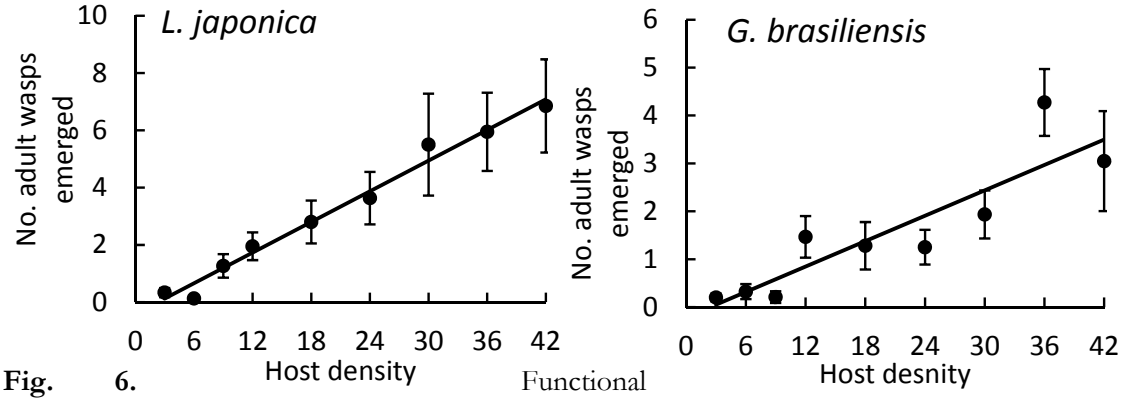


Fig. 6. Functional response by *L. japonica* and *G. brasiliensis* on *D. suzukii*. Values are means ± SE of number of adult parasitoids emerged at each host densities during a 24h exposure.

2.7. Other studies

In addition, we have investigated the temperature range and functional response (fixed or varying time functional response) of *P. vindemiae* and *T. drosophilae*. These results are not reported here.

3. Assessment of host-specificity

3.1 Host range test

We assessed the host specificity of all five major parasitoid species (*A. japonica*, *G. brasiliensis*, *L. japonica*, *T. drosophilae* and *P. vindemiae*). Give the diversity of non-target drosophila species (> 2000 described species), we focused on the physiological host range and tested phylogenetically related species to the target host species (i.e., using centrifugal phylogeny as a basis for non-target test). Although the physiological host range may not reflect the ecological host range in the nature, simply because a parasitoid may be unable to recognize a host's microhabitat, this method would narrow down further host test range if necessary by first examining host acceptance and suitability.

A wide range of 24 Drosophilidae species were selected, by taking into consideration of diverse geographical origins (Palaearctic, Cosmopolitan and especially Nearctic), phylogenetic relationships (7 clades, 7 genus, 8 subgenus, 22 species groups) and ecological niches (fruits, mushrooms, wood, flowers, sap) of testing species. All fly species were purchased at the University of California's San Diego Drosophila Stock Center, where the different species were originally collected from different states in the US, except one Japanese species (*Scaptomyza elmoi*) that was selected as a close representative of the endangered Hawaiian drosophilae and another species (*Samoaia leonensis*) from the Samoa islands (this genus occurs only on the pacific islands).

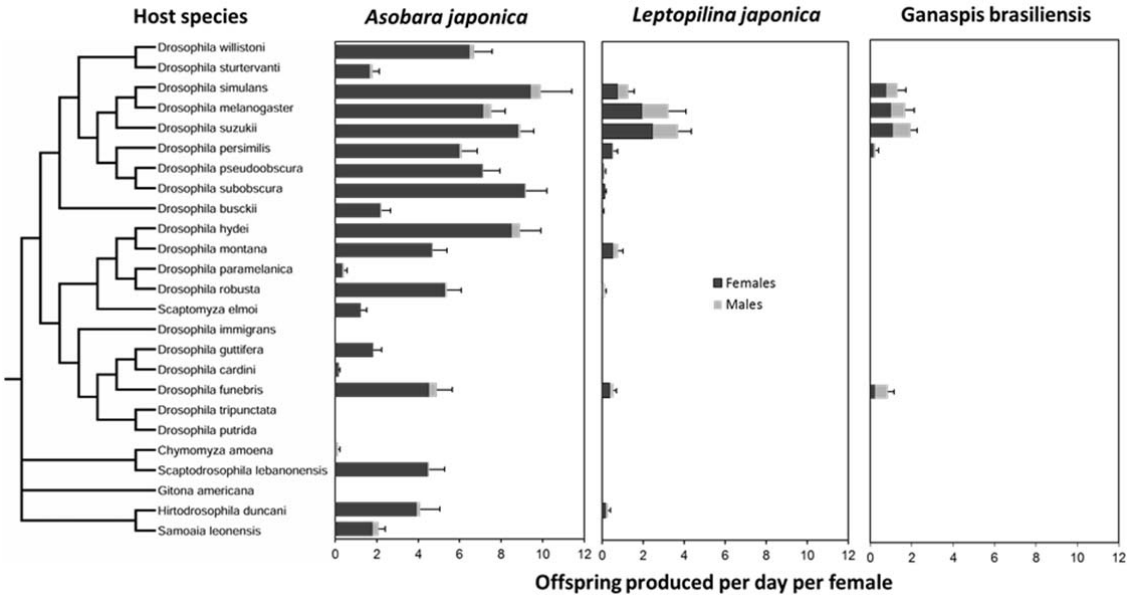


Fig. 7. Mean ± SE offspring produced per female per 24 h by three larval parasitoids from each tested drosophila species.

For the test of each larval parasitoid, twenty 2 d fly larvae were placed in a diet vail and exposed to one mated female wasp for 24 h. Exposed fly larvae were reared until the emergence of flies and wasps. Each test consisted of 30 replicates. Some *Drosophila* species larvae are able to defend themselves from parasitoid eggs placed inside their bodies by surrounding the egg with blood cells that eventually melanize and form a black capsule surrounding the egg, resulting in the immature parasitoid death by asphyxiation and the capsules are visible in developed adult flies. We estimated the 'Degree of infestation', measured as the proportion of hosts that were successfully parasitized, and estimated as (T- di)/T (T= the number of emerging flies in the absence of the parasitoids, di = the number of emerged flies in the presence of parasitoids) and 'Success rate of parasitism', measured as the probability that an infested host will give rise to an adult wasp, and estimated as pi/ (T-di) (pi = the number of emerged parasitoids). We also measured the effects of host species on the parasitoids' fitness (e.g. developmental time, body size and mature egg load).

A. japonica developed from 19 out of 24 tested non-target species while *G. brasiliensis* was able to develop only from four tested species (three of them are closely related to *D. suzukii* as they all belong to the *Melanogaster* group species) and *L. japonica* developed mainly from the *Melanogaster* group species (Fig. 7). A few (< 5) *L. japonica* individuals developed from other species, but either produced primarily males or was a rare event. Overall, more progeny was produced by *A. japonica* than *L. japonica* or *G. brasiliensis* on these three common host species (Fig. 7). These results clearly showed that *L. japonica* and *G. brasiliensis* are more host-specific than *A. japonica*, but are also less effective in the laboratory than *A. japonica*. The failure of development of *G. brasiliensis* and *L. japonica* in most of tested host species appear to correspond with more frequent encapsulation of parasitoids by the hosts. As a result, although *G. brasiliensis* and *L. japonica* can attack these no-target species, they failed to develop from most of attacked host species (Fig. 8). In contrast, both pupal parasitoids *P. vindemiae* and *T. drosophilae* were able to develop from all tested non-target species. Overall, the level of host specificity of the larval parasitoids is higher than the pupal parasitoids. *G. brasiliensis* and *L. japonica* are the most specialized species on *D. suzukii*. Therefore, a petition to release *G. brasiliensis* and *L. japonica* has been submitted to USDA APHIS. We note here that parasitism rates of figitids in the 2016

collections in China and Korea suggest that these figitids are far more common and effective than the braconid early in the season.

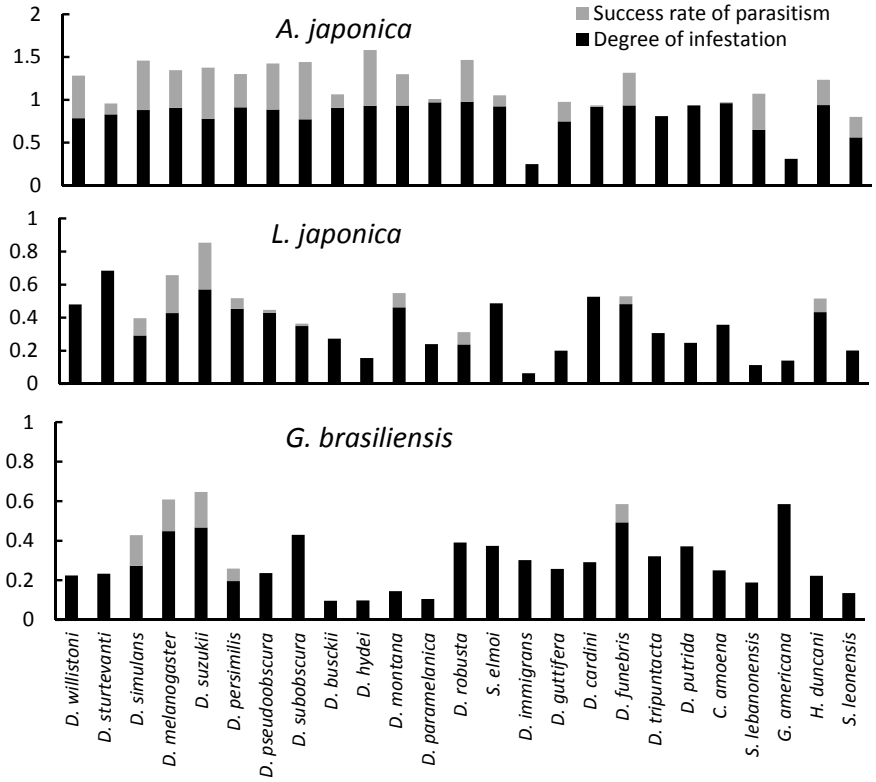


Fig. 9. Degree of infestation and success rate of parasitism by each larval parasitoid on different host species.

3.2. Host species effect on the parasitoid's fitness

Host species affected the fitness of these parasitoids. Both the size and developmental time varied among different host species. The developmental time of these different host species varied from 15 to 35 days and the developmental time of either *A. japonica* or *L. japonica* was positively related to the host developmental time. These results suggest that these endoparasitic koinobiont larval parasitoids are able to maximize the use of their host sources.

As a result, parasitoids developed from the large hosts had large body size (hind tibia or ovipositor length) at the cost of prolonged developmental time (used the most generalist *A. japonica* as an example. The length of hind tibia was positively related to the length of ovipositor in female *A. japonica*. Large female wasps contained more mature eggs (Fig. 10). Thus, there is a trade-off between the body size and developmental time or mature egg load in these larval parasitoids.

Similarly, female body size of *T. drosophilae* or *P. vindemiae* was also positively related to the body size of host pupae, and larger female wasps contained more mature eggs in both pupal parasitoids (Fig. 11). However, developmental time in both idiobiont parasitoid was not affected by the host size (data were not present here), suggesting the plasticity of growth and development (i.e., grow faster on large host species).

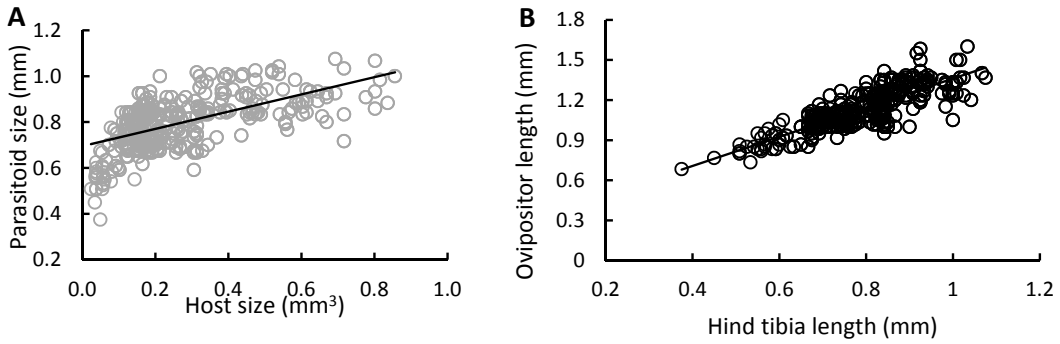


Fig. 10. The relationships between the size of host species and the body size of *A. japonica* (A) and between the hind tibia length and ovipositor length (B) or between the body size and mature egg load (C) of female *A. japonica.*

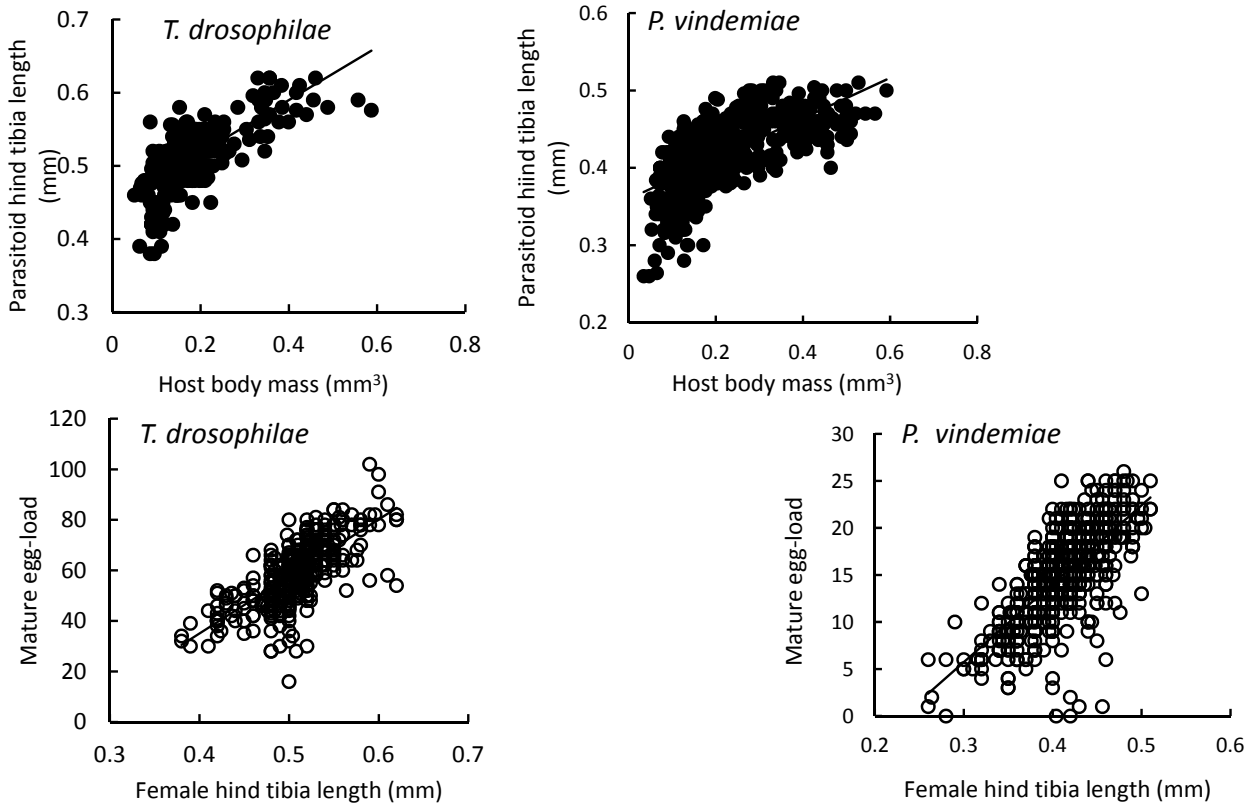


Fig. 11. The effect of host species size on the body size of the *T. drosophilae* and *P. vindemiae* and the relationship between the female parasitoid's body size and mature egg load in both parasitoid species.

4. Foreign exploration in South Korea and China

In collaboration with USDA-ARS, Oregon State University, Institute for Sustainable Plant Protection (Italy) and Yunnan Academy of Agricultural Science (China), we conducted collections of native *D. suzikii* parasitoids at 17 locations and two provinces in South Korea

during June 2016 and at four locations in Yunnan, China during July 2016. In South Korea, several wild *Rubus* fruits were collected. A total of 11,575 *D. suzukii* pupae were collected from the fruits and imported into the University of California Quarantined Facility. Of the collected pupae, 9,745 adult *D. suzukii*, 149 figitids, 22 braconids and 3 diapriids emerged. In Yunnan China, several different wild host fruits, including wild blackberries (*Rubus*), western strawberries (*Fragaria moupinensis*) and *Sambucus* sp. were collected. A total of 11,683 *D. suzukii* pupae were collected, imported and sorted in the insect quarantine facilities of the University of California Berkeley. A total of 2,091 adult *D. suzukii*, 929 figitids, 22 braconids and 3 diapriids emerged from the collected pupae. All originally collected specimen have been sent out for identification after they have been used for the establishment of quarantine colonies.

The majority of emerged parasitoids were figitids. The total parasitism of *D. suzukii* by all parasitoid species (estimated based on the numbers of emerged adult flies and recognizable immature flies or wasps from the dissection of dead fly pupae) was generally very low (< 6%) from the South Korean surveys in the early fruit seasons (June) but was as high as 75% from the surveys in China.

5. Ongoing studies and future plans

We expect to discover more effective and specialist larval parasitoid species or strains from this year's collections in China (still under identification) as some of these species appeared to be very effective based on the parasitism. We will continue foreign exploration in unexplored regions in Asia to discover, import and select more effective and safest parasitoids for future field release in North America. At the same time, in order to design a better strategy for the release of selected and approved parasitoid species, we will continually investigate biotic and abiotic factors that could affect their efficiency and establishment potential (e.g., fruit species effect, climatic adaptability, thermal performance, diapause, intra-guild competition, host shift, response to different host habitats and tri-trophic interactions etc.), and develop effective rearing methods for these parasitoids.

Acknowledgements

We thank Riki York and Kyoo Park (Oregon State University), Hyun Jung Kim and Jongwoo Nam (Seoul Women's University), Uk-Jin Park and Rameswor Maharjan (Andong National University), Oh-Gyeong Kwon (Kyungpook National University), Deuk So Choi (Department of Plant Quarantine, Anyang-si), and Iksoo Kim (Chonnam National University, Gwangju) for assistance with collections in South Korea and Hongyin Chen and Chenxi Liu (Chinese Academy of Agricultural Science), Zon Qi Chen and Yang Wang (Yunnan Academy of Agricultural Science) for collections in China; John Hutchins and Brandy Chavez (University of California, Berkeley) for assistance with insect rearing in the quarantine; and Ken Bloem (Biological Control Coordinator, USDA, APHIS, PPQ) for program design. Antonio Biondi was supported through the People Programme of the European Union's Seventh Framework Programme FP7/2007-2013/ under REA grant agreement 318246 and from the Italian Ministry of Education, University and Research (PRIN project GEISCA, 2010CXXHJE_004). Funding for research was supported in the US by the USDA-NIFA award # 2010-51181-21167, the USDA APHIS (Farm bill, fund 14-8130-0463), and the California Cherry Board, and in Italy by the FP7 project Ameliorating the Sustainable Control of Invasive Insects (ASCII) PIRSES 318246.

Hickner et al. BMC Genomics (2016) 17:648
DOI 10.1186/s12864-016-2983-9

BMC Genomics

RESEARCH ARTICLE Open Access

The making of a pest: Insights from the evolution of chemosensory receptor families in a pestiferous and invasive fly, *Drosophila suzukii*

Paul V. Hickner, Chissa L. Rivaldi, Cole M. Johnson, Madhura Siddappaji, Gregory J. Raster and Zainulabeuddin Syed*

Abstract

Background: *Drosophila suzukii* differs from other *melanogaster* group members in their proclivity for laying eggs in fresh fruit rather than in fermenting fruits. Olfaction and gustation play a critical role during insect niche formation, and these senses are largely mediated by two important receptor families: olfactory and gustatory receptors (*Ors* and *Grs*). Earlier work from our laboratory has revealed how the olfactory landscape of *D. suzukii* is dominated by volatiles derived from its unique niche. Signaling and reception evolve in synchrony, since the interaction of ligands and receptors together mediate the chemosensory behavior. Here, we manually annotated the *Ors* and *Grs* in *D. suzukii* and two close relatives, *D. biarmipes* and *D. takahashii*, and compared these repertoires to those in other *melanogaster* group drosophilids to identify candidate chemoreceptors associated with *D. suzukii*'s unusual niche utilization.

Results: Our comprehensive annotations of the chemosensory genomes in three species, and comparative analysis with other *melanogaster* group members provide insights into the evolution of chemosensation in the pestiferous *D. suzukii*. We annotated a total of 71 *Or* genes in *D. suzukii*, with nine of those being pseudogenes (12.7 %). Alternative splicing of two genes brings the total to 62 genes encoding 66 *Ors*. Duplications of *Or23a* and *Or67a* expanded *D. suzukii*'s *Or* repertoire, while pseudogenization of *Or74a*, *Or85a,* and *Or98b* reduced the number of functional *Ors* to roughly the same as other annotated species in the *melanogaster* group. Seventy-one intact *Gr* genes and three pseudogenes were annotated in *D. suzukii*. Alternative splicing in three genes brings the total number of *Grs* to 81. We identified signatures of positive selection in two *Ors* and three *Grs* at nodes leading to *D. suzukii*, while three copies in the largest expanded *Or* lineage, *Or67a*, also showed signs of positive selection at the external nodes.

Conclusion: Our analysis of *D. suzukii*'s chemoreceptor repertoires in the context of nine *melanogaster* group drosophilids, including two of its closest relatives (*D. biarmipes* and *D. takahashii*), revealed several candidate receptors associated with the adaptation of *D. suzukii* to its unique ecological niche.

Keywords: Olfaction, Gustation, Odorant receptors, Gustatory receptors, Niche utilization, Adaption, Molecular evolution, Episodic selection

* Correspondence: zainulabeuddin.syed.5@nd.edu
Department of Biological Sciences & Eck Institute for Global Health, University of Notre Dame, Notre Dame, IN 46556, USA

BioMed Central

© 2016 The Author(s). **Open Access** This article is distributed under the terms of the Creative Commons Attribution 4.0 International License (http://creativecommons.org/licenses/by/4.0/), which permits unrestricted use, distribution, and reproduction in any medium, provided you give appropriate credit to the original author(s) and the source, provide a link to the Creative Commons license, and indicate if changes were made. The Creative Commons Public Domain Dedication waiver (http://creativecommons.org/publicdomain/zero/1.0/) applies to the data made available in this article, unless otherwise stated.

Background

Chemoreception, broadly encompassing olfaction and gustation, is essential to a number of insect life history traits such as host detection and discrimination, mate location, and predator avoidance. Chemoreception in insects is largely mediated by two divergent protein families, olfactory receptors (Ors) and gustatory receptors (Grs). A third family described in 2009 by Benton et al. [1] as the ionotropic receptors (Irs) has been implicated in multiple sensory modalities, including chemosensation [2]. Insect chemoreceptors (Ors and Grs) are seven transmembrane proteins expressed on the surface of chemosensory neurons housed in hair-like structures called sensilla [3, 4]. The genome of *Drosophila melanogaster* contains 60 *Ors* encoding 62 proteins through alternative splicing [4, 5], and each *Or* is expressed in a specific sub-set of olfactory receptor neurons (ORNs), with very few exceptions. All ORNs expressing the same *Or* merge into a single glomerulus [6]. While the basic principles and mechanisms of olfaction remain conserved across phyla [3, 7, 8], insect *Ors* have little homology to *Caenorhabditis elegans* or vertebrates, and the membrane topology is quite distinct [9]. Moreover, all canonical *Ors* are co-expressed with a single noncanonical olfactory receptor co-receptor (*Orco*), and together appear to define the response characteristic of an ORN [10]. The sense of taste in *D. melanogaster* is defined by 60 *Grs* encoding 68 proteins through alternative splicing [5]. In contrast to Ors, there is no clear evidence for a non-canonical co-receptor, and the membrane topology remains poorly defined [11].

The number of chemoreceptors often varies widely among insects, broadly reflecting their environment and function [3]. For example, the tsetse fly, *Glossina morsitans*, is estimated to have 40–46 *Ors* and 11–14 *Grs* [12, 13], while the red flour beetle, *Tribolium castaneum*, has 259 *Ors* and 220 *Grs* [14] (*Tribolium* Genome Sequencing Consortium 2008), and the honey bee, *Apis mellifera*, has 163 *Ors* and only 10 functional *Grs* [15]. The largest chemoreceptor repertoires (over 350 Ors) are reported in eusocial insects, such as ants [16]. In *Drosophila, Or* repertoires reflect the niche specialization patterns, such that a restricted spectrum of host/diet choice can be correlated with changes in chemoreceptor repertoire, such as specific losses and/or duplications in a set of receptors [17–21]. These changes can be further correlated with structural changes to the peripheral olfactory apparatus such as an altered number of specialized sensilla/ORNs [22–24]. Since, signaling and reception evolve in synchrony and in parsimony [25], an overall understanding of both these aspects will provide insights into the chemosensory basis of host utilization.

The recent (*Drosophila* 12 Genomes Consortium, Drosophila modENCODE Project) sequencing and subsequent annotation of multiple *Drosophila* spp. provides us with an excellent opportunity to connect the natural history of drosophilids [26] with the evolutionary history of chemosensation [27]. Recently, a member of the *melanogaster* group, *Drosophila suzukii* (Matsumura), has gained immense attention due to its invasion of the western hemisphere from its original endemic zone of South East Asia and emergence as a serious economic pest. A reduction in the yield of berry and soft fruit crops in newly invaded areas of North America and Europe are reported to reach as high as 80 % in the absence of any management practices, although a current and comprehensive economic assessment is lacking [28, 29].

Among the Drosophilidae, comprising over 1,500 known species [30], *D. suzukii* is one of only a few *Drosophila* with a highly evolved serrated ovipositor [31] that enables gravid females to pierce the skin of fresh fruits and lay their eggs inside the flesh. Though *D. suzukii* has been recognized as a pest of cherries in Japan since 1931, they were found infesting strawberries and cranberries in California, USA in 2008 [29]. They have since been discovered in at least a dozen states in the USA, as well as areas of Canada, Mexico, Italy, Spain and France [28, 29, 32]. We recently conducted a comprehensive analysis of the suite of volatile organic chemicals (VOCs) that define the unique olfactory landscape of *D. suzukii*, and compared it with that of *D. melanogaster* [33]. We demonstrated that *D. suzukii's* unique attraction to fresh fruits may be associated with the distinctive volatile repertoire originating from the host fruit-fly associated yeast complex. Recent studies are providing exciting insights into the complex interactions of *D. Suzukii* with yeast and fruits [34].

Here, we explored the role of olfaction and gustation in *D. suzukii's* unique ecological niche. We first manually annotated the Ors and Grs in the recently sequenced *D. suzukii* genomes [35, 36], and two closely related species, *D. biarmipes* and *D. takahashii* (Drosophila modENCODE Project), herein collectively referred to as the *suzukii-takahashii* clade. The latter two species occur in geographically overlapping regions with *D. suzukii* [30] but are mostly saprophytic and do not have the pointed ovipositor that enables them to lay eggs in fresh fruits [31]. We then compared these repertoires to those in six other previously annotated *melanogaster* group *Drosophila* [5, 19, 37]. Following our earlier comprehensive analysis of ligand repertoires for *D. suzukii* [33], we present the associated chemoreceptor repertoire that together defines *D. suzukii*'s unique ability to exploit diverse niches, and in turn pose a serious threat to fruit crops. This study further adds to ongoing efforts in understanding the chemosensory basis of host and mate finding in *D. suzukii* [33, 34, 38–40].

Methods

Manual curation of *Or* and *Gr* repertoires

D. suzukii gene models were manually curated based on the *D. melanogaster Or* and *Gr* annotations in FlyBase version FB2015_03 [41]. In short, *D. melanogaster* peptide sequences were used to screen the *D. suzukii* genome scaffolds using tBLASTn analysis in SpottedWingFlyBase v1.0 (last accessed on 4 September, 2015) [35]. To help predict start and stop codons, and exon-intron boundaries, scaffold regions containing putative chemoreceptors were aligned with their homologous *D. melanogaster* coding sequences (CDS) in MultAlin [42]. Where exon-intron boundaries were ambiguous, intron donor and accepter sites were evaluated using the splice site prediction tool [43] on the Berkeley Drosophila Genome Project web site (http://www.fruitfly.org/seq_tools/splice.html). Complementary strands were generated using the Reverse Compliment tool in the Sequence Manipulation Suite [44] (http://www.bioinformatics.org/sms/rev_comp.html), and coding sequences were translated using the ExPASy translate tool [45]. The *D. suzukii Or* and *Gr* annotations were then used to screen the *D. biarmipes* (Dbia_2.0, GCA_000233415.2) and *D. takahashii* (Dtak_2.0, GCA_000224235.2) genome assemblies with the methods described for *D. suzukii* using the BLAST tools on the National Center for Biotechnology Information (NCBI) web server. The *D. biarmipes* and *D. takahashii* genome assemblies were generated and made publicly available by the Drosophila ModENCODE project and the Baylor College of Medicine-Human Genome Sequencing Center (BCM-HGSC).

Gap filling and sequence validation

Gap filling

We filled gaps in the genome scaffolds that prevented the building of complete gene models using the sequence read archive (SRA) databases in NCBI. In those gene models where this method failed, PCR and capillary sequencing were used to fill the gaps.

Validation of duplications

Two approaches were used to evaluate duplications. When possible, tandem repeats were confirmed by amplifying and sequencing a region spanning the proximal ends of the duplicates. However, when the copies were greater than ~4,000 nucleotides apart or on a different scaffold we sequenced the individual genes.

Validation of pseudogenes

Predicted pseudogenes were resequenced to confirm the predictions from the initial tBLASTn analysis for *D. suzukii*, *D. takahashii* and *D. biarmipes.*

Genomic DNA (gDNA) for resequencing was extracted from the strains used for genome sequencing that are presently available at the UC San Diego Drosophila Stock Center: *D. suzukii* (stock # 14023–0311.03), *D. biarmipes* (stock # 14023–0361.10) and *D. takahashii* (stock # 14022–0311.13). A cetyltrimethylammonium bromide (CTAB) protocol [46] was modified for the extraction of genomic DNA from insects. Ten adult flies (5 males and 5 females) were ground with a pestle in 1.5 ml microcentrifuge tubes containing 200 μl 2 % CTAB solution (100 mM Tris HCl pH 8.0, 10 mM EDTA, 1.4 M NaCl, and 2 % CTAB). Samples were incubated for 5 min at 65 °C, followed by the addition of 200 μl chloroform and mixing by inverting 10 times. Samples were then centrifuged for 5 min at 13,000 x g. The aqueous phase was removed and placed in a new tube containing 200 μl isopropanol, mixed by inverting 10 times, and centrifuged for 5 min at 13,000 x g. The supernatant was poured off, 500 μl of 70 % ethanol was added, and the sample was centrifuged for 5 min at 13,000 x g. The supernatant was removed and the pellet was allowed to dry at room temperature for 15 min. DNA was resuspended in 50 μl deionized water, and all samples were normalized to 50 ng/μl using a Nanodrop ND-2000 (ThermoScientific, USA).

Primers flanking the gaps were designed using the Primer3plus program [47]. PCR was carried out in 50 μl reaction volumes using GoTaq® reagents (Promega). Each reaction contained a final concentration of 0.2 μM of each primer, 1.0 units of *Taq* polymerase and 2 ng/μl of genomic DNA. The thermal cycle included an initial denaturation of 94 °C for 2 min, 35 cycles of 94 °C for 1 min, 58 °C for 1 min, and 72 °C for 2 min, with a final extension time of 5 min at 72 °C. PCR products were visualized with agarose gel electrophoresis using SYBR® Safe gel stain (ThermoScientific). PCR products were cleaned-up using the Wizard® SV Gel and PCR Clean-Up System (Promega). Sequencing was performed using the ABI 3730xl (Life Technologies) and BigDye® chemistry (Life Technologies) at the University of Notre Dame Genomics Core Facility. Genes with sequence gaps filled using the SRA databases or by PCR and sequencing were suffixed with "fixSRA" or "fixPCR", respectively. Nucleotides that were fixed based on SRA or PCR are in bold or underlined, respectively (Additional file 1: Table S1-S6).

Gene nomenclature

Ors and *Grs* were named based on homology to *D. melanogaster* using standard *Drosophila* community gene nomenclature [48]. Each gene was prefixed with 'D' and the first three letters of the specific epithet (Dsuz, Dbia or Dtak), and named based on a combination of phylogenetic and reciprocal BLASTp analyses with the *D. melanogaster* annotated protein database in FlyBase

version FB2015_03 [41]. Duplications were suffixed with a unique numeral (e.g. *DsuzOr23a-1* and *DsuzOr23a-2*). Splice variants were predicted solely on genomic sequence (no transcript evidence) and suffixed using the capital letter designation in accordance with the homologous splice variant in FlyBase for *D. melanogaster* (e.g. *DmelOr69aA* and *DsuzOr69aA*). However, where novel splice variants were predicted, splice variants were designated based on their order on the scaffold rather than homology to *D. melanogaster*.

Pseudogenes were suffixed with 'P', and are defined here as genes with a mutated start codon, premature stop codon, or frameshift mutation leading to loss of ≥20 % of the original protein and ≥1 transmembrane domain [19] compared to the *D. melanogaster* homolog. The number of transmembrane domains was predicted using the topology prediction program, OCTOPUS [49]. Pseudogenes that were not excessively degraded were reconstructed for phylogenetic analysis by repairing mutated start codons, exon-intron boundaries or frameshift mutations to a functional state based on an intact homolog in the *suzukii* or *takahashii* subgroup. Repaired nucleotides are in lowercase in Table S1. All genes other than pseudogenes and partial gene models are assumed to be functional and are referred to here as intact. We refer to a lineage as lost when pseudogenizations or deletions (no apparent vestiges) resulted in the absence of at least one intact gene in one of 47 *Gr* or 54 *Or* orthologous groups (OGs) present in the *melanogaster* group as defined by Almeida et al. 2014 [50] (see Additional file 2: Table S3 and S4).

Comparisons were made to the previously annotated chemoreceptor repertoires of *D. melanogaster* [5], *D. ananassae*, *D. erecta*, *D. sechellia*, *D. simulans*, and *D. yakuba* [19, 37]. To better characterize lineages that were lost in the *suzukii-takahashii* clade, we screened the genomes of six additional *Drosophila* genome assemblies (*D. bipectinata*, *D. elegans*, *D. eugracilis*, *D. ficusphila*, *D. kikkawai*, and *D. rhopaloa*), generated and made publicly available by the BCM-HGSC, using the methods described above. Evolutionary inferences were based on phylogeny reconstruction by Chiu et al. [35], while divergence times were based on earlier estimates [36]. Reconciliation of gene trees with the species tree for the expanded lineages was performed using the parsimony-based method in NOTUNG v2.8.1.6 [51]. Gene trees were estimated using Mega version 6 [52] where the maximum likelihood approach with the Jones, Taylor, Thornton (JTT) substitution model [53], a Gamma distribution (+G) with five discrete categories, and complete deletion of gaps was implemented. The edge weight thresholds were 0.9 and based on bootstrap support following 500 iterations, while the loss and duplication costs were 1.0 and 1.5, respectively. No branches were collapsed for NOTUNG analysis.

Measures of divergence

Two proxies were used to describe divergence, the percent of identical amino acids in a peptide sequence alignment to *D. melanogaster* (%ID) and the ratio of nonsynonymous (*dN*) to synonymous (*dS*) substitution rates (*dN*/*dS*). %ID was calculated using Clustal Omega [54] on the European Bioinformatics Institute (EMBL-EBI) web server [55]. Nonsynonymous and synonymous substitution rates were calculated using the Nei and Gojobori method [56] implemented in SNAP v2.1.1 [57] (http://www.hiv.lanl.gov/content/sequence/SNAP/SNAP.html). *D. melanogaster* was used as an outgroup for *dN* and *dS* calculations for all three species (i.e. *suzukii-melanogaster, biarmipes-melanogaster,* and *takahashii-melanogaster*). Differences were determined using paired (between species) and unpaired (*Ors* vs *Grs*) Wilcoxon Signed-Rank tests with the MASS package [53] in the R statistical environment.

Tests for positive selection

Positive selection acting on a small proportion of sites is often hard to detect using the ratio of nonsynonymous to synonymous substitution rates across the entire length of a gene (*dN*/*dS*). Therefore, we used the adaptive branch-site random effects likelihood (aBSREL) approach [52] to identify signatures of diversifying selection at the codon level within a phylogenetic framework comprised of 9 species in the *melanogaster* group: *D. ananassae*, *D. biarmipes*, *D. erecta*, *D. melanogaster*, *D. sechellia*, *D. simulans*, *D. suzukii*, *D. takahashii* and *D. yakuba. Or* sequences for *D. ananassae*, *D. erecta*, *D. sechellia*, *D. simulans* and *D. yakuba* were from Guo and Kim [37] while *Gr* sequences were kindly provided by Michael Ritchie (University of St Andrews, UK). Only functional genes were used in the positive selection analysis.

Peptide sequences of homologous chemoreceptors (gene sets) were aligned in MAFFT v7 using the Blosum62 scoring matrix, a gap penalty of 1.53, and the G-INS-1 refinement method [58]. Each alignment was visually inspected and manually edited, when necessary, and used to estimate a phylogeny for each homologous gene set. The maximum likelihood approach with the Jones, Taylor, Thornton (JTT) substitution model [59] and a Gamma distribution (+G) with five discrete categories, and complete deletion of gaps was implemented in Mega version 6 [60]. Codon alignments were generated using PAL2NAL [61]. The aBSREL method [52] was implemented in HyPhy [62], where all internal and external nodes were tested for signatures of diversifying selection using likelihood ratio tests (LRTs). The Holm-Bonferroni method was used to control the familywise error rate for multiple tests within a gene set [63], whereas the Benjamini-Hochberg false discovery rate

method was used for corrections across all gene sets [64]. Chemoreceptors showing positive selection based on the aBSREL method were further tested by using the stringent M1–M2 models of the codeml program in PAML [65]. Values >0.95 from Bayes empirical Bayes (BEB) method were considered sites under diversifying selection [66].

Phylogenetic analysis

Phylogenies were estimated for Ors and Grs to help reconstruct evolutionary events and to assist in the naming of the genes. Peptide sequences of *D. suzukii*, *D. biarmipes*, *D. takahashii* and *D. melanogaster* ≥ 360 aa (Ors) or ≥ 340 (Grs) in length were multiply aligned using MUSCLE v3.8.31 [67]. Maximum likelihood trees were inferred using the PROTGAMMA model of protein substitution, JTT matrix, and 500 bootstrap replications in RAxML v.8 [68]. RAxML analysis was conducted on the CIPRES Science Gateway and XSEDE [69]. Figures were prepared using the FigTree program for visualization and annotation of phylogenetic trees [70]. The *Or* and *Gr* trees were rooted with *Orco* and *Gr21a*, respectively. The aligned peptide sequences files (Phylip) and phylogenetic tree files (Nexus) for both the OR and Gr families are in the additional files (Additional files 3, 4, 5 and 6)

Scanning electron microscopy (SEM)

Freshly emerged *D. suzukii* were placed in acetone for at least 24 h until they could be processed by scanning electron microscopy (SEM). After undergoing critical point drying, flies were mounted both dorsally and ventrally on carbon tape attached to an aluminum stub mount, and coated with 4 μM of iridium using a Cressington 208 HR sputter coater (Cressington Scientific Instruments, Watford, UK) in conjunction with the Cressington MTM 20 thickness monitor. Images were taken with a FEI-Magellan 400 FESEM (FEI, Hillsboro, OR, USA).

Results

Chemosensory organs and receptor repertoires

Scanning Electron Microscopy (SEM) of the olfactory organs in *D. suzukii* revealed striking morphological similarity to the well-defined *D. melanogaster* structures (Fig. 1) [71, 72]. Maxillary palps were adorned with a single class of olfactory sensilla, basiconic (Fig. 1c, d), whereas an additional two types, trichodea and coeloconic, are seen on the antenna (Fig. 1e). One unusual feature we noted in the large basiconic class was the presence of two distinct pore patterns. The single pattern reported earlier in *D. melanogaster* (Fig. 1f; circle) [71, 72] was observed *in D. suzukii*, but we also noted an additional unique pore pattern (Fig. 1f; circle).

Next, we annotated the *Ors* and *Grs* from the genome assemblies of *D. suzukii* and two closely related members, *D. biarmipes* and *D. takahashii*. A summary of the *Or* and *Gr* repertoires, along with those previously annotated in *D. melanogaster*, are reported in Table 1. Phylogenetic relationships among the *Ors* in these four species are represented in Fig. 2, illustrating several clade specific and species specific expansions. The total number of *Or* loci ranged from 64 in *D. biarmipes* to 71 in *D. suzukii and D. takahashii*. However, pseudogenizations reduced the

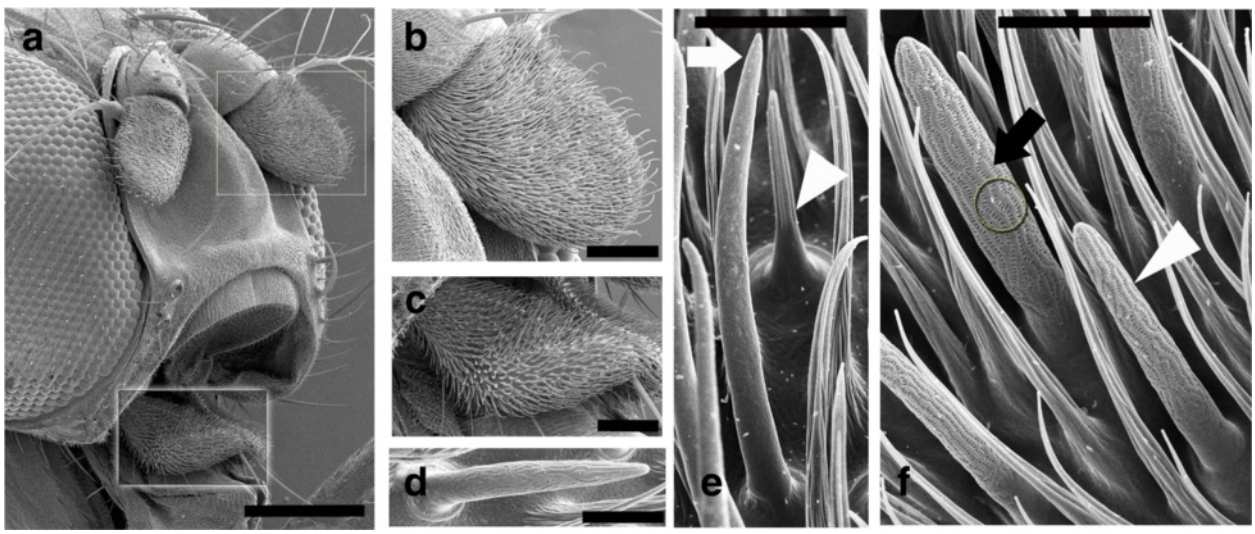


Fig. 1 Olfactory structures in *D. suzukii* viewed under SEM. An adult head with antenna and maxillary palp, highlighted in the insets. **a** Magnified antenna (**b**) and a maxillary palp (**c**). Palps have only one type of multiporous basiconic sensillum type (**d**). Antennal surface shows: long pointed trichoid sensillum (white arrow) and distinct coeloconic (arrow head) (**e**); multiporous basiconic sensilla (black arrow points to the large basiconic and short basiconic are indicated by white arrow head) (**f**). Scale bar is 200 μM in (**a**), 50 μM in (**b**) and (**c**); 5 μM for (**d**), (**e**) and (**f**)

Table 1 Summary of the chemoreceptor repertoires in *D. suzukii* (Dsuz), *D. biarmipes* (Dbia), and *D. takahashii* (Dtak), along with those previously annotated in *D. melanogaster* (Dmel)

	ORs				GRs			
	Dsuz	Dbia	Dtak	Dmel*	Dsuz	Dbia	Dtak	Dmel*
Loci	71	64	71	62	74	74	88	62
Functional genes	62	60	70	60	71	74	82	60
Pseudogenes	9	4	1	2	3	0	6	2
Genes w/splice variants	2	2	2	2	3	3	3	3
Splice variants	6	7	9	4	13	12	12	11
Total functional proteins	66	65	77	62	81	83	91	68

Total functional proteins include predicted splice variants
*Data from [5, 77, 97–99]

number of functional *Or* genes to 62, 60, and 70 in *D. suzukii*, *D. biarmipes* and *D. takahashii*, respectively. We predicted alternative splicing in two *Or* genes (*Or46a* and *Or69a*) in all three species, the same genes with splice variants in *D. melanogaster* [5]. *Or46a* encodes two splice variants that are moderately conserved, with percent identity of *D. suzukii* to *D. melanogaster* ranging from 80.7 to 83.4 % (Additional file 1: Table S1). Conversely, *Or69a* is predicted to encode four to seven splice variants in the *suzukii-takahashii* clade, compared to only two isoforms in *D. melanogaster*. The number of functional genes in *D. suzukii* and *D. biarmipes* is roughly the same as *D. melanogaster*, whereas *D. takahashii*, with 70 genes, is more than the 66 predicted in *D. ananassae,* the largest *Or* repertoire among the *melanogaster* group *Drosophila* annotated prior to this study.

We predicted a total of 74 *Gr* genes in *D. suzukii,* of which 71 are functional and three are pseudogenes, while 74 intact genes and no pseudogenes were predicted in *D. biarmipes*, and 88 genes were predicted in *D. takahashii* of which six are pseudogenes (Table 1). Phylogenetic relationships among the *Grs* in four species showed several unique expansions (Fig. 3). In *D. suzukii*, three genes encode 13 splice variants, bringing the total to 81 functional *Grs* (Table 1). *D. suzukii's* repertoire of *Grs* is nearly identical to *D. biarmipes*, which has 74 genes encoding 83 Grs. While these two *Gr* repertoires are larger than any other *Drosophila* annotated thus far, *D. takahashii's* repertoire is even larger with 82 intact genes encoding 91 Grs (Table 1).

The number of introns in *Ors* and *Grs* was consistent with those in *D. melanogaster*, with the exception of *Gr85a. D. suzukii*, *D. biarmipes* and *D. takahashii* each have two copies of *Gr85a*, and *Gr85a-1* has one intron while *Gr85a-2* has two introns. Furthermore, the peptide sequences are notably shorter (374–381 aa) than *Gr85a* in *D. melanogaster* (397 aa). The functional state of *Or42a* in both *D. suzukii* and *D. biarmipes* was initially unclear due to an unusually long first intron. *Or42a* resides on two different scaffolds in both species where it is fragmented in the 1st intron. Attempts to amplify and sequence the gene region were unsuccessful. *Or42a* in *D. takahashii* has a large first intron (2511 nucleotides) compared to *D. ananassae* (66 nucleotides) and *D. melanogaster* (185 nucleotides), so next we examined *Or42a* in other *melanogaster* group genomes and found that the first intron is also large in *D. kikkawai* (4,475), and on two different scaffolds in the *D. eugracilis* assembly. Consequently, failure to amplify the gene could have been due to the size of the amplicon. Screening of the SRA from transcriptome sequencing by Chiu et al. [35], however, shows that *Or42a* is being transcribed in *D. suzukii*; therefore, we considered *Or42a* intact in *D. suzukii* and *D. biarmipes*.

Evolutionary events

Expansions and losses

Gene tree reconciliation revealed complex birth-and-death evolutionary patterns, wherein the *suzukii* and *takahashii* subfamilies (Fig. 4a and b; shaded box) underwent changes in copy numbers in a subset of *Ors* and *Grs* as they diverged from their common ancestor (CA1 in Fig. 4a). The later split of *D. suzukii* and *D. biarmipes* from CA2 was accompanied by similar changes. Three *Or* lineages, *Or74a*, *Or85a* and *Or98b* were lost in *D. suzukii* but were functional in *D. biarmipes* and *D. takahashii,* while *Or33c* was lost in *D. biarmipes*, and none were lost in *D. takahashii* (Fig. 4). Based on previous annotations, and the screening of five additional *melanogaster* group genomes, the loss of *Or74a* is unique to *D. suzukii,* while *Or85a* was lost independently in *D. ananassae* and *D. suzukii.*

The two largest expansions in the *D. suzukii* and *D. takahashii Or* lineages were *Or23a* and *Or67a* (Fig. 4b; Additional file 7: Figure S1). *D. suzukii* and *D. takahashii* have four and five copies of *Or23a*, respectively, while *D. biarmipes* has only one (Fig. 4b). Four intact and one *Or67a* pseudogene were found in *D. suzukii*, while four intact copies were found in *D. biarmipes*, and six copies plus a pseudogene were found in *D. takahashii* (Fig. 4b). The

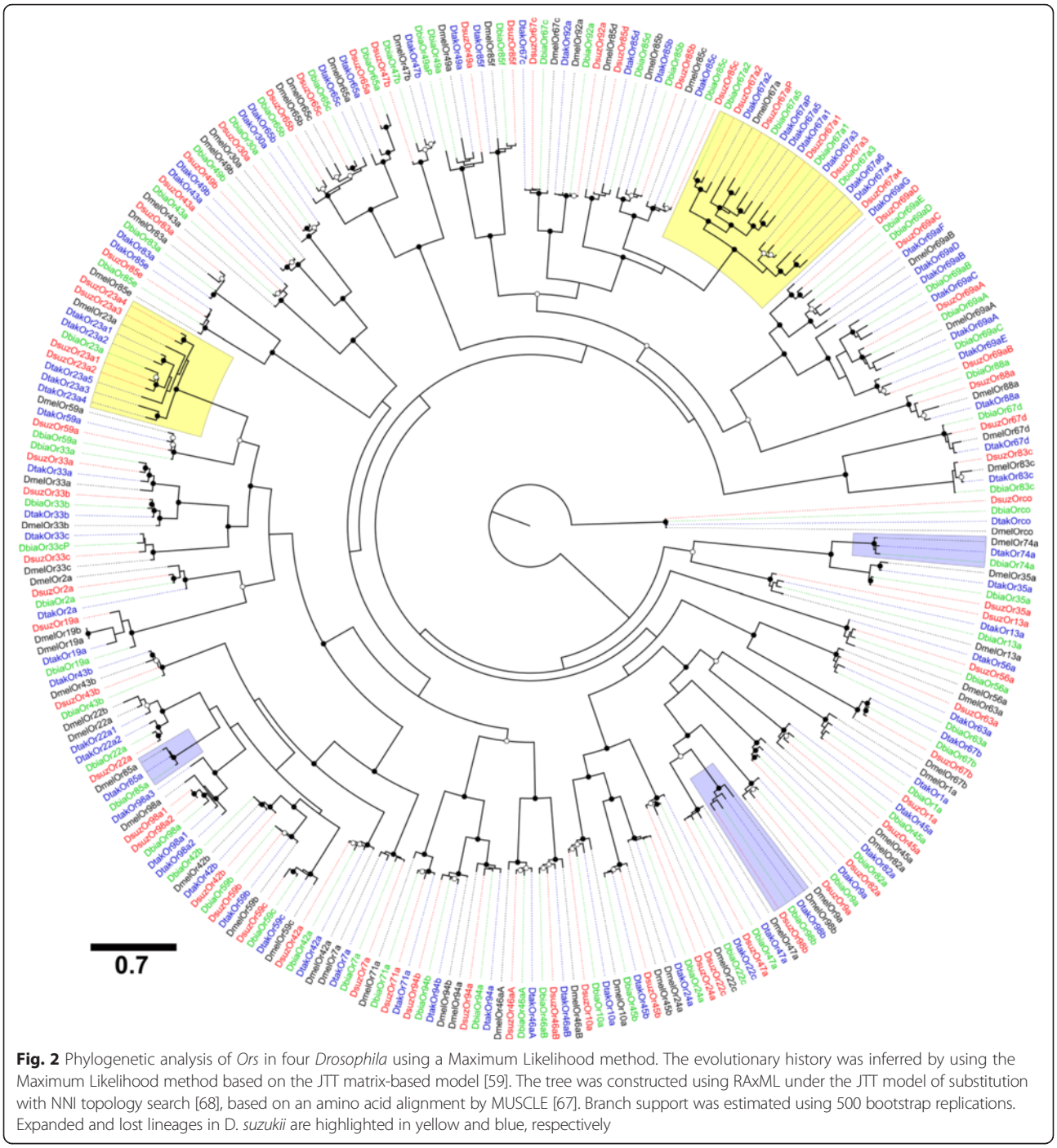


Fig. 2 Phylogenetic analysis of *Ors* in four *Drosophila* using a Maximum Likelihood method. The evolutionary history was inferred by using the Maximum Likelihood method based on the JTT matrix-based model [59]. The tree was constructed using RAxML under the JTT model of substitution with NNI topology search [68], based on an amino acid alignment by MUSCLE [67]. Branch support was estimated using 500 bootstrap replications. Expanded and lost lineages in D. *suzukii* are highlighted in yellow and blue, respectively

Gr lineages showed by far the largest expansions in the *suzukii-takahashii* clade compared to all of the annotated *melanogaster* group *Drosophila.* Four lineages were expanded in *D. suzukii*, two in *D. biarmipes*, and six in *D. takahashii* (Fig. 4a and b; Additional file 7: Figure S1). One lineage, *Gr59cd*, was expanded in all three members of the *suzukii-takahashii* clade, whereas *Gr36a-c* was uniquely expanded in the *D. suzukii* and *D. biarmipes.* The only other shared expansion was between *D. suzukii* and *D. takahashii* for *Gr59ab*. The largest number of unique expansions in the *suzukii-takahashii* clade was in *D. takahashii* and includes *Gr22a-f*, *Gr64a*, *Gr64f* and *Gr98b-d*. Interestingly, no *Gr* lineages were lost in any of the three species annotated in the present study.

Next, we used the parsimony-based gene tree reconciliation method in NOTUNG v2.8.1.6 [51] to analyze the two largest expanded lineages in both *Ors* and *Grs*. Among the *Ors*, *Or23a* duplicated

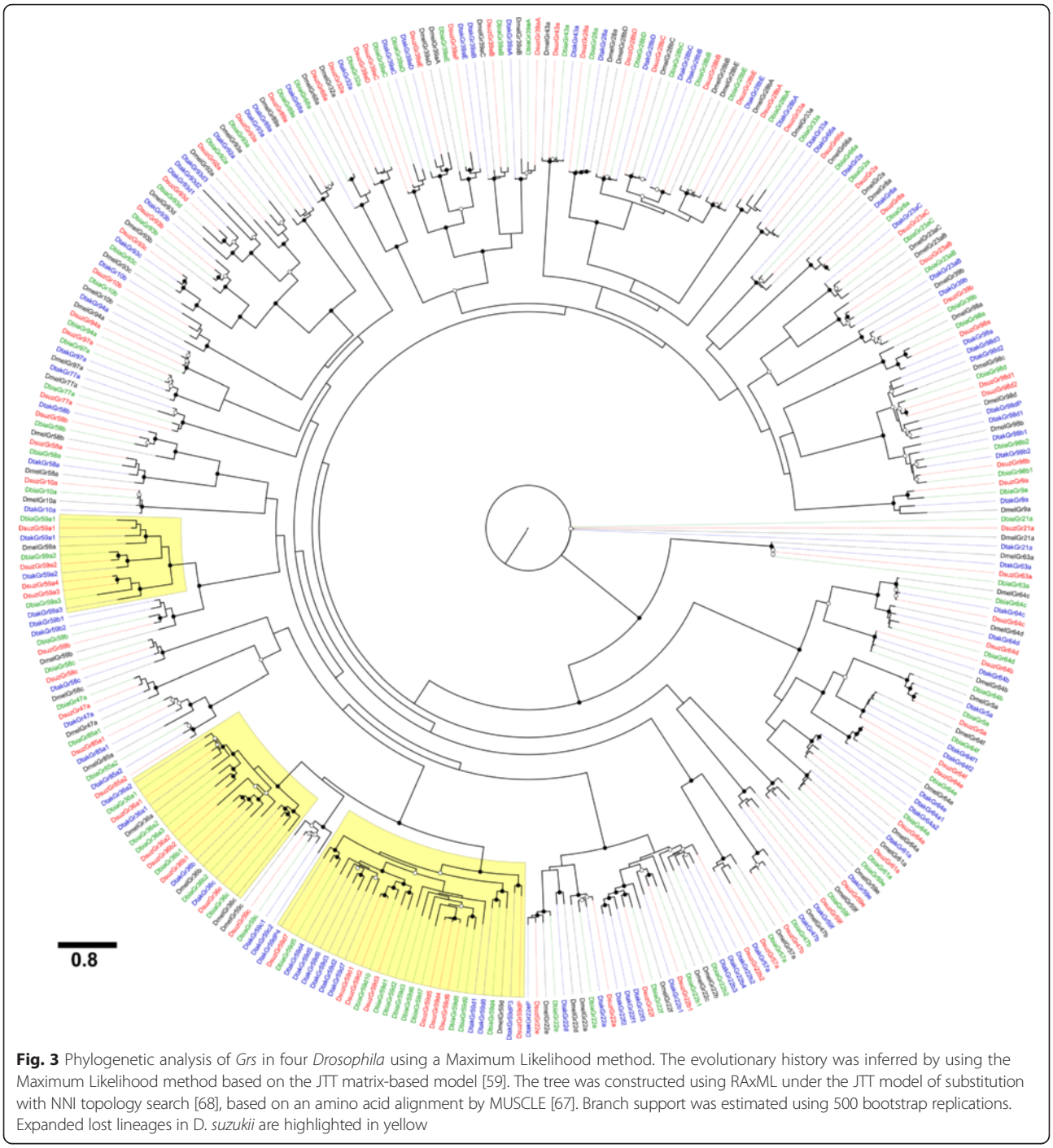


Fig. 3 Phylogenetic analysis of *Grs* in four *Drosophila* using a Maximum Likelihood method. The evolutionary history was inferred by using the Maximum Likelihood method based on the JTT matrix-based model [59]. The tree was constructed using RAxML under the JTT model of substitution with NNI topology search [68], based on an amino acid alignment by MUSCLE [67]. Branch support was estimated using 500 bootstrap replications. Expanded lost lineages in D. *suzukii* are highlighted in yellow

several times, and the common ancestor to the *suzukii-takahashii* clade probably had three copies, indicating that *D. biarmipes* lost two copies while *D. suzukii* and *D. takahashii* gained one and two copies, respectively (Additional file 7: Figure S1). The expansion of the *Or67a* lineage was already present prior to the *suzukii-takahashii* split, except for one later duplication in *D. takahashii* (Fig. S1). The two largest expanded *Gr* lineages were *Gr59a* and *Gr59d* in all three species. The *Gr59a* duplication pattern was comparable to *Or67a*, whereas *Gr59d* showed by far the most complex pattern of evolution resulting in 27 total copies in the three species (Fig. S1).

Divergence

Having annotated the genomes of three species that include the pest, *D. suzukii*, we estimated divergence and

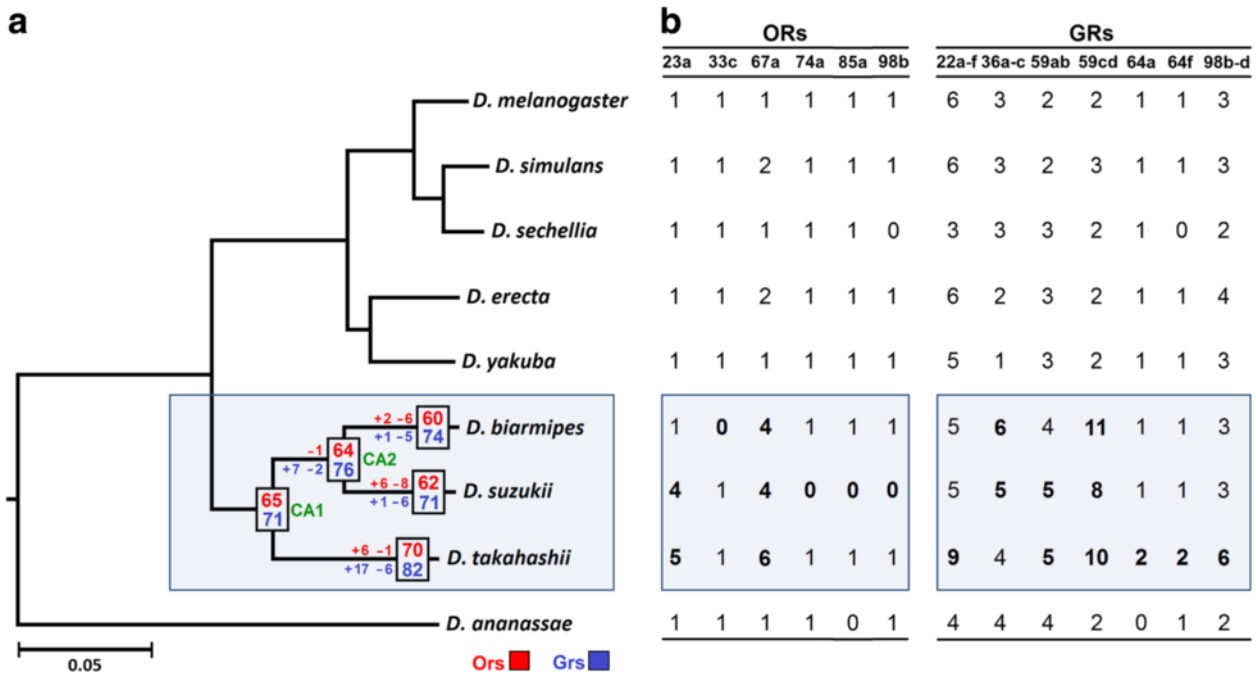


Fig. 4 Evolutionary events in the *suzukii-takahashii* clade chemoreceptor families. **a** Evolutionary changes in the number of functional *Or* and *Gr* genes in the clade derived from common ancestors, CA1 and CA2. **b** The number of intact genes in the expanded and lost lineages in the clade (shaded) is shown in bold, and is compared with the number in six other *melanogaster* group *Drosophila*. Phylogeny adapted from Chiu et al. [35]

selection in the chemosensory receptor families using the percent of identical amino acids to homologous *D. melanogaster* peptide sequences (%ID) and the ratio of nonsynonymous (*dN*) to synonymous (*dS*) substitution rates (*dN*/*dS)* (Table 2). The *dN/dS* ratios in *Ors* ranged from 0.0125 in *DsuzOrco* to 0.3670 in *DbiaOr19a* (mean of three species = 0.111), while the %IDs ranged from 44.72 % in *DbiaOr67a*-2 to 98.77 % in *DsuzOrco* (mean = 81.9 %). The %ID of Grs ranged from 33.06 % in *DbiaGr59a*-3 to 99.55 % in *Gr21a* (mean = 74.8 %). The *Gr* dN/dS ratios ranged from 0.002 in *Gr21a* to 0.370 in *Gr10b* (mean = 0.138). These low *dN*/*dS* values imply that both chemoreceptor families have evolved under strong purifying selection. These values are larger than the reported genome wide estimates of 0.095 for X chromosome genes and 0.090 for autosomal genes [35],. Differences in the means between species, based on paired Wilcoxon Signed-Rank tests, are shown in Table 2. Comparisons between *dN*, *dS*, and *dN/dS* of *Ors* and *Grs* using unpaired tests showed that *Grs* are more divergent than *Ors* in all three species (Table 3).

Table 2 Substitution rate analysis of *Ors* and *Grs*

		species			*p*-values		
Genes	Parameter	Dsuz	Dbia	Dtak	suz-bia	suz-tak	bia-tak
Ors	*dN*	0.094	0.103	0.089	0.023	0.001	<0.001
	dS	0.936	1.009	0.945	0.007	0.790	0.048
	dN/dS	0.102	0.104	0.093	0.679	0.006	0.009
Grs	*dN*	0.146	0.152	0.141	0.000	0.004	<0.001
	dS	1.036	1.039	1.018	0.373	0.489	0.707
	dN/dS	0.141	0.142	0.131	0.003	0.008	0.014

Differences in mean nonsynonymous (*dN*) and synonymous (*dS*) substitution rates, and *dN*/*dS* are indicated by asterisks

Genes with the highest and lowest *dN/dS* values in *D. suzukii* provide insights into highly divergent or conserved functions. Among the most conserved *Ors*, *Orco* tops the list, followed by *Or47a*, *Or92a*, *Or42b* and *Or24a*, and whereas *Gr21a*, *Gr28a*, *Gr28bB*, *Gr63a* and *Gr64c* were the most conserved *Grs*. Most divergent *Ors* were *Or19a*, *Or23a*, *Or69aA*, *Or65a* and *Or33a* and the *Grs* included *Gr10b*, *Gr93d*, *Gr92a*, *Gr85a* and *Gr22c* (Additional file 2: Tables S1 and S2). This trend was comparable in *D. takahashii* and *D. biarmipes*.

Selection

Next we tested for the signatures of positive selection acting on a small proportion of sites that are often difficult to detect using the *dN*/*dS* ratio across the entire gene. The adaptive branch-site random effects likelihood (aBSREL) approach [52] on homologous gene sets revealed two *Ors* and three *Grs* showing evidence of positive selection the *suzukii-takahashii* clade in the phylogenetic framework comprising nine *melanogaster* group drosophilids (Fig. 5; Additional file 2, Table S5). The number of tests for each gene set ranged from a small set of 11 (singletons with

Table 3 Differences in substitution rates between *Ors* and *Grs*. Mean synonymous (*dS*) and nonsynonymous (*dN*) substitution rates and ratios (*dN/dS*) for *Ors* and *Grs* using *D. melanogaster* as an outgroup

	Dsuz			Dbia			Dtak		
Parameter	*Ors*	*Grs*	*p*-value	*Ors*	*Grs*	*p*-value	*Ors*	*Grs*	*p*-value
dN	0.094	0.146	0.004	0.103	0.152	0.005	0.089	0.141	0.001
dS	0.936	1.036	0.924	1.009	1.039	0.681	0.945	1.018	0.623
dN/dS	0.102	0.141	0.005	0.104	0.142	0.002	0.093	0.131	0.001

Mean *dN* and *dN/dS* was greater for *Grs* suggesting that, overall, *Grs* were more divergent than *Ors* in all three species

losses in some lineages) to as many as 43 for a gene with large expansion across spp. (*Gr59d*). A total of five lineages showed signatures of positive selection, four of those being at internal nodes and one being at an external node (Fig. 5). In all cases, the percentage of sites exhibiting signatures of positive selection (ω_2%) was small, ranging from 1.1 % to 7.2 % (Fig. 5). Selection at the remaining sites (ω_1) ranged from very high purifying selection ($\omega_1 < 0.01$) to neutral selection ($\omega_1 = 1$).

In the *suzukii-takahashii* clade, positive selection was detected in *Or2a*, *Gr5a* and *Gr97a* along branches leading to both the *suzukii* and *takahashii* subgroups, while *Gr58a* showed signatures of positive selection along the branch leading to the *suzukii* subgroup (Fig. 5). In *Or2a*, positive selection was found at a very small percentage of codons (1.9 %), while the remaining sites exhibit signatures of purifying selection ($\omega_1 = 0.225$) (Fig. 5). Strong purifying selection ($\omega_1 < 0.01$) was evident at 96.4 % of *Gr5a*, while 3.6 % of the codons showed evidence of positive selection ($\omega_2 = 11$; $p = 0.024$). *Gr58a* also exhibited strong purifying selection ($\omega_1 < 0.01$) at the majority of sites (92.8 %), while the remaining 7.2 % exhibited signatures of positive selection (; $p = 0.031$). The vast proportion of *Gr97a* (98.6 %) shows no signs of selection pressure ($\omega_1 = 1.0$) while 1.6 % of the sites show evidence for positive selection ($p = 0.004$).

Next, the two largest expanded *Or* and *Gr* lineages were subjected to aBSREL analysis by restricting the phylogeny to the three species in the *takahashii-suzukii* clade. Only the *Or67a* lineage had genes with signatures of positive selection, of which two genes were in *D. takahashii* (*DtakOr67a-4* and *DtakOr67a-4*) and one in *D. suzukii* (*DsuzOr67a-3*) (Fig. 6a). These results were independently confirmed using the branch-site test in PAML that further identified codons under positive selection (Fig. 6b).

Finally, we would like to state that the reason for reporting the less stringent p-values from Holm-Bonferroni corrections within gene sets was to extract candidates with some (any) evidence of positive selection.

Discussion

Olfactory structures

Peripheral olfactory structures in *D. melanogaster* have been studied over the years and have revealed stereotypic pattern of sensillary organization [6, 71, 73]. These studies laid a solid foundation to the functional mapping of sensilla [74, 75]. More advanced molecular techniques have correlated the morphological and functional sensillary patterns with that of chemosensory gene expression [76, 77]. A broadly conserved pattern emerged in our *D. suzukii* SEM studies as compared to *D. melanogaster*. Limited single sensillum recordings (SSR) from *D. suzukii* antennal basiconic (ab) sensilla in combination with high resolution gas chromatography (GC-SSR) suggested

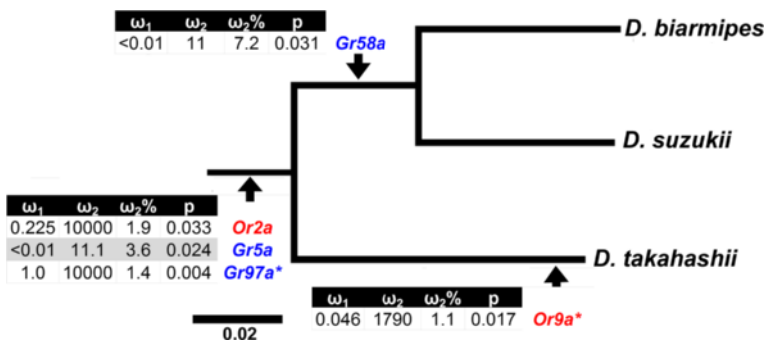


Fig. 5 Genes with signatures of positive selection in the *suzukii-takahashii* clade based on the adaptive branch-site random effects likelihood (aBSREL) method [52] performed across 59 *Or* and 61 *Gr* homologous gene sets within a phylogenetic framework comprised of nine *melanogaster* group *Drosophila*. P-values were corrected for multiple tests within each gene set using the Holm-Bonferroni method [63]. *Genes also showing signatures of positive selection using the branch site method in PAML [65]

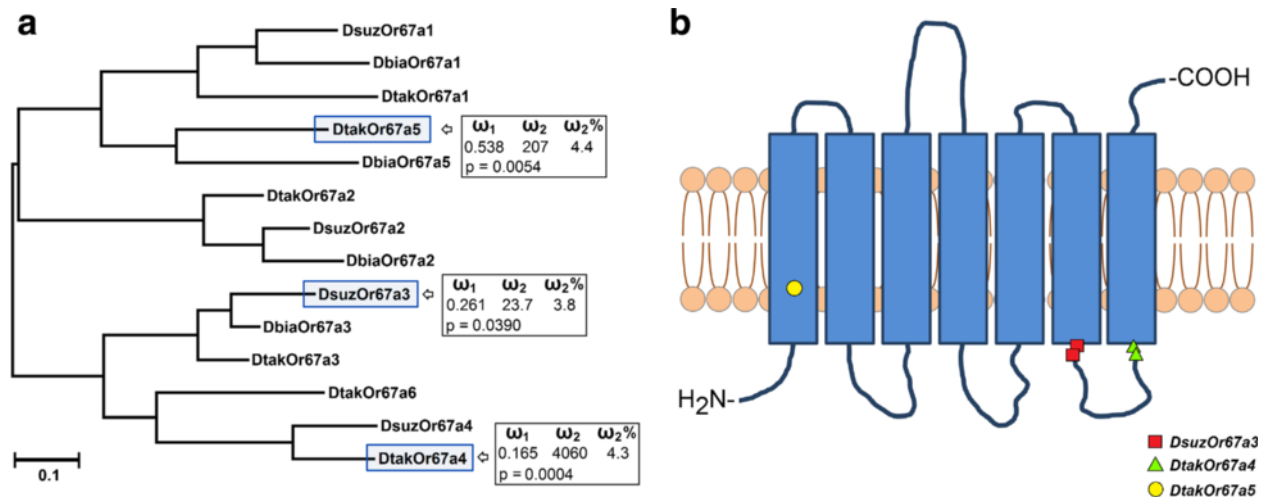


Fig. 6 Signatures of positive selection in the suzukii-takahashii Or67a lineage. **a** Three gene showed signatures of positive selection in the highly expanded Or67a lineage. Phylogeny adapted from Chiu et al. [35]. **b** The approximate position of the sites under positive selection based on branch site tests in PAML and the transmembrane domain predictions by OCTOPUS

a high conservation in response profile from the ab1 sensilla as compared to *D. melanogaster*, whereas other two large sensilla (ab2 and ab3) had significantly altered physiological profiles [39]. This could be due to the alteration in *Or* sequences and/or expression profiles.

Repertoire size

Unlike vertebrates and many insects, in which there has been extensive variation in the number of genes in the chemoreceptor families, the size of the *Or* and *Gr* repertoires in *Drosophila* have changed little during the last ~70 million years [78] despite their extensive distribution and diverse life history traits [79] that range from primitive sap and slime feeding (*virilis-repleta*) to more recent adaptations in the *melanogaster* group that utilize decaying and fermenting fruits [26]. Of ~30 *Drosophila* genomes that have been sequenced, the *melanogaster* group is the most represented. Furthermore, this group has been well characterized in terms of chemosensory repertoire annotation.

A number of previous studies have described a balanced birth-and-death process of evolution, wherein the number of genes gained through duplication roughly equals the number of genes lost through pseudogenization, thus maintaining *Or* and *Gr* repertoires comprising approximately 60 genes each [5, 21, 37, 80]. Our chemoreceptor annotations in the *suzukii-takahashii* clade revealed similar patterns (Fig. 4; Additional file 2: Tables S3 and S4). The size of the *Or* repertoires in *D. suzukii* and *D. biarmipes* are roughly the same as other *melanogaster* group flies, while *D. takahashii* has several additional *Ors* that make its repertoire the largest among all the annotated species in this group with 77 intact Ors. The size of the *Gr* repertoires in *D. suzukii*, *D. biarmipes* and *D. takahashii* are all relatively large compared to other *melanogaster* group members, with 81, 83 and 91 total proteins, respectively. Analysis of the evolutionary history of duplications and losses revealed that the expansions of the *Gr* lineages in the *suzukii-takahashii* clade occurred prior to *D. suzukii's* divergence from *D. biarmipes,* ~7.3 mya [36]. Thus, the expanded *Gr* lineage is not a direct consequence of *D. suzukii's* adaptation to its expanded ecological niche, but could have simply helped facilitate the shift by providing ample variation for evolution to act upon.

Expansions and losses

Despite the maintenance of a standard repertoire size, gene births and deaths during trophic shifts can produce unique and rapidly evolving chemosensory repertoires. A study by McBride [19] showed that *D. sechellia,* a species endemic to the Seychelles and a specialist on the fruit of *Morinda citrifolia,* experienced an accelerated rate of chemoreceptor gene loss during its evolution to a specialist life style. A similar trend in the *Grs* was found in *D. erecta*, a specialist on *Pandanus candelabrum* [20]. A recent study further demonstrated a relationship between host-choice and chemoreceptor repertoire wherein four widely conserved *Ors* (*Or9a, Or22a, Or42b and Or85d*) that detect yeast-derived and fruit related compounds were uniquely lost in an herbivorous *Drosophila*, *Scaptomyza flava*, while *Or67b*, a receptor shown to enhance the sensitivity and detection of plant derived green leaf volatiles, was uniquely expanded [18]. These unique changes in the *Or* repertoire were considered as adaptive losses and gains towards the evolution of herbivory in *Scaptomyza* from its ancestral drosophilids that feed on yeast [18, 81].

The *D. suzukii* and *D. takahashii Or* repertoires are distinct in having two large expansions, *Or23a* and *Or67a*, while only the *Or67a* expansion was retained in *D. biarmipes*. In *D. melanogaster*, *Or23a* is expressed on the surface of the B cell in antennal intermediate 2 (ai2) sensilla [82], formerly classified as antennal trichoid 2 (at2) sensilla [76]. And despite screening with a large panel of compounds using SSRs and the *Δ-halo* system in *D. melanogaster*, no strong ligands for *Or23a* have been identified [83, 84]. In *D. melanogaster*, *Or67a* is expressed on the surface of the B cell in ab10 sensilla (Couto et al. 2005), where methyl benzoate and ethyl benzoate elicited strong excitatory responses (≥100 spikes/s) at a low dose of 10^{-4} dilutions [83]. Five functional copies of *Or67a* in the *D. suzukii* strain from Italy have been found [40], while we identified only four intact copies and one pseudogene in the North American isolate, suggesting that the number of functional genes in the *Or67a* lineage can be variable across geographical regions. This group also suggested that *D. suzukii's* increased sensitivity to isoamyl acetate [33, 39], a yeast-derived and fresh fruit volatile, could be due to the expanded *Or67a* copy-numbers [40].

Interestingly, of the three species annotated here, *D. suzukii's* repertoire of *Ors* underwent the most gene deaths, with losses of *Or74a*, *Or85a* and *Or98b*. This results in the smallest number of *Or* lineages (51) among the nine drosophilids studied here (Additional file 2: Table S3). It is worth mentioning that this number of lineages is even smaller than *D. sechellia's* and *D. erecta's*, both of which have a very restricted diet. Of the three lost lineages, *Or74a* in *D. melanogaster* is a larval specific receptor expressed in a subset of ORNs in the larval dorsal organ (LDO) [85, 86] (Table 4). A heterologous expression using *Δ-halo* system revealed excitatory responses to linear aliphatic compounds such as 1-hexanol, (*E*)-2-hexenal, 1-heptanol and 1-nonanol (≥100 spikes/s), compounds commonly associated with fruits [86] (Table 4). The second, *Or85a*, is a narrowly tuned receptor expressed on the B cell of ab2 sensilla in *D. melanogaster* where ethyl 3-hydroxybutyrate elicits a strong excitatory response [87]. Single sensillum recordings (SSRs) by Keesey et al. [39] showed similar response profiles for the B cell in ab2 sensilla in *D. biarmipes* and *D. melanogaster*, but not for *D. suzukii*. Ethyl 3-hydroxybutyrate still elicited a strong response, but 2-heptanone elicited the strongest response in *D. suzukii* (Table 4). However, 2-heptanone did not elicit a response in *D. biarmipes* or *D. melanogaster*, suggesting that a different, more broadly tuned Or is being expressed in *D. suzukii's* ab2 sensillum, which lends physiological evidence for the loss of *Or85a* from *D. suzukii's* repertoire of functional *Ors*. Very little is known

Table 4 Ligands and chemosensory organs, based on studies in *D. melanogaster*, are shown for lost and expanded lineages, and genes with signatures of positive selection (a = antenna, *p* = palp, b = basiconic, t = trichoid, LDO = larval dorsal organ)

	Gene	Species	Ligands	Expression
Losses	*Or33cP*	bia	Ethyl acetate, Cyclohexanone, Fenchone [100]	pb2A [76, 100]
	Or74aP	suz	*E,E*-2-4-nonadienal [101]	LDO [86]
	Or85aP	suz	Ethyl 3-hydroxybutyrate [87]	ab2B [76]
	Or98bP	suz	unknown	ab6B*[76]
Expansions	*Or23a*	suz, tak	Isoamyl acetate [83]	ai2B [76]
	Or67a	suz, bia, tak	Ethyl benzoate, Methyl benzoate [83]	ab10B [76]
	Gr22a-f	tak	bitter compounds [102]	Labellum [103], larvae [104] legs [105]
	Gr36a-c	suz, bia, tak	bitter compounds [102]	Larvae [104], legs [105]
	Gr59ab	suz, bia, tak	bitter compounds [102]	Larvae [104], legs [105]
	Gr59cd	suz, bia, tak	bitter compounds [102]	Larvae n [104], legs [105]
	Gr93a	suz	bitter compounds [102]	unknown
	Gr98b	suz	bitter compounds [102]	unknown
	Gr98d	suz	bitter compounds [102]	Legs [105]
Positive sel.	*Or2a*	suz, bia, tak	Ethyl 3-hydroxybutyrate, Isoamyl acetate [83]	ai3A [76]
	Or9a	tak	2-acetoin, 2,3-butanediol [84]	ab8B [76]
	Gr5a	suz, bia, tak	Trehalose [93, 94]	Labellum [103], legs [105]
	Gr58a	suz, bia	unknown	unknown
	Gr97a	suz, bia, tak	unknown	Larvae [104]

*Not confirmed

about the function of *Or98b*, except for its co-expression with *Or85b* in the A cell of the ab6 sensillum in *D. melanogaster* [76].

Finally, we made numerous attempts to sequence all three lost lineages in the *D. suzukii* genome. Our sequencing of *Or74a* and *Or85a* confirmed the highly degraded state of the loci in the North American isolate [35]. However, these two genes were considerably less degraded in the genome assembly from the Italian isolate, but pseudogenizations were still apparent [36]. Conversely, we were unsuccessful in sequencing the *Or98b* locus in *D. suzukii*. Amplicon size was consistent with that of a full length gene, but sequencing indicated that the locus is polymorphic in the North American assembly. However, we were able to build an intact gene model for *Or98b* in the genome assembly from the Italian assembly [36], and that sequence is provided in Additional file 1, Table S1. Polymorphism in *Or98b* among *D. melanogaster* strains was also reported, wherein several functional and pseudogene alleles were found in the Ives strain, a single pseudogene was found in the New Jersey strain, and no allele could not be amplified in the Oregon R strain [5].

Divergence

Measures of divergence provide insights into the molecular evolution which can often be correlated with conserved and divergent physiological processes. Our measure of divergence (*dN*/*dS)* implies that both chemoreceptor families have evolved under strong purifying selection. However, these values are larger than the genome wide estimates of 0.095 for X chromosome genes and 0.090 for autosomal genes [35], demonstrating that these gene families are more divergent than average. Comparisons between *dN*, *dS*, and *dN/dS* of *Ors* and *Grs* using unpaired tests showed that *Grs* are more divergent than *Ors* in all three species (Table 3).

Among the most conserved Ors, *Orco* tops the list, followed by *Or47a*, *Or92a*, *Or42b* and *Or24a*. These genes are also highly conserved in *D. takahashii* and *D. biarmipes.* Expression studies in *D. melanogaster* have revealed *Orco* to be a non-canonical receptor with a wide distribution [9, 10, 77], whereas expression of the remaining *Ors* is confined to basiconic sensilla [76] except for *Or24a* which is larval specific in *D. melanogaster* [85]. Interestingly *Or92a* and *Or42b* are expressed in ab1 sensilla on the A and B ORNs, respectively. This high level of conservation corresponds with the electrophysiological data of ab1 that showed similar responses to a panel of ab1-sensitive odorants in *D. melanogaster*, *D. biarmipes* and *D. suzukii* [39]. An earlier study showed similar findings comparing nine species in the *melanogaster* subgroup [24]. Combined, these findings suggest that the role of ab1 sensilla has largely been conserved during at least the last ~13 million years of *melanogaster* group evolution. In fact, McBride and Arguello [20] proposed this phenomenon to be applicable for all the large basiconic sensilla (ab1-3) in five members of the *melanogaster* subgroup.

On the other hand, the expression of the most divergent receptors in *D. suzukii* is predicted to be among three different sensilla types. Of these, both *Or19a* and *Or23a* are expressed in intermediate sensilla [71, 82], *Or33a* and *Or69aA* are restricted to a basiconic [71, 82], and *Or65a* is expressed in a trichoid [76]. Potential response characteristic and the significance of these *Ors* in *D. suzukii* remains an exciting avenue to explore. Three of these five homologues in *D. melanogaster* (*Or23a*, *Or65a*, and *Or69aA*) did not respond with high sensitivity to any of the odorants tested heterologously [83]. Physiological data is lacking for *DmelOr33a*. Two different studies reported *DmelOr19a* responding to limonene, a major citrus fruit volatile [83, 88].

Among the gustatory receptors in the *suzukii-takahashii* clade, *Gr21a* was the most conserved, surpassing even *Orco*. The other highly conserved *Grs* include *Gr28bB*, *Gr28a*, *Gr63a* and *Gr64c*. It is worth mentioning that *Gr21a* and *Gr63a* are highly conserved among insects [5, 80], and together confer the sensitivity to carbon dioxide [89, 90], whereas *Gr28bB* and *Gr28a* are part of the bitter receptor family and are shown to be ubiquitously expressed in a wide array of sensory and non-sensory tissue [91, 92]. The five most divergent *Grs* include *Gr10b*, *Gr93d*, *Gr92a*, *Gr85a* and *Gr22c*; little is known about their expression or response characteristics.

Selection

Our set of 11 chemoreceptor lineages with signatures of positive selection in the nine species is smaller than the reported 20 in an earlier study that compared chemosensory repertoires in 12 *Drosophila,* even though two genes (*Or9a* and *Gr5a*) were common in both studies [17]. These differences could be due to multiple reasons. Our study focused on the drosophilids from the *melanogaster* group that have a relatively comparable host range [26], while the other study included six species outside the *melanogaster* group. In addition, we adjusted the *p*-values based on more stringent Holm-Bonferroni corrections which reduced the number of significant candidates. However, we note that our corrections were performed within, but not across gene sets; therefore, these results should be interpreted with caution.

Of the 11 genes, we found four genes (*Or2a*, *Gr5a*, *Gr58a* and *Gr97a*) that were significant in the branches leading to *D. suzukii*. In *D. melanogaster*, *Or2a* is expressed in ai3 sensilla [76, 82] and has been shown to respond to ethyl 3-hydroxybutyrate and isoamyl acetate

eliciting only moderate responses (~50 spikes/s) [83]. It is interesting to note that isoamyl acetate has been identified as a strong ligand from *suzukii*-associated yeasts [33] and host fruits [40]. Among the *Grs*, *DmelGr5* has been studied in detail. Molecular, physiological and behavioral studies identified it as a sugar receptor with a strong selectivity and sensitivity to trehalose [93, 94]. Importance of sugars in *D. suzukii* is more pronounced since this fly also uses a variety of non-conventional sugar sources such as nectar and cherry blossom in the field [95]. Functional data on *DmelGr58a* and *DmelGr97a* is lacking [91]. Our restricted aBSREL analysis of the four largest expanded lineages (*Or23a*, *Or67a*, *Gr59a* and *Gr59d*) in the *suzukii-takahashii* clade revealed evidence for positive selection only in *Or67a*, where three copies showed signatures of positive selection (Fig. 6a). Overall, adaptation of *D. suzukii* to novel niches appears to be facilitated by unique expansions and losses of chemosensory lineages. Together with our earlier that described the volatile chemical landscapes of *D. suzukii* [33], present study further provides novel insights into the synchronous evolution of signaling and reception in flies.

Conclusions

We manually annotated the olfactory and gustatory receptor families of the pest fly, *D. suzukii* to complement our earlier analysis of the evolution of olfactory signals in this fly that showed salience of a set of yeast derived odorants enriched in the *D. suzukii* landscape [33]. We further annotated two close relatives, *D. biarmipes* and *D. takahashii* to compare and contrast their chemosensory repertoire with that of *D. suzukii*. This revealed three unique losses of *Ors* (*Or74a*, *Or85*, *Or98b*) in *D. suzukii* among the three species in the *suzukii-takahashii* clade, and two large expansions in the olfactory receptors, *Or23a* and *Or67a*. There was an overall pattern of purifying selection in both chemoreceptor families, with *Ors* exhibiting greater conservation. The gustatory genome repertoire size in this clade was by far the largest among all the annotated species of the *melanogaster* group. Finally, our analysis for the signature of positive episodic selection in *D. suzukii* led to the identification of *Or2a* and one copy of *Or67a* as strong candidates. Taken together, this study provides detailed insights into the molecular evolution of the two major chemoreceptor families in an invasive and pestiferous fly. The evolution of a serrated ovipositor for piercing the skin of fresh fruits is a unique innovation that conferred a distinct advantage in fruit flies to exploit fruits of varying ripeness. In tephritids, this innovation facilitated the radiation of thousands of species [96]. Surprisingly, this innovation exists in only two known drosophilids, *D. suzukii* and *D. subpulchrella*, both of which are members of the *suzukii* subgroup [31]. The recent sequencing of *D. suzukii* (pest) and *D. biarmipes* (non-pest) within the *suzukii* subgroup provided us with an excellent opportunity to explore the contribution of chemosensation in the evolution of pestilence in *D. suzukii*.

Additional files

Additional file 1: Table S1. *D. suzukii* Or coding and peptide sequences. **Table S2.** *D. suzukii* Gr coding and peptide sequences. **Table S3**. *D. biarmipes* Or coding and peptide sequences. **Table S4.** *D. biarmipes* Gr coding and peptide sequences. **Table S5.** *D. takahashii* Or coding and peptide sequences. **Table S6.** *D. takahashii* Gr coding and peptide sequences. (XLSX 379 kb)

Additional file 2: Table S1. Non-synonymous (*dN*) and synonymous (*dS*) substation rates and ratios (*dN*/*dS*) of *Ors* in members of the *suzukii-takahashii* clade using *D. melanogaster* as an outgroup. **Table S2.** Non-synonymous (*dN*) and synonymous (*dS*) substation rates and ratios (*dN*/*dS*) of *Grs* in members of the *suzukii-takahashii* clade using *D. melanogaster* as an outgroup. **Table S3.** The number of functional *Or* genes in each orthologous group for nine species in the *melanogaster* group based on Almeida et al. [50]. **Table S4.** The number of functional *Gr* genes in each orthologous group for nine species in the *melanogaster* group based on Almeida et al. [50]. **Table S5.** Summary of the chemoreceptors in the *suzukii-takahashii* clade under episodic positive selection as revealed by aBSREL analyses. (XLSX 35 kb)

Additional file 3: Or alignment file (Phylip). Olfactory receptor peptide sequences of *Drosophila suzukii* (Dsuz), *D. biarmipes* (Dbia), *D. takahashii* (Dtak) and *D. melanogaster* (Dmel) ≥ 360 aa in length were multiply aligned using MUSCLE v3.8.31. (PHY 229 kb)

Additional file 4: Or phylogenetic tree file (Nexus). Phylogenetic analysis of Ors in *Drosophila suzukii* (Dsuz), *D. biarmipes* (Dbia), *D. takahashii* (Dtak) and *D. melanogaster* (Dmel) using a Maximum Likelihood method. Evolutionary history was inferred using a Maximum Likelihood method based on the JTT matrix-based model. The tree was constructed using RAxML under the JTT model of substitution with NNI topology search, based on an amino acid alignment by MUSCLE. Branch support was estimated using 500 bootstrap replications. The tree is rooted with *Orco*. (NEX 26 kb)

Additional file 5: Gr alignment file (Phylip). Gustatory receptor peptide sequences of *Drosophila suzukii* (Dsuz), *D. biarmipes* (Dbia), *D. takahashii* (Dtak) and *D. melanogaster* (Dmel) ≥ 340 aa in length were multiply aligned using MUSCLE v3.8.31. (PHY 274 kb)

Additional file 6: Gr phylogenetic tree file (Nexus). Phylogenetic analysis of Grs in *Drosophila suzukii* (Dsuz), *D. biarmipes* (Dbia), *D. takahashii* (Dtak) and *D. melanogaster* (Dmel) using a Maximum Likelihood method. Evolutionary history was inferred using a Maximum Likelihood method based on the JTT matrix-based model. The tree was constructed using RAxML under the JTT model of substitution with NNI topology search, based on an amino acid alignment by MUSCLE. Branch support was estimated using 500 bootstrap replications. The tree is rooted with *Gr21a*. (NEX 31 kb)

Additional file 7: Figure S1. Evolutionary history of duplications and losses in four expanded lineages in the chemoreceptor families based on the parsimony-based method of gene tree reconciliation in NOTUNG v2.8.1.6 [51]. (TIF 2581 kb)

Abbreviations

aBSREL, adaptive branch-site random effects likelihood; *Gr*, gustatory receptor; LRT, likelihood ratio tests; *Or*, odorant receptor; *Orco*, olfactory receptor co-receptor; ORN, olfactory receptor neuron; SEM, scanning electron microscopy; VOC, volatile organic compounds

Acknowledgements
The authors thank Frank Zalom and Johanna Chiu (both at the University of California, Davis), Michael Prender, Yiyuan Li and RJ Nowling (all at the University of Notre Dame) for advice and helpful discussions. We gratefully acknowledge Hugh Robertson (The University of Illinois at Urbana–Champaign) for much discussions, advice, and exchange of ideas. We also thank Julien Pelletier (Keele University, UK), Johanna Chiu (UC-Davis), and Carolyn (Lindy) McBride (Princeton University) for constructive comments on an earlier version of this manuscript.

Funding
This work was supported by grants to ZS from the California Cherry Marketing and Research Board grant (G00001951), UC Davis-Notre Dame Research Agreement award (201224810), and USDA National Institute for Food and Agriculture (NIFA) Specialty Crops Research Initiative (SCRI) Award (2015-51181-24252) as a subcontract between the University of Notre Dame and North Carolina State University.

Availability of data and material
All relevant data are available within the manuscript and its additional files (Additional file 1 has Table S1 through S6 that contain all the annotated *Or* and *Gr* coding and peptide sequences for *D. suzukii*, *D. biarmipes* and *D. takahashii*. Additional file 3, 4, 5 and 6 are aligned peptide sequence and phylogeny tree files for *Or* and *Grs,* respectively. The phylogenetic data and the support files have been deposited at the: http://datadryad.org/review?doi=doi:10.5061/dryad.5q1h4view?doi=doi:10.5061/dryad.5q1h4.

Authors' contributions
PVH carried out the gene annotation and molecular evolution analysis and drafted the manuscript. CLR participated in the phylogenetic and molecular evolution analysis. CMJ and GJR assisted in PCR amplifications and sequencing. MS participated in early annotations of *D. suzukii* receptors. ZS had the research idea, and participated in developing the study design and drafting the manuscript along with PVH. All authors read and approved the final manuscript.

Competing interests
The authors declare that they have no competing interests.

Consent for publication
Not applicable.

Ethics approval and consent to participate
Not applicable.

Received: 28 May 2016 Accepted: 29 July 2016
Published online: 17 August 2016

References

1. Benton R, Vannice KS, Gomez-Diaz C, Vosshall LB. Variant ionotropic glutamate receptors as chemosensory receptors in Drosophila. Cell. 2009; 136(1):149–62.
2. Rytz R, Croset V, Benton R. Ionotropic receptors (IRs): chemosensory ionotropic glutamate receptors in Drosophila and beyond. Insect Biochem Mol Biol. 2013;43(9):888–97.
3. Hansson BS, Stensmyr MC. Evolution of insect olfaction. Neuron. 2011;72(5): 698–711.
4. Joseph RM, Carlson JR. Drosophila Chemoreceptors: A Molecular Interface Between the Chemical World and the Brain. Trends Genet. 2015;31(12): 683–95.
5. Robertson HM, Warr CG, Carlson JR. Molecular evolution of the insect chemoreceptor gene superfamily in Drosophila melanogaster. Proc Natl Acad Sci U S A. 2003;100:14537–42.
6. Stocker RF. The organization of the chemosensory system in *Drosophila melanogaster* - a review. Cell Tissue Res. 1994;275(1):3–26.
7. Ache BW, Young JM. Olfaction: diverse species, conserved principles. Neuron. 2005;48(3):417–30.
8. Hildebrand JG, Shepherd GM. Mechanisms of olfactory discrimination: converging evidence for common principles across phyla. Ann Rev Neurosci. 1997;20:595–631.
9. Benton R, Sachse S, Michnick SW, Vosshall LB. Atypical membrane topology and heteromeric function of Drosophila odorant receptors in vivo. Plos Biol. 2006;4(2):240–57.
10. Larsson MC, Domingos AI, Jones WD, Chiappe ME, Amrein H, Vosshall LB. Or83b encodes a broadly expressed odorant receptor essential for Drosophila olfaction. Neuron. 2004;43(5):703–14.
11. Zhang H-J, Anderson AR, Trowell SC, Luo AR, Xiang Z-H, Xia Q-Y. Topological and Functional Characterization of an Insect Gustatory Receptor. PLoS One. 2011;6(8), e24111.
12. Macharia R, Mireji P, Murungi E, Murilla G, Christoffels A, Aksoy S, Masiga D. Genome-Wide Comparative Analysis of Chemosensory Gene Families in Five Tsetse Fly Species. PLoS Negl Trop Dis. 2016;10(2), e0004421.
13. Obiero GF, Mireji PO, Nyanjom SR, Christoffels A, Robertson HM, Masiga DK. Odorant and gustatory receptors in the tsetse fly Glossina morsitans morsitans. PLoS Negl Trop Dis. 2014;8(4), e2663.
14. Engsontia P, Sanderson AP, Cobb M, Walden KKO, Robertson HM, Brown S. The red flour beetle's large nose: An expanded odorant receptor gene family in Tribolium castaneum. Insect Biochem Mol Biol. 2008;38(4):387–97.
15. Robertson HM, Wanner KW. The chemoreceptor superfamily in the honey bee, Apis mellifera: Expansion of the odorant, but not gustatory, receptor family. Genome Res. 2006;16(11):1395–403.
16. Zhou X, Rokas A, Berger SL, Liebig J, Ray A, Zwiebel LJ. Chemoreceptor Evolution in Hymenoptera and Its Implications for the Evolution of Eusociality. Genome Biol Evol. 2015;7(8):2407–16.
17. Gardiner A, Barker D, Butlin RK, Jordan WC, Ritchie MG. Drosophila chemoreceptor gene evolution: selection, specialization and genome size. Mol Ecol. 2008;17(7):1648–57.
18. Goldman-Huertas B, Mitchell RF, Lapoint RT, Faucher CP, Hildebrand JG, Whiteman NK. Evolution of herbivory in Drosophilidae linked to loss of behaviors, antennal responses, odorant receptors, and ancestral diet. Proc Natl Acad Sci U S A. 2015;112(10):3026–31.
19. McBride CS. Rapid evolution of smell and taste receptor genes during host specialization in Drosophila sechellia. Proc Natl Acad Sci U S A. 2007;104(12): 4996–5001.
20. McBride CS, Arguello JR. Five drosophila genomes reveal nonneutral evolution and the signature of host specialization in the chemoreceptor superfamily. Genetics. 2007;177(3):1395–416.
21. Nozawa M, Nei M. Evolutionary dynamics of olfactory receptor genes in Drosophila species. Proc Natl Acad Sci U S A. 2007;104(17):7122–7.
22. Dekker T, Ibba I, Siju KP, Stensmyr MC, Hansson BS. Olfactory shifts parallel superspecialism for toxic fruit in *Drosophila melanogaster* sibling, *D. sechellia*. Curr Biol. 2006;16(1):101–9.
23. Linz J, Baschwitz A, Strutz A, Dweck HKM, Sachse S, Hansson BS, Stensmyr MC. Host plant-driven sensory specialization in *Drosophila erecta*. P Roy Soc B-Biol Sci. 2013;280(1760):20130626.
24. Stensmyr MC, Dekker T, Hansson BS. Evolution of the olfactory code in the Drosophila melanogaster subgroup. P Roy Soc B-Biol Sci. 2003;270(1531): 2333–40.
25. Syed Z. Chemical ecology and olfaction in arthropod vectors of diseases. Curr Opin Insect Sci. 2015;10:83–9.
26. Markow TA. The secret lives of Drosophila flies. eLife. 2015;4.
27. Vosshall LB. Into the mind of a fly. Nature. 2007;450(7167):193–7.
28. Rota-Stabelli O, Blaxter M, Anfora G. Drosophila suzukii. Curr Biol. 2013;23(1): R8–9.
29. Walsh DB, Bolda MP, Goodhue RE, Dreves AJ, Lee JC, Bruck DJ, Walton VM, O'Neal SD, Zalom FG. *Drosophila suzukii* (Diptera: Drosophilidae): invasive pest of ripening soft fruit expanding its geographic range and damage potential. J Integr Pest Manag. 2011;2(1):G1–7.
30. Markow TA, O'Grady PM. Drosophila: a guide to species identification and use. London: Academic; 2006.
31. Atallah J, Teixeira L, Salazar R, Zaragoza G, Kopp A. The making of a pest: the evolution of a fruit-penetrating ovipositor in *Drosophila suzukii* and related species. P Roy Soc B-Biol Sci. 2014;281(1781):20132840.
32. Cini A, Ioriatti C, Anfora G. A review of the invasion of Drosophila suzukii in Europe and a draft research agenda for integrated pest management. Bull Insectology. 2012;65(1):149–60.
33. Scheidler NH, Liu C, Hamby KA, Zalom FG, Syed Z. Volatile codes: Correlation of olfactory signals and reception in Drosophila-yeast chemical communication. Sci Rep. 2015;5:14059.

34. Hamby KA, Becher PG. Current knowledge of interactions between Drosophila suzukii and microbes, and their potential utility for pest management. J Pest Sci. 2016;1–10.
35. Chiu JC, Jiang X, Zhao L, Hamm CA, Cridland JM, Saelao P, Hamby KA, Lee EK, Kwok RS, Zhang G, et al. Genome of Drosophila suzukii, the Spotted Wing Drosophila. G3. 2013;3(12):2257–71.
36. Ometto L, Cestaro A, Ramasamy S, Grassi A, Revadi S, Siozios S, Moretto M, Fontana P, Varotto C, Pisani D, et al. Linking Genomics and Ecology to Investigate the Complex Evolution of an Invasive Drosophila Pest. Genome Biol Evol. 2013;5(4):745–57.
37. Guo S, Kim J. Molecular evolution of Drosophila odorant receptor genes. Mol Biol Evol. 2007;24(5):1198–207.
38. Dekker T, Revadi S, Mansourian S, Ramasamy S, Lebreton S, Becher PG, Angeli S, Rota-Stabelli O, Anfora G. Loss of Drosophila pheromone reverses its role in sexual communication in Drosophila suzukii. Proc R Soc Lond B Biol Sci. 2015;282(1804):20143018.
39. Keesey IW, Knaden M, Hansson BS. Olfactory Specialization in Drosophila suzukii Supports an Ecological Shift in Host Preference from Rotten to Fresh Fruit. J Chem Ecol. 2015;41(2):121–8.
40. Revadi S, Vitagliano S, Stacconi MVR, Ramasamy S, Mansourian S, Carlin S, Vrhovsek U, Becher PG, Mazzoni V, Rota-Stabelli O, et al. Olfactory responses of Drosophila suzukii females to host plant volatiles. Physiol Entomol. 2015; 40(1):54–64.
41. dos Santos G, Schroeder AJ, Goodman JL, Strelets VB, Crosby MA, Thurmond J, Emmert DB, Gelbart WM, FlyBase C. FlyBase: introduction of the Drosophila melanogaster Release 6 reference genome assembly and large-scale migration of genome annotations. Nucleic Acids Res. 2015; 43(Database issue):D690–697.
42. Corpet F. Multiple sequence alignment with hierarchical-clustering. Nucleic Acids Res. 1988;16(22):10881–90.
43. Reese MG, Eeckman FH, Kulp D, Haussler D. Improved splice site detection in Genie. J Comput Biol. 1997;4(3):311–23.
44. Stothard P. The sequence manipulation suite: JavaScript programs for analyzing and formatting protein and DNA sequences. BioTechniques. 2000;28(6):1102. 1104.
45. Gasteiger E, Gattiker A, Hoogland C, Ivanyi I, Appel RD, Bairoch A. ExPASy: The proteomics server for in-depth protein knowledge and analysis. Nucleic Acids Res. 2003;31(13):3784–8.
46. Doyle JJ. A rapid DNA isolation procedure for small quantities of fresh leaf tissue. Phytochem Bull. 1987;19:11–5.
47. Untergasser A, Cutcutache I, Koressaar T, Ye J, Faircloth BC, Remm M, Rozen SG. Primer3-new capabilities and interfaces. Nucleic Acids Res. 2012;40(15), e115.
48. Drosophila Odorant Receptor Nomenclature Committee. A unified nomenclature system for the Drosophila odorant receptors. Cell. 2000; 102(2):145–46.
49. Viklund H, Elofsson A. OCTOPUS: improving topology prediction by two-track ANN-based preference scores and an extended topological grammar. Bioinformatics (Oxford, England). 2008;24(15):1662–8.
50. Almeida FC, Sanchez-Gracia A, Luis Campos J, Rozas J. Family Size Evolution in Drosophila Chemosensory Gene Families: A Comparative Analysis with a Critical Appraisal of Methods. Genome Biol Evol. 2014;6(7):1669–82.
51. Stolzer M, Lai H, Xu M, Sathaye D, Vernot B, Durand D. Inferring duplications, losses, transfers and incomplete lineage sorting with nonbinary species trees. Bioinformatics (Oxford, England). 2012;28(18):I409–15.
52. Smith MD, Wertheim JO, Weaver S, Murrell B, Scheffler K, Kosakovsky Pond SL. Less is more: an adaptive branch-site random effects model for efficient detection of episodic diversifying selection. Mol Biol Evol. 2015;32(5):1342–53.
53. Venables WN, Ripley BD. Modern Applied Statistics with S. 4th ed. New York: Springer; 2002.
54. Sievers F, Wilm A, Dineen D, Gibson TJ, Karplus K, Li W, Lopez R, McWilliam H, Remmert M, Soeding J, et al. Fast, scalable generation of high-quality protein multiple sequence alignments using Clustal Omega. Mol Syst Biol. 2011;7.
55. McWilliam H, Li W, Uludag M, Squizzato S, Park YM, Buso N, Cowley AP, Lopez R. Analysis Tool Web Services from the EMBL-EBI. Nucleic Acids Res. 2013;41(W1):W597–600.
56. Nei M, Gojobori T. Simple methods for estimating the numbers of synonymous and nonsynonymous nucleotide substitutions. Mol Biol Evol. 1986;3(5):418–26.
57. Korber B. HIV Signature and Sequence Variation Analysis. In: Rodrigo AG, Learn GH, editors. Computational Analysis of HIV Molecular Sequences. Dordrecht: Kluwer Academic Publishers; 2000. p. 55–72.
58. Katoh K, Standley DM. MAFFT multiple sequence alignment software version 7: improvements in performance and usability. Mol Biol Evol. 2013; 30(4):772–80.
59. Jones DT, Taylor WR, Thornton JM. The rapid generation of mutation data matrices from protein sequences. Comput Appl Biosci. 1992;8(3): 275–82.
60. Tamura K, Stecher G, Peterson D, Filipski A, Kumar S. MEGA6: Molecular Evolutionary Genetics Analysis version 6.0. Mol Biol Evol. 2013;30(12):2725–9.
61. Suyama M, Torrents D, Bork P. PAL2NAL: robust conversion of protein sequence alignments into the corresponding codon alignments. Nucleic Acids Res. 2006;34(Web Server issue):W609–612.
62. Kosakovsky Pond SL, Posada D, Gravenor MB, Woelk CH, Frost SD. GARD: a genetic algorithm for recombination detection. Bioinformatics (Oxford, England). 2006;22(24):3096–8.
63. Holm S. A simple sequentially rejective multiple test procedure. Scand J Stat. 1979;6(2):65–70.
64. Benjamini Y, Hochberg Y. Controlling the false discovery rate - a practical and powerful approach to multiple testing. J R Stat Soc Ser B Methodol. 1995;57(1):289–300.
65. Yang Z. PAML: a program package for phylogenetic analysis by maximum likelihood. Comput Appl Biosci. 1997;13(5):555–6.
66. Yang Z, Wong WS, Nielsen R. Bayes empirical Bayes inference of amino acid sites under positive selection. Mol Biol Evol. 2005;22(4):1107–18.
67. Edgar RC. MUSCLE: multiple sequence alignment with high accuracy and high throughput. Nucleic Acids Res. 2004;32(5):1792–7.
68. Stamatakis A. RAxML version 8: a tool for phylogenetic analysis and post-analysis of large phylogenies. Bioinformatics. 2014;30(9):1312–13.
69. Miller MA, Pfeiffer W, Schwartz T. The CIPRES science gateway: enabling high-impact science for phylogenetics researchers with limited resources. Proceedings of the 1st Conference of the Extreme Science and Engineering Discovery Environment: Bridging from the eXtreme to the campus and beyond. Chicago: ACM; 2012. p. 1-8.
70. Rambaut A. FigTree, a graphical viewer of phylogenetic trees. 2007. http://tree.bio.ed.ac.uk/software/figtree.
71. Shanbhag SR, Muller B, Steinbrecht RA. Atlas of olfactory organs of Drosophila melanogaster - 1. Types, external organization, innervation and distribution of olfactory sensilla. Int J Insect Morphol Embryol. 1999;28(4): 377–97.
72. Steinbrecht RA. Structure and function of insect olfactory sensilla. CIBA Found Symp. 1996;200:158–74. discussion 174–157.
73. Carlson JR. Olfaction in Drosophila: From odor to behavior. Trends Genet. 1996;12(5):175–80.
74. de Bruyne M, Clyne PJ, Carlson JR. Odor coding in a model olfactory organ: the Drosophila maxillary palp. J Neurosci. 1999;19(11):4520–32.
75. de Bruyne M, Foster K, Carlson JR. Odor coding in the Drosophila antenna. Neuron. 2001;30(2):537–52.
76. Couto A, Alenius M, Dickson BJ. Molecular, anatomical, and functional organization of the Drosophila olfactory system. Curr Biol. 2005;15(17):1535–47.
77. Vosshall LB, Amrein H, Morozov PS, Rzhetsky A, Axel R. A spatial map of olfactory receptor expression in the Drosophila antenna. Cell. 1999;96(5): 725–36.
78. Nei M, Niimura Y, Nozawa M. The evolution of animal chemosensory receptor gene repertoires: roles of chance and necessity. Nat Rev Genet. 2008;9(12):951–63.
79. Markow TA, O'Grady PM. Evolutionary genetics of reproductive behavior in Drosophila: connecting the dots. Annu Rev Genet. 2005;39:263–91.
80. Robertson HM, Kent LB. Evolution of the gene lineage encoding the carbon dioxide receptor in insects. J Insect Sci (Online). 2009;9:19.
81. Whiteman NK, Pierce NE. Delicious poison: genetics of Drosophila host plant preference. Trends Ecol Evol. 2008;23(9):473–8.
82. Lin CC, Potter CJ. Re-Classification of Drosophila melanogaster Trichoid and Intermediate Sensilla Using Fluorescence-Guided Single Sensillum Recording. PLoS One. 2015;10(10), e0139675.
83. Hallem EA, Carlson JR. Coding of odors by a receptor repertoire. Cell. 2006; 125(1):143–60.
84. Hallem EA, Ho MG, Carlson JR. The molecular basis of odor coding in the drosophila antenna. Cell. 2004;117(7):965–79.

85. Fishilevich E, Domingos AI, Asahina K, Naef F, Vosshall LB, Louis M. Chemotaxis behavior mediated by single larval olfactory neurons in Drosophila. Curr Biol. 2005;15(23):2086–96.
86. Kreher SA, Kwon JY, Carlson JR. The molecular basis of odor coding in the Drosophila larva. Neuron. 2005;46(3):445–56.
87. Stensmyr MC, Giordano E, Balloi A, Angioy AM, Hansson BS. Novel natural ligands for Drosophila olfactory receptor neurones. J Exp Biol. 2003; 206(Pt 4):715–24.
88. Dweck HK, Ebrahim SA, Farhan A, Hansson BS, Stensmyr MC. Olfactory proxy detection of dietary antioxidants in Drosophila. Curr Biol. 2015;25(4): 455–66.
89. Jones WD, Cayirlioglu P, Kadow IG, Vosshall LB. Two chemosensory receptors together mediate carbon dioxide detection in Drosophila. Nature. 2007;445(7123):86–90.
90. Kwon JY, Dahanukar A, Weiss LA, Carlson JR. The molecular basis of CO2 reception in Drosophila. Proc Natl Acad Sci U S A. 2007;104(9):3574–8.
91. Freeman EG, Dahanukar A. Molecular neurobiology of Drosophila taste. Curr Opin Neurobiol. 2015;34:140–8.
92. Thorne N, Amrein H. Atypical expression of Drosophila gustatory receptor genes in sensory and central neurons. J Comp Neurol. 2008;506(4):548–68.
93. Chyb S, Dahanukar A, Wickens A, Carlson JR. Drosophila Gr5a encodes a taste receptor tuned to trehalose. Proc Natl Acad Sci U S A. 2003;100 Suppl 2:14526–30.
94. Dahanukar A, Foster K, van Naters W, Carlson JR. A Gr receptor is required for response to the sugar trehalose in taste neurons of Drosophila. Nat Neurosci. 2001;4(12):1182–6.
95. Tochen S, Walton V, Lee J. Impact of floral feeding on adult Drosophila suzukii survival and nutrient status. J Pest Sci. 2016.
96. Díaz-Fleischer F, Papaj DR, Prokopy RJ, Norrbom AL, Aluja M. Evolution of Fruit Fly Oviposition Behavior. In: Fruit Flies (Tephritidae): Phylogeny and Evolution of Behavior. Aluja M, Norrbom AL, editors. CRC Press; 2001: 811–849.
97. Clyne PJ, Warr CG, Carlson JR. Candidate taste receptors in Drosophila. Science. 2000;287(5459):1830–4.
98. Clyne PJ, Warr CG, Freeman MR, Lessing D, Kim JH, Carlson JR. A novel family of divergent seven-transmembrane proteins: Candidate odorant receptors in Drosophila. Neuron. 1999;22(2):327–38.
99. Dunipace L, Meister S, McNealy C, Amrein H. Spatially restricted expression of candidate taste receptors in the Drosophila gustatory system. Curr Biol. 2001;11(11):822–35.
100. Goldman AL, Van der Goes van Naters W, Lessing D, Warr CG, Carlson JR. Coexpression of two functional odor receptors in one neuron. Neuron. 2005;45(5):661–6.
101. Mathew D, Martelli C, Kelley-Swift E, Brusalis C, Gershow M, Samuel AD, Emonet T, Carlson JR. Functional diversity among sensory receptors in a Drosophila olfactory circuit. Proc Natl Acad Sci U S A. 2013;110(23): E2134–2143.
102. Weiss LA, Dahanukar A, Kwon JY, Banerjee D, Carlson JR. The Molecular and Cellular Basis of Bitter Taste in Drosophila. Neuron. 2011;69(2):258–72.
103. Thorne N, Chromey C, Bray S, Amrein H. Taste perception and coding in Drosophila. Curr Biol. 2004;14(12):1065–79.
104. Kwon JY, Dahanukar A, Weiss LA, Carlson JR. Molecular and Cellular Organization of the Taste System in the Drosophila Larva. J Neurosci. 2011;31(43):15300–9.
105. Ling F, Dahanukar A, Weiss LA, Kwon JY, Carlson JR. The Molecular and Cellular Basis of Taste Coding in the Legs of Drosophila. J Neurosci. 2014; 34(21):7148–64.

Submit your next manuscript to BioMed Central and we will help you at every step:

- We accept pre-submission inquiries
- Our selector tool helps you to find the most relevant journal
- We provide round the clock customer support
- Convenient online submission
- Thorough peer review
- Inclusion in PubMed and all major indexing services
- Maximum visibility for your research

Submit your manuscript at
www.biomedcentral.com/submit

Annual Report - 2016

Prepared for the California Cherry Advisory Board

Project Title:	Management and Epidemiology of Pre- and Postharvest Foliar and Fruit Diseases of Sweet Cherry
Project Leader:	Dr. James E. Adaskaveg, Department of Plant Pathology, University of California, Riverside, CA 92521 (951) 827-7577
Cooperators:	Dr. H. Förster, D. Thompson, L. Wade, and J. Grant (Farm Advisor)

SUMMARY

Our research emphasized blossom, preharvest, and postharvest treatments for the management of major foliar and fruit diseases of sweet cherry in California. We continued our work on bacterial canker, powdery mildew, blossom blights and fruit rots caused by *Monilinia* and *Botrytis* spp., as well as postharvest decays including brown rot, gray mold, and Rhizopus rot.

1) Studies on bacterial canker caused by *Pseudomonas syringae* pv. *syringae*:

   a. Susceptibility of injuries to infection by the pathogen was evaluated over time and was found to decrease over time. In 2015 studies, canker size was greatly reduced on 8-day-old wounds made in mid- to late January. When wounds were made in mid-February in this year's study, susceptibility still decreased over time, however, wounds were susceptible for at least 16 weeks. Thus, wound healing proceeds differently among physiological host stages and environmental conditions in each year.

   b. The incidence of blossom blast was very low in the spring of 2016. Kasugamycin is currently under review for full registration on cherry. Oxytetracycline is currently in the IR-4 program for registration on cherry with support from the registrants, the California Cherry Board, and other researchers in the North Central and North Eastern regions of the US.

2) In powdery mildew studies, the disease developed at high incidence on leaves of water sprouts and then on new shoots on terminal branches. The incidence of fruit infections was low in 2016.

   a. In a trial in San Joaquin Co., the most effective treatments included the SDHI (FG 7)-containing fungicides Fontelis, Luna Sensation, Luna Experience, and Merivon, selected DMI (FG 3)-containing fungicides such as Rhyme, Procure, and Quadris Top, as well as the experimental fungicides UC-1, UC-2B, EXP-A, -AD, and -AF. Quintec continued to show reduced performance, but selected integrated programs with Quintec in mixture or rotation with other FRAC Groups were effective.

3) For brown rot and gray mold blossom blight, highly effective fungicides with excellent pre- and post-infection activity included FG 3 (e.g. Quash) and the pre-mixture treatments FG 7/11 (e.g., Merivon) and FG 3/11 (e.g., Quadris Top), as well as the experimentals R-106506, UC-1, UC-2B, and IL54111. The experimental EXP-A was very effective against brown rot, but was not very effective against Botrytis blossom blight at the rate tested. The biocontrol Serenade Opti was moderately effective against brown rot, and also showed some efficacy against Botrytis blossom blight.

4) Two field studies were conducted on the efficacy of preharvest fungicide treatments.

   a. Brown rot: In applications at 6- or 7-days PHI, the pre-mixture Quadris Top and the experimental compounds UC-1, UC-2B, UC-AD provided excellent protection in wound-inoculations of non-washed fruit. Efficacy was generally reduced on washed fruit. When harvested fruit were non-wound drop-inoculated, all treatments evaluated, including the bio-fungicide pimaricin, were highly effective on non-washed and washed fruit.

   b. Gray mold: A mixture of Elevate and Procure and the experimentals UC-1, EXP-AD, and EXP-AF were most effective in inoculation studies. Natural incidence of gray mold of non-washed fruit was

most effectively reduced by Ph-D + Elevate, Procure + Elevate, Luna Experience, R106506, UC-2B, and EXP-AF. Thus, these studies possibly identified new effective gray mold treatments.

5) In studies on the evaluation of postharvest fungicides, we focused on the newly registered bio-fungicide BioSpectra (pimaricin, natamycin, marketed as Zivion on other crops). As in 2015, this treatment showed consistent high efficacy in reducing brown rot, gray mold, and Rhizopus rot when using rates as low as 250 ppm in spray or drench applications. Pimaricin also demonstrated very high efficacy in a study on a commercial packingline. With increasing emphasis on food safety and consumer concerns, this treatment like with 'exempt from tolerance status' will become important in the future.

INTRODUCTION

Management of bacterial blast and canker. The main bacterial pathogen that causes blossom blast and cankers of woody tissue of sweet cherry and other stone fruit crops is *Pseudomonas syringae* pv. *syringae*, but other pathovars have been also associated with the disease namely *Pseudomonas syringae* pv. *morsprunorum*. Blossom blast develops after cold injury, and with subsequent infection, blossoms become dark to black in color, wilt, and die. The disease is more commonly found on early-blooming varieties or trees treated with rest-breaking treatments that experience cooler, wet environments in the spring. The disease can also occur on leaf and flower buds where it causes bud death; and on leaves and fruit where it causes spots and specks.

Based on our efforts, recent advances have been made in bacterial disease control with the identification and development of the antibiotic kasugamycin (commercial name: Kasumin) for fire blight management on pome fruit and other bacterial diseases of agronomic crops in the United States and elsewhere. This antibiotic is not used in animal or human medicine and the US-EPA registration for pome fruit was granted in 2014. Registration of Kasumin on sweet cherry in California is pending CDPR approval. Kasumin has high activity against *Erwinia* and *Pseudomonas* species and moderate activity against *Xanthomonas* species and other plant pathogenic bacteria. In our studies using Kasumin for managing bacterial blast of sweet cherry, we were able to reduce the disease in inoculation studies. The natural incidence of disease was also significantly reduced after commercial applications with Kasumin. Furthermore, using an increased rate of 200 ppm, kasugamycin was the only compound that consistently reduced the severity of bacterial canker of inoculated branches.

Our screening of compounds led to the identification of several other materials that look quite promising. We are also pursuing registration of oxytetracycline on cherry in California as dormant and bloom treatments. Oxytetracycline (Fireline, Mycoshield) was successfully accepted into the IR-4 program in Sept 2013 for residue trials on bacterial blast of cherry. Other compounds included the biocontrol Actinovate (fermentation product of *Streptomyces lydicus*) and Blossom Protect/Botector (*Aureobasidium pullulans*) that inconsistently reduced both the blossom and canker phase of the disease. In 2016, we focused on the temporal susceptibility of lateral and terminal injuries of cherry branches to infection by *P. syringae*.

Management of powdery mildew, blossom blight, and fruit rot. Powdery mildew of sweet cherry is an ongoing problem for growers in California especially in southern production areas. Leaves and fruit may be infected. In some export markets, powdery mildew is a quarantine disease and fruit for shipment may have to be certified as disease-free. With decreased powdery mildew sensitivity to Quintec, new, highly effective materials are being evaluated. Alternative fungicides that we evaluated over several years in our field trials on sweet cherry in California include the FG 3 (DMI) Procure (triflumizole), the FG 7 (SDHI) fungicides (e.g., fluopyram, fluxapyroxad, and penthiopyrad), and the pre-mixtures Luna Sensation (fluopyram/trifloxystrobin), Merivon (fluxapyroxad/pyraclostrobin) (FG 7/11), and Quadris Top (azoxystrobin/difenoconazole) (FG 3/11). Still, other new powdery mildew fungicides such as metrafenone (FG U8; Vivando), polyoxin-D, UC-1, UC-2B and Syngenta's new FTH 545 (e.g., EXP A, -AD, and -AF) (in the IR-4 residue program as of Sept. 2015) are being developed, and we are seeking their registration on

cherry in California. This will allow alternatives to DMI, QoI, and SDHI fungicides used during bloom, petal fall, and preharvest.

For management of brown rot blossom blight and fruit rot of sweet cherry caused by *Monilinia fructicola* and *M. laxa* and Botrytis blossom blight and fruit rot caused by *Botrytis cinerea,* compounds of different modes of action (QoIs, DMIs, anilinopyrimidines, phenylpyrroles, hydroxyanilides, and SDHIs) have been evaluated by us over the years and were found to be effective. The pre-mixtures Quilt Xcel, Quadris Top, Pristine, Merivon, and Luna Sensation represent some of the top treatments along with tank mixtures of fenhexamid and DMI fungicides. Still, more new fungicides are being developed. Thus, we continued to evaluate the efficacy, spectrum of activity, and persistence of residues of the new classes of fungicides, as well as the integration of these materials into a comprehensive management program. Information on the preventative and post-infection activity of fungicides is helping to develop our delayed bloom fungicide application model for improved timing in low to moderate disease pressure years and for optimizing fungicide treatments. Although DMI fungicides are highly effective against brown rot, they have to be complemented with other materials to obtain a high efficacy against gray mold.

Management of postharvest fruit decay with postharvest treatments. We are also continuing our efforts to provide effective and economical treatments for management of postharvest fruit decays such as brown rot, gray mold, Rhizopus rot, as well as powdery mildew lesions from field infections. Powdery mildew on fruit is a quarantine disease with selected trade partners and moreover, powdery mildew infections can be entryways for secondary infections by other fruit pathogens. Currently, five postharvest fungicides, Tebucon (the Elite replacement- Note: Tebucon label has changed to a maximum rate of 8 oz), Mentor (propiconazole), Scholar (fludioxonil), and Penbotec (pyrimethanil) are registered on sweet cherry. In 2016, Judge (fenhexamid) was withdrawn from postharvest use, however, the new postharvest fungicide BioSpectra SC (natamycin) was registered in California. This is the first biofungicide and first postharvest fungicide that is exempt from tolerance on fruit crops in the United States. Penbotec is effective against brown rot and gray mold, whereas Scholar and BioSpectra are also active against Rhizopus rot. The DMI propiconazole (Mentor) is mainly effective against brown rot, but also against sour rot, a less common decay on sweet cherry. The Scholar-Mentor combination has the broadest spectrum of activity with controlling four decays. Of the four classes or FRAC groups (e.g., 3, 9, 12, and U) registered, Tebucon and Mentor (FG 3) are not ‘reduced-risk’ fungicides. Scholar in 2011 and Penbotec in 2013 received Food Additive Tolerances in Japan, and the registrants of Mentor and BioSpectra have applied for FATs in Japan. Thus, continued studies on how to use Scholar, Tebucon, Penbotec, Mentor, and BioSpectra most efficiently for the Japanese export market are critical to the industry.

OBJECTIVES

1. Study the epidemiology of bacterial canker and evaluate new products against bacterial blast in flower inoculation studies and against canker in twig inoculation studies.
2. Evaluate, under field conditions, bloom and preharvest applications of new compounds (e.g., Fontelis), pre-mixtures (e.g., Luna Sensation, Merivon, Quadris Top), EXP-A, -AD, -and -AF, as well as UC-1 and UC-2B, R106506, polyoxin-D, and biologicals for control of brown rot and Botrytis blossom blight, powdery mildew, and pre- and postharvest brown rot and gray mold fruit decays.
3. Evaluate new fungicides as postharvest treatments including the newly registered BioSpectra and develop cost-effective application methods.

MATERIALS AND METHODS

Evaluation of the susceptibility of branch injuries over time to infection by the bacterial canker pathogen. In mid-February 2016, branches of cv. Coral cherry were puncture-wounded laterally (3 wounds per branch) using a nail and cut at the end. There six branch replications per timing. Wounds

Table 1: Fungicides, bactericides, and biologicals used in 2016 studies*.

Pesticide	FRAC group	Trade name	Active ingredient
Fungicides	***Single active ingredients***		
	3	Indar	fenfuconazole
	3	Procure	triflumizole
	3	Quash	metconazole
	3	Rally	myclobutanil
	3	Rhyme	flutriafol
	7	Fontelis	penthiopyrad
	7	Kenja	isofetamid
	11	Intuity	mandestrobin
	12	Scholar	fludioxonil
	13	Quintec	quinoxyfen
	17	Elevate	fenhexamid
	19	Ph-D, Oso/Tavano	polyoxin-D
	U	Zivion, Delvocid, Biospectra	pimaricin/natamycin
	Experimentals	EXP-A	not disclosed
		EXP-AD	not disclosed
		EXP-AF	not disclosed
		R-106506	not disclosed
		UC-1	not disclosed
		UC-2B	not disclosed
		IL-54111	not disclosed
	Double (Premixtures)		
	7 + 11	Luna Sensation	fluopyram + trifloxystrobin
	7 + 3	Luna Experience	fluopyram + tebuconazole
	7 + 11	Merivon	fluxapyroxad + pyraclostobin
	3 + 11	Quadris Top	difenoconazole + azoxystrobin
Biologicals	Bacterium	Serenade Opti	*Bacillus subtilis* QST713
	Plant extract	Fracture	protein from *Lupinus* sp.

* - Alphabetical by trade name for each Fungicide Resistance Action Committee (FRAC) group or mode of action. Some fungicides were used with adjuvants such as Breakthru or Dyne-Amic.

were spray-inoculated with *P. syringae* pv. *syringae* (approximately 2 x 10^7 cfu/ml) after selected time periods. Branches were sampled in mid-April 2016 and evaluated for gumming, and canker length was measured. Data were analyzed using analysis of variance and mean separation procedures of SAS 9.4.

Evaluation of new fungicides for control of powdery mildew of sweet cherry. A field trial in San Joaquin Co. was conducted to evaluate fungicides for powdery mildew control. Treatments were done on 3-8-16 for protection from primary inoculum (ascospores from overwintering chasmothecia), and were followed by two additional treatments on 3-30 and 4-21-16 (early fruit development) for protection from secondary infection from conidia. Single fungicides, pre-mixtures, and two rotation programs were evaluated (Fig. 3). The incidence of powdery mildew was evaluated on 20 leaves from four random shoots each from inside the tree or from the outer tree perimeter for each of the four single-tree replications on May 25, 2016. Severity was rated using the following scale: 0 = healthy, 1 = 1-3 lesions, 2 = <25%, 3 = up to 50%,

4 = >50% of leaf area affected. Disease intensity was calculated as the multiplication product of disease incidence and severity. Data were analyzed using analysis of variance and mean separation procedures of SAS 9.4.

Evaluation of new fungicides for control of brown rot and Botrytis blossom blight and fruit decay. Laboratory experiments were conducted to evaluate the pre-and post-infection activity of fungicides against brown rot and gray mold blossom blight. For pre-infection activity (protection), blossoms were collected at white bud, allowed to open in the laboratory, and treated using a hand sprayer. After 12 h, blossoms were inoculated with a spore suspension of *M. fructicola* or *B. cinerea* (30,000 conidia/ml) until water droplets formed on anther filaments. To evaluate the post-infection ("kick-back') activity, blossoms were collected, inoculated, and treated after 16 h with a hand-sprayer. Blossoms were evaluated for stamen infection after 4-5 days of incubation at 20 C, >95% relative humidity. Disease incidence was evaluated as the number of stamens infected divided by the total number of stamens per blossom. Three replications of 8 blossoms were used for each treatment and data were analyzed using analysis of variance and mean separation procedures (SAS 9.4).

To evaluate preharvest fungicide applications for control of fruit decay, orchards were used in San Joaquin Co. (commercial orchard) and at UC Davis (experimental orchard). In the UC Davis trial, treatments were applied 6 days PHI using a back-pack sprayer calibrated to deliver 100 gal/A. Fruit (8 fruit from each of three single-tree replication) were harvested, wounded with a glass rod (1 x 1 x 0.5 mm), and inoculated with 20 µl of a conidial suspension of *M. fructicola* or *B. cinerea* (30,000 conidia/ml); or 16 fruit from each replication were non-wound, drop-inoculated with a spore suspension of *M. fructicola* (50,000 spores/ml). In the San Joaquin trial, fungicides were applied 7 days before harvest. Fruit were harvested and 8 fruit from each of four single-tree replication were wound-inoculated with *M. fructicola* or *B. cinerea* as described above. In non-wound inoculations, approximately 50 to 60 fruit from each replication were sprayed with conidia of *M. fructicola* (20,000 spores/ml). All fruit were incubated for 5-7 days at 20 C, >95% RH. Percent incidence of infection was determined as the number of fruit infected of the total number of fruit evaluated. Data were analyzed as described above.

To evaluate preharvest fruit treatments for postharvest decay management and the persistence of the fungicides on the fruit that were treated in San Joaquin orchard, fruit were washed by spraying with high-volumes of water for 3 minutes prior to wound- and non-wound inoculations. Fruit were inoculated with *M. fructicola* or *B. cinerea* and decay was evaluated as described above.

Efficacy of new and registered postharvest treatments for managing brown rot, gray mold, and Rhizopus rot fruit rots of sweet cherry. Five laboratory studies and one commercial packingline study focused on the efficacy of several formulations of the new compound pimaricin against brown rot, gray mold, and Rhizopus rot and efficacy was compared to that of Scholar. Fruit were wound-inoculated with 20 µl of a spore suspension of *M. fructicola, B. cinerea,* or *R. stolonifer* (30,000 spores/ml each). In the laboratory, fungicides were applied as aqueous solutions using an air-nozzle sprayer or as drenches 11-14 h after (Inoculated-Treated) inoculation with the respective fungal pathogens. In the commercial packingline study, aqueous spray treatments were done to fruit on moving netted belts using two sequential T-Jets. Between the two T-Jets, there was a step on the belt so that fruit slightly tumbled and turned. Fruit were then incubated for 4-7 days at 20 C, >95% RH. Incidence of decay was determined as the number of fruit infected of the total fruit evaluated. Data were analyzed using analysis of variance procedures of SAS 9.4.

RESULTS AND DISCUSSION

Evaluation of the susceptibility of branch injuries over time to infection by the bacterial canker pathogen. In our studies in 2016, we focused on the canker phase of the disease. We conducted studies on the temporal susceptibility of injuries on cv. Coral cherry. Branches that were wounded in mid-February 2016 gradually became less susceptible over time to disease development by *P. syringae* pv. *syringae* and developed smaller cankers at evaluation time in mid-April. However, inoculations 15 days after wounding still resulted in disease. Thus, in contrast to 2015 when injuries were done in mid- to late January, wound healing proceeded much more slowly in 2016. In 2015, canker formation was significantly reduced in

inoculations 4 days after wounding, and after 8 days, only very small lesions developed. Thus, wound healing appears to be greatly affected by environmental conditions and possibly by different wound types (punctures, branch cuts). A comparison of climatic conditions at the trial sites in 2015 and 2016 is shown in Fig. 2. There was more rainfall during the trial period in 2016 than in 2015 and additionally, temperatures were higher. These factors could have contributed to the difference in wound healing. The natural incidence of blossom blast was very low in the spring of 2016 and no data were obtained.

With widespread copper resistance in the bacterial pathogen (*Pseudomonas syringae* pv. *syringae*), no effective treatments are currently available to manage bacterial canker and blast. These are important diseases of sweet cherry that can impact cherry production in seasons with favorable environmental conditions and can also have long-term effects on tree health. We identified the antibiotic kasugamycin as effective against both phases of the disease, whereas the biocontrol Actinovate in some trials significantly reduced the incidence of blossom blast. Due to the known defined period of high susceptibility for flower infections, blossom blast will be easier to manage once effective treatments are available to the industry. Infection periods for woody tissues that result in the canker phase of the disease are much more difficult to predict because they are determined by multiple causes including pruning, frost damage, wetness conditions, as well as wound healing processes.

In summary, our research on the management of bacterial canker and blossom blast, we identified Kasumin as the most effective and consistent treatment. Oxytetracycline was only evaluated in some trials but was identified as a very promising bactericide against *P. syringae*. Registrants of both of these antibiotics are supportive of a registration on sweet cherry and this is currently pursued. Oxytetracycline is in the IR-4 program and kasugamycin (i.e., Kasumin) is being reviewed both federally and by the state for use on sweet cherry. The state's 18-month review period concludes in January 2017, and the registrant has supplied additional data at the request of CA Department of Pesticide registration and has proposed a modified label reducing the number of applications per season. The antibiotic is federally registered on pome fruit since 2014 for management of fire blight. Over the years in our evaluations, Actinovate showed moderate efficacy in reducing blossom blast (but was less or not effective against canker). Because biocontrol agents are potentially more persistent than organo-chemical treatments, optimization of biocontrol treatments, possibly in combination with amendments is worth pursuing.

Evaluation of new fungicides for control of powdery mildew of sweet cherry. Our epidemiological studies have shown that mildew sequentially develops on: 1) leaves of inside shoots (water sprouts); 2) leaves of outer shoots; 3) green stems of fruit; and 4) on ripening fruit (fruit with color). The disease has not been found on green fruit mesocarp tissue. We have shown that young leaves are more susceptible than old leaves. The efficacy of new fungicides and new pre-mixtures was evaluated in a trial in San Joaquin Co. Three applications were done in ca. three-week intervals over a 6-week period starting at full bloom with fungicide applications for brown rot blossom blight. At evaluation time, all sampled leaves on trunk shoots (water sprouts) and 80% of leaves of the outside canopy showed symptoms of powdery mildew in the untreated control. The most effective treatments in both evaluations included selected DMI (FG 3)-containing fungicides such as Rhyme, Procure, Luna Experience, and Quadris Top, SDHI (FG 7)-containing fungicides such as Fontelis, Luna Sensation, Luna Experience, and Merivon, the mixture of the FG 19 Ph-D and the FG 3 Procure, as well as the experimental fungicides R106506, EXP-A, EXP-AD, EXP-AF, UC-2B (Fig. 3). Treatments with Fracture were not effective in 2016. Quintec (FG 13) that was highly effective in the first years after its registration on cherry and that has become less effective over time, was used in the first application of two rotation/mixture treatments (Fig. 3). In the program where Fontelis was used at the 20-oz rate, good disease control was obtained, whereas when using the 14-oz rate, good disease control was only achieved for the inside water sprouts.

Thus, this research demonstrated excellent activity of several newly registered, as well as of several numbered compounds against powdery mildew. We show that the disease can be reduced to acceptable levels by properly timed applications. Because of the potential of resistance to single-site mode of action fungicides, pre-mixtures or tank mixtures of FG 3, FG 7, FG 11, and FG 19 fungicides will be most sustainable. This limits the use of any single-site mode of action fungicide (i.e., single FG number) and reduces the selection pressure for selecting for fungicide resistance. Limiting the number of applications

of any one mode of action (i.e., FG) will also reduce the residue and ensure that MRLs are not exceeded with any of the trade partners of the cherry industry.

Under conditions where fungicides have to be used as post-infection treatments when visible symptoms are already present on fruit, we showed previously that Ph-D can be used with a multi-site fungicide like Kaligreen or with DMI fungicides like Procure for effective suppression of the disease.

Efficacy of new fungicides for control of brown rot and Botrytis blossom blight. Fungicide treatments were evaluated on detached opened blossoms in comparative laboratory studies. In pre- and post-infection studies, new and registered fungicides were very effective against brown rot and Botrytis blossom blights (Fig. 4). Highly effective fungicides with excellent pre- and post-infection activity against both blossom diseases included FG 3 (e.g. Quash) and the pre-mixture treatments FG 7/11 (e.g., Merivon) and FG 3/11 (e.g., Quadris Top), as well as the experimentals R-106506, UC-1, UC-2B, and IL54111. The experimental EXP-A was very effective against brown rot, but was not very effective against Botrytis blossom blight at the rate tested. The biocontrol Serenade Opti was moderately effective against brown rot, and also showed some efficacy against Botrytis blossom blight. Due to the good pre- and post-infection activity of most of the conventional fungicides, the practice of a single delayed-bloom application when environmental conditions are not favorable for disease development is an excellent strategy for obtaining highly effective blossom disease management and result in a minimal number of blossom treatments on sweet cherry.

Evaluation of preharvest treatments for fruit decay control without postharvest washes and for postharvest decay control after postharvest washes. Two preharvest efficacy trials with 6- and 7-day PHI applications were done in 2016 (Figs. 5, 6). In wound inoculation studies using non-washed fruit, several fungicides provided excellent protection against brown rot and these included the registered pre-mixture Quadris Top and the experimental compounds UC-1, UC-2B, UC-AD. When harvested fruit were washed and then inoculated, most treatments were less effective (Fig. 6A). In contrast, when harvested fruit were non-wound drop-inoculated, all treatments evaluated, including the bio-fungicide pimaricin (when applied 0-day PHI), were highly effective on non-washed and washed fruit (Fig. 5, 6A). This emphasizes the importance of care in handling fruit to prevent injuries that by-pass the protective fungicides.

For gray mold, the experimentals UC-1, EXP-AD and EXP-AF were most effective after inoculation in the first study (Fig. 5), whereas a mixture of Elevate and Procure gave the lowest incidence in the second study, but only on non-washed fruit (Fig. 6B). Natural incidence of gray mold developed on 30% of non-treated, non-washed fruit in the second study and was most effectively reduced by Ph-D + Elevate, Procure + Elevate, Luna Experience, R106506, UC-2B, and EXP-AF. Thus, these results possibly identified new effective gray mold treatments. These fungicides will be evaluated again in 2017.

These studies demonstrate that preharvest treatments can protect fruit from infections before and during harvest. Postharvest decays, however, can still develop due to minor injuries that occur during the bulk handling of fruit and lack of local systemic action of many fungicides.

Efficacy of new postharvest treatments for managing brown rot, gray mold, and Rhizopus rot of sweet cherry. In postharvest decay management in 2016, we focused on evaluating the newly registered bio-fungicide pimaricin also known as natamycin (Zivion – registered as BioSpectra in Aug. 2016). The fermentation product polyoxin-D (Oso, Tavano) was included in some of these studies. Although highly effective against brown rot and gray mold using low rates as a postharvest treatment (Fig. 7), registration of polyoxin-D is currently not being pursued by any US registrant. Both polyoxin-D and pimaricin are of interest to us because they are exempt-from-tolerance in the US but need maximum residue limits (MRLs) in many export countries.

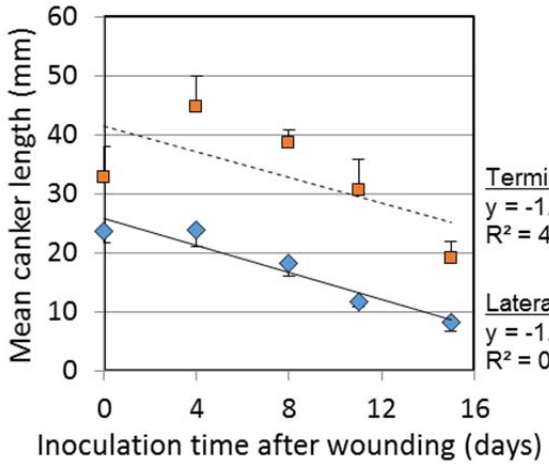
We also evaluated several formulations of pimaricin, a WP formulation that is used in the food industry, and two liquid formulations (i.e., Zivion which is the same formulation as BioSpectra and a formulation from another potential registrant). In all studies, pimaricin significantly and effectively reduced brown rot, gray mold, and Rhizopus rot when treatments were applied 11-14 h after inoculation of fruit as sprays (Figs. 7-10) or drench treatments (Fig. 11) in the laboratory at concentrations as low as 250 ppm (Fig. 9). There was no difference in efficacy using different formulations when used as aqueous

applications, however, we noted in other studies that one of the formulations is not compatible with certain fruit coatings (e.g., petroleum-based coatings). In our studies, application in a vegetable-based fruit coating, however, was highly effective (Fig. 8). Mixtures of pimaricin and Scholar also performed very well (Figs. 7, 10). Using very ripe fruit, treatments applied 4 h after inoculation were more effective than those after 14 h (Fig. 11).

In a study conducted on a commercial packingline, Zivion or a Zivion-Scholar mixture was applied using two T-Jet sprayers in immediate succession. This application method was done over two netted belts with one T-Jet over each belt. As fruit were transferred from one belt to the other, the fruit gently rolled over and thus, they were thoroughly treated on all sides. This treatment effectively reduced the incidence of all three decays (Fig. 12).

Thus, in our postharvest studies, we identified, optimized, and helped registered a new postharvest treatment BioSpectra for sweet cherry. Excitingly, resistance has never been reported to the active ingredient pimaricin (natamycin). Still, combination treatments of BioSpectra with other postharvest fungicides such as Scholar or Tebucon will be most beneficial in providing consistent, high efficacy. This strategy will also reduce the risk of selecting resistant sub-populations of the decay pathogens to other registered postharvest fungicides. At this time, MRLs have not been established in many countries and use is suggested only for domestic markets (including Canada). Excitingly, FATs for pimaricin are established in Japan for other food products and we expect an expedited review. We will continue our evaluations of these treatments in 2017 in cooperation with commercial packinghouses.

Fig. 1. Temporal susceptibility of cv. Coral cherry branch injuries to infection by *P. syringae* pv. *syringae*– Field studies at UC Davis 2016



In February of 2016, branches were puncture-wounded laterally using a nail and cut at the end. Wounds were inoculated with *P. syringae* pv. *syringae* (approximately 2 x 10^7 cfu/ml) after selected time periods. Branches were evaluated for gumming and canker formation in mid-April 2016.

Fig. 2. Climatic conditions at trial sites for bacterial canker in 2015 and 2016

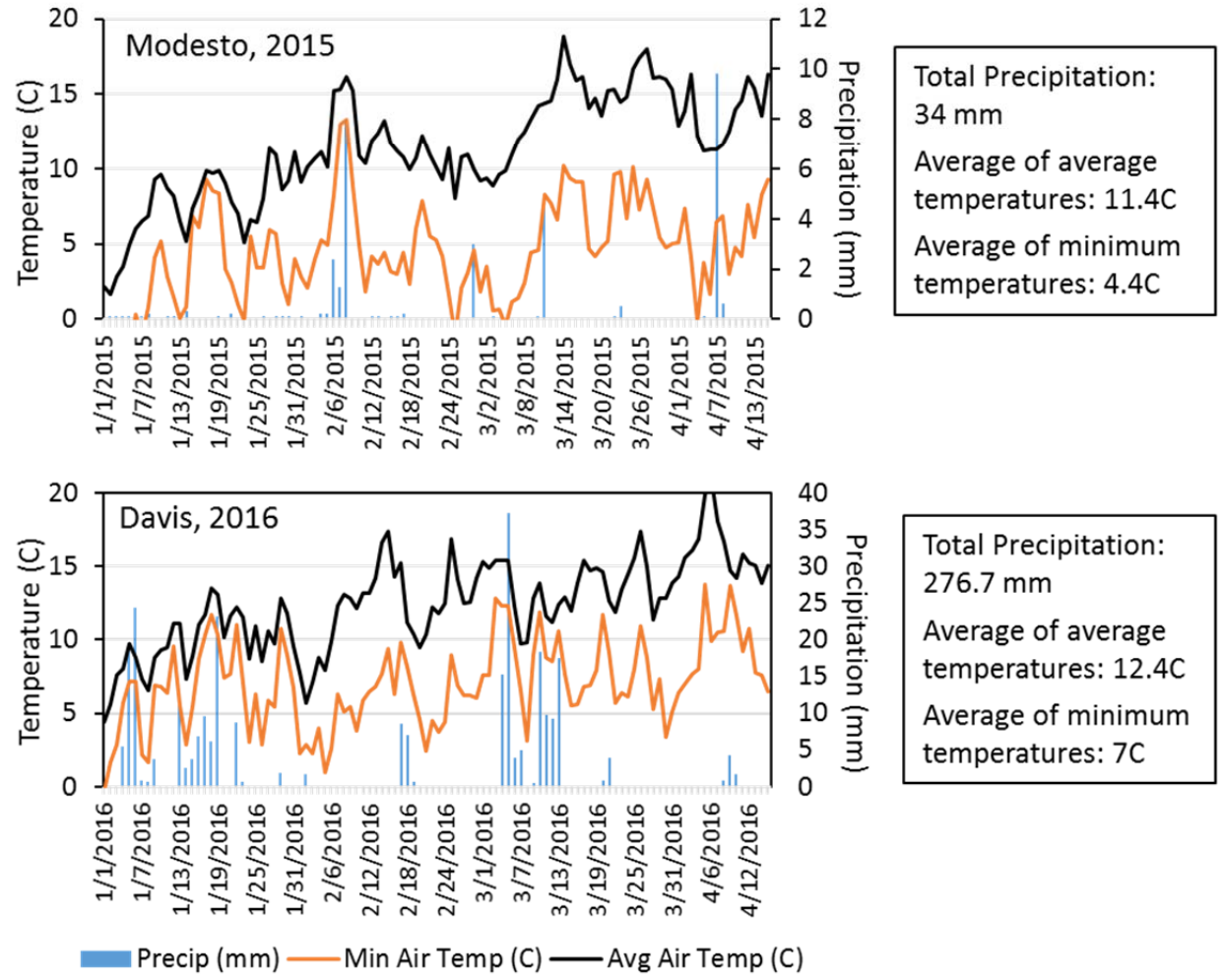
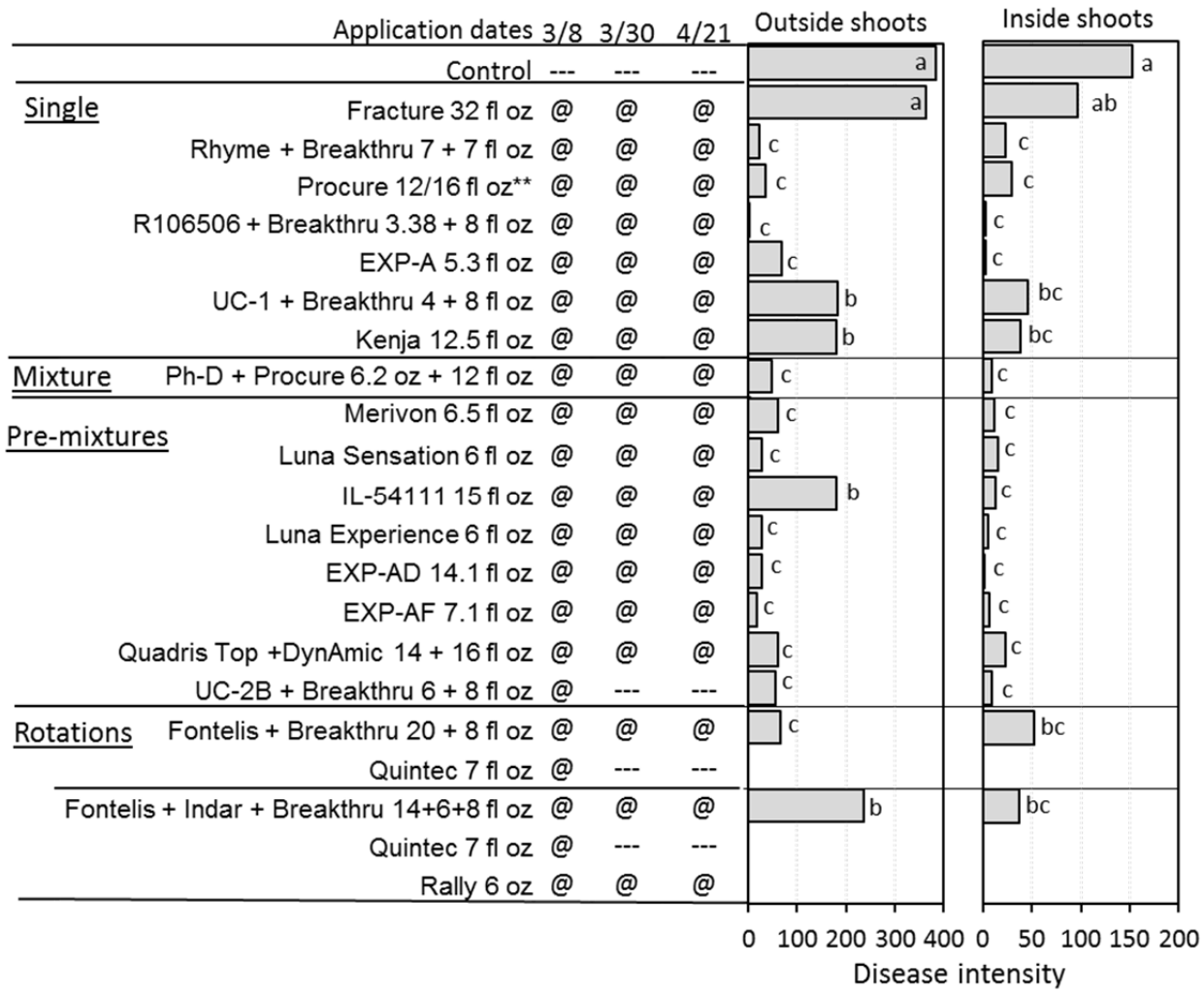
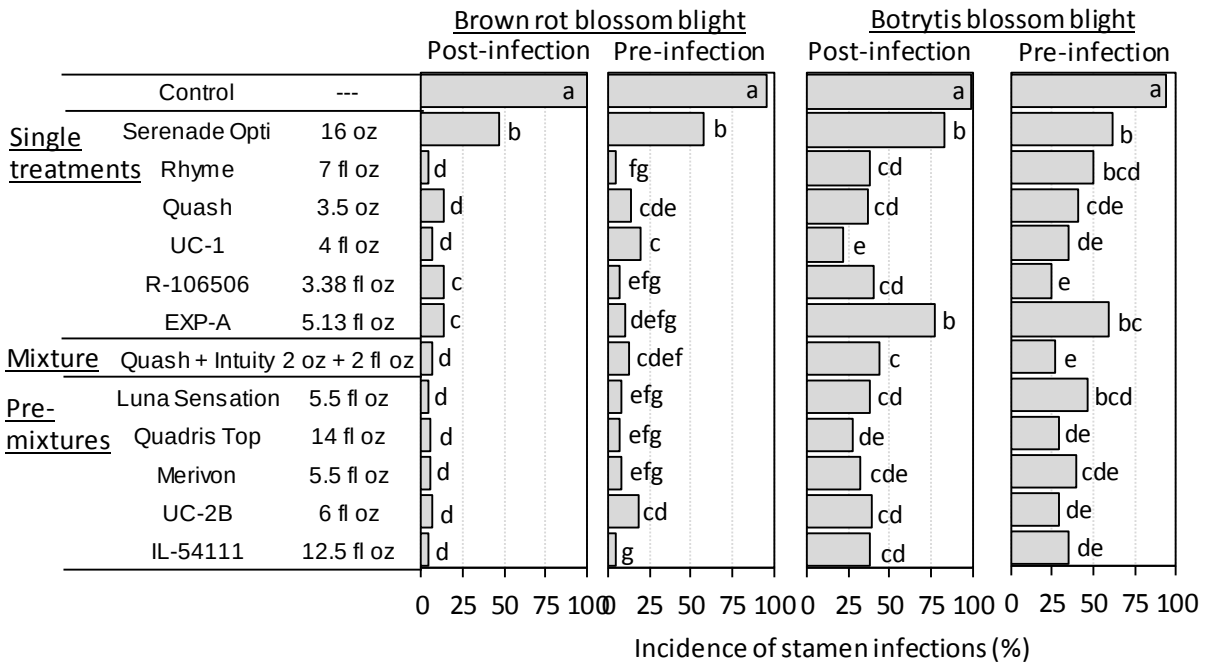


Fig. 3. Evaluation of preharvest fungicide treatments for management of powdery mildew of Bing cherries in San Joaquin Co. 2016



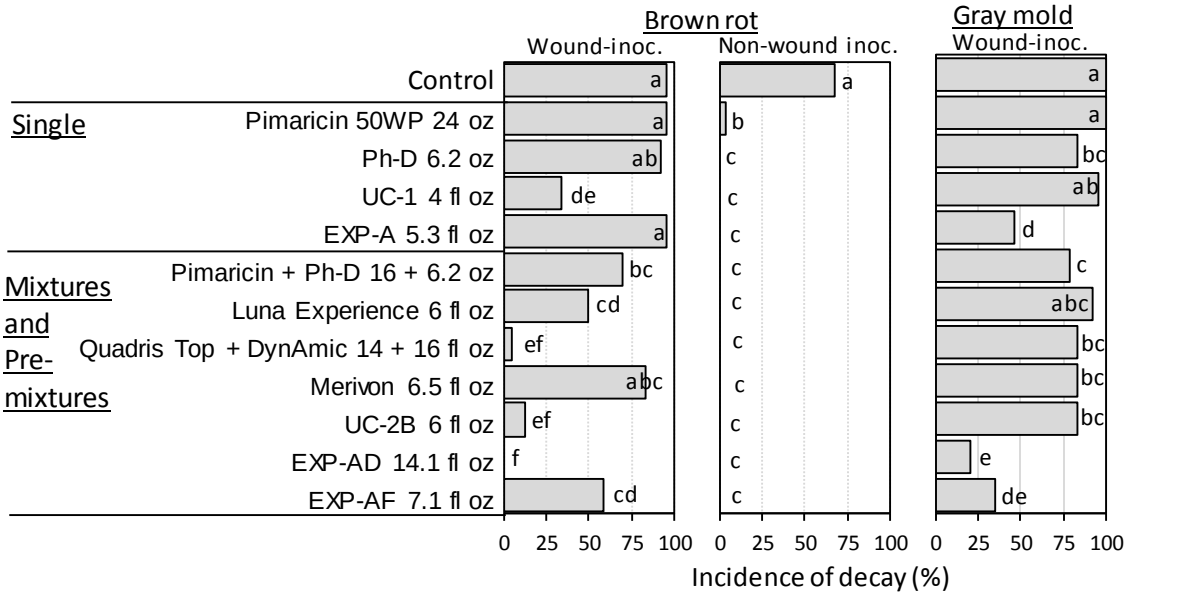
Applications were done using an airblast sprayer at 100 gal/A. For evaluation on 5-25-16, 20 leaves from 4 shoots each from inside or outside of the tree were sampled. The rating scale was: 0=healthy, 1=1-3 lesions/leaf, 2=<25%, 3=26-50%, 4 = >50% of leaf area diseased. Disease intensity is the multiplication product of disease incidence and disease severity.

Fig. 4. Efficacy of pre- and post-infection treatments for control of brown rot and Botrytis blossom blight of Bing cherry – Laboratory studies 2016



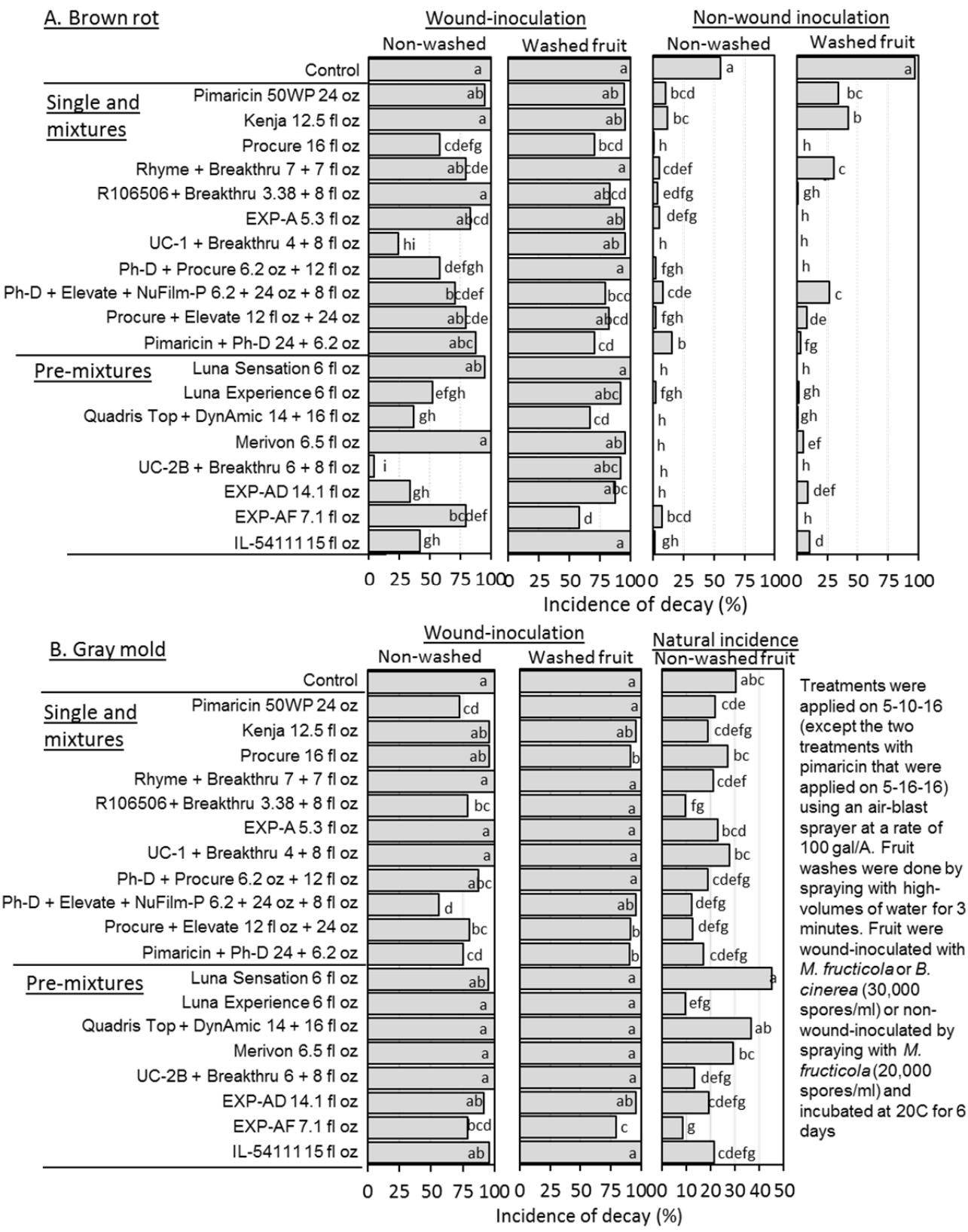
For evaluation of the pre-infection activity, closed blossoms were collected in the field, allowed to open, and treated in the laboratory using a hand sprayer. After 12 h blossoms were inoculated with a spore suspension of *M. fructicola* or *B. cinerea* (30 K/ml). For post-infection activity, blossoms were inoculated, incubated at 22 C, and treated after 16 h. Blossoms were evaluated for stamen infections after 4-5 days of incubation at 20 C.

Fig. 5. Efficacy of 6-day preharvest fungicide treatments for management of postharvest brown rot and gray mold of Bing cherries - Orchard 1 UC Davis - 2016



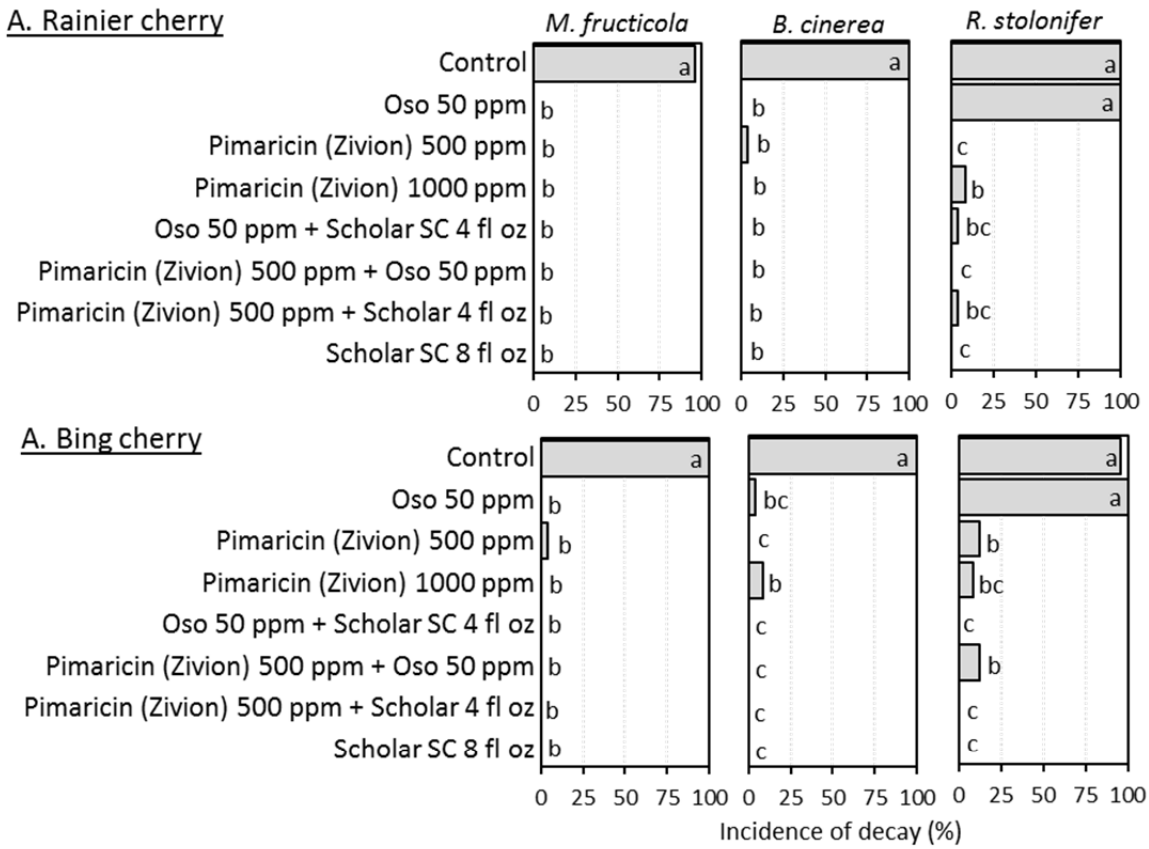
Treatments were applied on 5-10-16 using an air-blast sprayer at a rate of 100 gal/A. Fruit were wound-inoculated with *M. fructicola* or *B. cinerea* (30,000 spores/ml) or non-wound-inoculated with *M. fructicola* (60,000 spores/ml) and incubated at 20C for 6 days.

Fig. 6. Efficacy of 7-day preharvest fungicide treatments for management of postharvest brown rot and gray mold of Bing cherries - Orchard 2 San Joaquin Co. - 2016



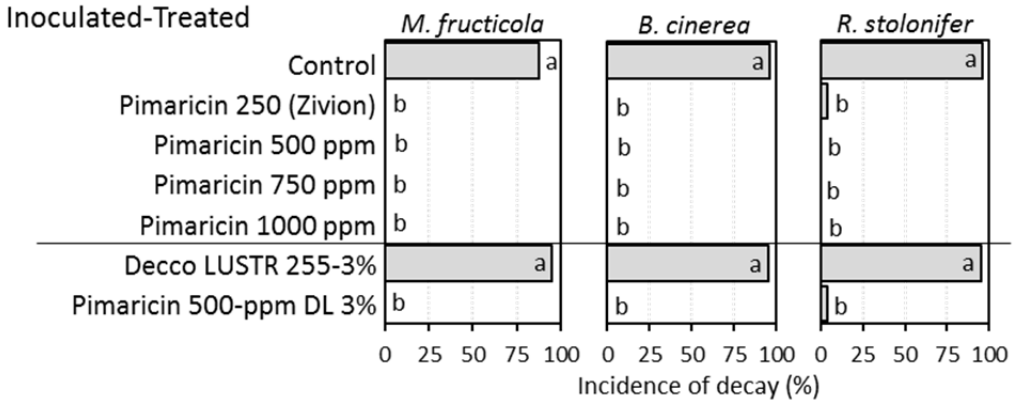
Treatments were applied on 5-10-16 (except the two treatments with pimaricin that were applied on 5-16-16) using an air-blast sprayer at a rate of 100 gal/A. Fruit washes were done by spraying with high-volumes of water for 3 minutes. Fruit were wound-inoculated with *M. fructicola* or *B. cinerea* (30,000 spores/ml) or non-wound-inoculated by spraying with *M. fructicola* (20,000 spores/ml) and incubated at 20C for 6 days

Fig. 7 Postharvest treatments with pimaricin, polyoxin-D (Oso), and Scholar for decay control of inoculated sweet cherry fruit in laboratory studies - 2016



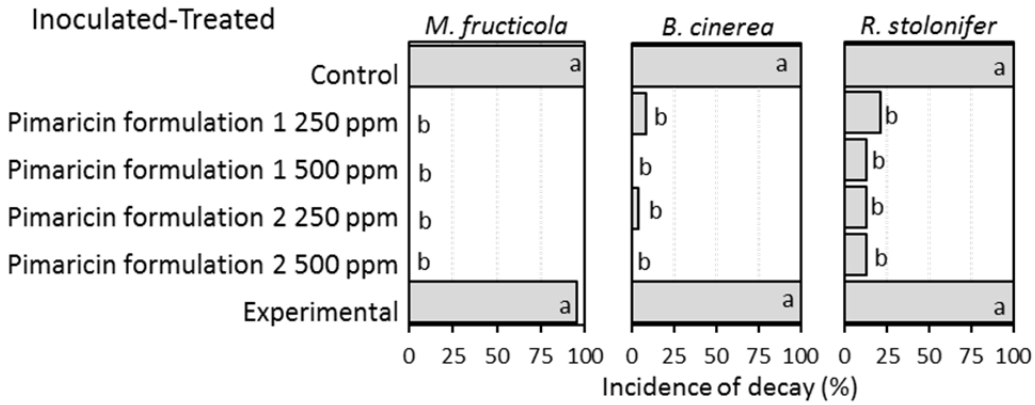
For the Inoculated-Treated procedure, fruit were wound-inoculated with spores of *M. fructicola*, *B. cinerea*, or *R. stolonifer* (30,000 spores/ml), treated with aqueous fungicide solutions after 14 h using an air-nozzle sprayer, and incubated at 20C for 4 to 6 days.

Fig. 8. Postharvest treatments with pimaricin in aqueous or wax solutions for decay control of inoculated Bing cherry fruit in laboratory studies - 2016



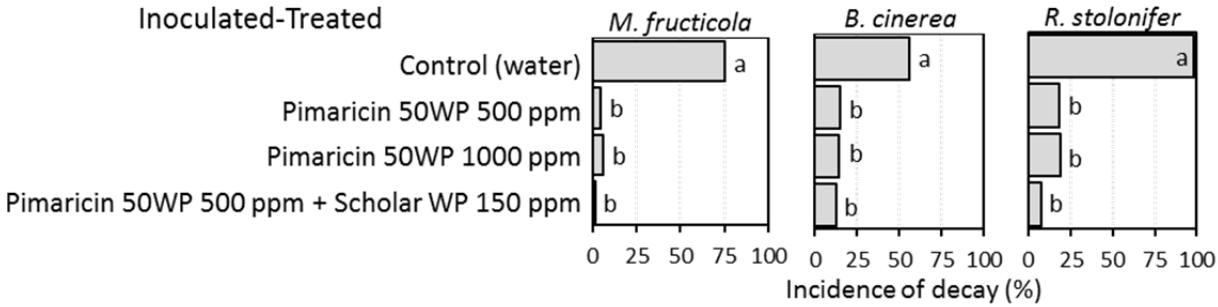
Fruit were wound-inoculated with spores of *M. fructicola*, *B. cinerea*, or *R. stolonifer* (30,000 spores/ml), incubated for 14 h at 20C, spray-treated with aqueous or wax fungicide solutions using an air-nozzle sprayer, and incubated at 20C for 6 days.

Fig. 9. Postharvest treatments with two formulations of pimaricin and an experimental treatment for decay control of inoculated Bing cherry fruit in laboratory studies - 2016



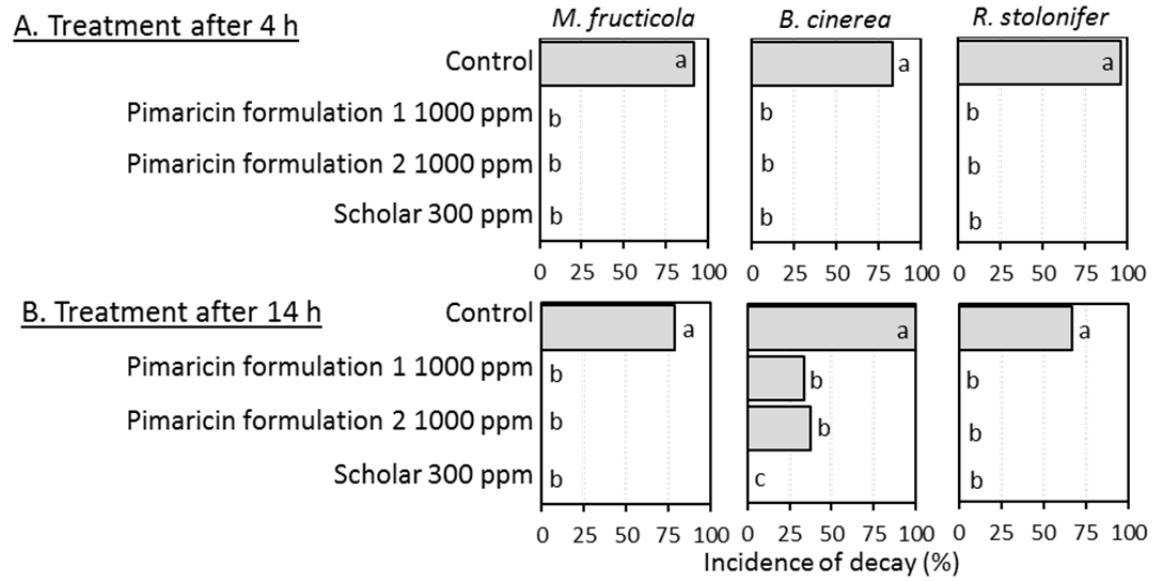
Fruit were wound-inoculated (30,000 spores/ml), incubated for 14 h at 20C, spray-treated with aqueous fungicide solutions using an air-nozzle sprayer, and incubated at 20C for 6 days.

Fig. 10. Postharvest drench treatments with pimaricin and Scholar for decay control of inoculated Bing cherry fruit in a laboratory study - 2016



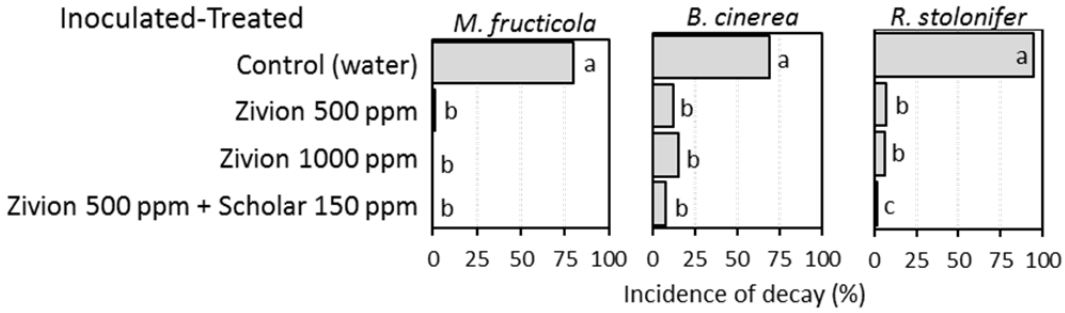
Fruit were wound-inoculated (30,000 spores/ml) and incubated for 11-13 h at 20C. Aqueous drench treatments were applied by hand over the fruit that were placed on a wire net, and incubated at 20C for 6 days.

Fig. 11. Effect of postharvest treatments with pimaricin and Scholar in combination with cold storage on decay control of inoculated sweet cherry fruit in laboratory studies - 2016



Fruit were wound-inoculated with spores of *M. fructicola*, *B. cinerea*, or *R. stolonifer* (30,000 spores/ml) and treated with aqueous fungicide solutions after 4 or 14 h of incubation at 20C. Fruit were then stored at 2C for 14 days, and then for 3 to 5 days until evaluation.

Fig. 12. Postharvest spray treatments with pimaricin and Scholar for decay control of inoculated Bing cherry fruit in a commercial packingline study - 2016



Fruit were wound-inoculated (30,000 spores/ml) and incubated for 14 h at 20C. Aqueous spray treatments were done to fruit on moving netted belts using two sequential T-Jets. Fruit were then incubated at 20C for 6 days.

Spotted Wing Drosophila 2016 Progress Report –Akbari and Hay

Project Title Engineered Transgenic *Drosophila suzukii* for wild population suppression and eradication: Production, Performance Assessment and Effective wild releases.

Collaborating PIs
Bruce A. Hay, California Institute of Technology
Omar S. Akbari, University of California, Riverside.

December 8th, 2016

Current Progress (2016) and Future work (2017) in *Drosophila Suzukii*

The final step in the development of a *D. suzukii* population suppression system is to combine all essential components including the gRNAs targeting the *D. suzukii* X chromosome in multiple positions, and Cas9 endonuclease driven by a male germline-specific promoter, onto the *D. suzukii* Y chromosome. In our first attempt, we successfully engineered the transgenes, however we failed to obtain germline integration events of these plasmids in *D. suzukii*. This result is likely due to a few possibilities: 1) It is conceivable that our gRNA's were not functional, as not all gRNAs function (Ran et al., 2013). 2) It is also feasible that our transgenes did integrate and were silenced on the Y chromosome. There is evidence in *Drosophila* that suggests that transgenes on the Y chromosome get rapidly silenced (Bachtrog 2013). Therefore to overcome these potential issues in *D. Suzukii*, we would like to redesign our transgenes to include chromosomal insulators known to prevent position effects and try to integrate them once again into the same genomic location on the Y (West, Gaszner, and Felsenfeld 2002). We will also design a few more transgenes with chromatin insulators, which will integrate into other locations on the Y – to rule out that our gRNAs are simply non-functional at certain Y-chromosome locations. Furthermore, we would like to also try to insert into an autosomal location in *Drosophila suzukii*. This would allow us to try different gRNAs, in addition to bypassing the Y-chromosome silencing issue altogether.

Parallel tests in *Drosophila melanogaster*

Given the low transformation rates in *D. Suzukii*, in parallel to the above experiments, we have been working to develop the above idea of X-shredding in *Drosophila melanogaster* and have made some considerable progress. Over the last several months we have developed a binary genetic system in *Drosophila melanogaster* to see if we can achieve effective X-chromosome shredding. In this system, we are essentially crossing flies that express Cas9 in their germlines (Gratz et al. 2013) (via the Vasa promoter) with flies expressing single gRNAs or multiplexed

gRNA	Sequence	Target sites on X Chromosome
1	ATTTTCGCATTTTTTGTAAGGGG	63
2	TCATCAAAATTTGCAAAAAATGG	62
3	AAATTTTCGCATTTTTTGTAAGG	43
Double	ATTTTCGCATTTTTTGTAAGGGG TCATCAAAATTTGCAAAAAATGG	125
Triple	ATTTTCGCATTTTTTGTAAGGGG TCATCAAAATTTGCAAAAAATGG AAATTTTCGCATTTTTTGTAAGG	168

gRNAs (double or triple) that we engineered to target the X-chromosome in up to 168 locations (see table).

While many of these crosses are still ongoing, preliminary results indicate that X-chromosome shredding is occurring in flies carrying the germline expression of cas9 and the autosomal guide RNA (#2). We believe this is the case since no progeny survive that contain the guide RNA, while 50% would be expected to carry it under normal Mendelian transmission, suggesting that these have been killed. This likely is a result of somatic expression of cas9 and the guide RNA resulting in X-chromosome shredding during embryogenesis. Importantly, this result is highly suggestive that we can destroy the X chromosome using Cas9 and guide RNAs! This would be very good news!!

From other work we already know that we can home a cas9 cassette into a specific location on the autosomes. Also, similar to work in *D. Suzukii* Figure 1: gRNA target sites for CRISPR mediated cleavage. gRNA sequences and frequency of target sites on the X chromosome are listed. (described above) building docking strains on the Y chromosome of *Drosophila melanogaster* using Cas9 has been difficult.

Development of Y-Docking strains

Furthermore, very recently using CRISPR/Cas9 we were able to develop 2 targeted insertions on the Y-chromosome that express!! This is a significant advancement for our approach, as we now have two Y-specific regions that we can position our X-shredders on that we know are not silenced. This was our major rate limiting step previously and we have overcome it.

Overall Progress Summary

We have a number of tools needed to get Y drive

1. Guide RNAs that target the X
2. Evidence that expression of these can cause killing of the X
3. We know we can home into the Y-chromosome and have our transgene expressed.
4. We also have a number of male-specific promoters.

Going forward with Y-Drive 2017

We were previously limited by the inability to insert genes on the Y-chromosome. Over the past year, we have overcome this limitation and now have the ability to insert/express at two separate locations on the Y-chromosome. This was a major rate limiting step. Now we can move forward with building everything on the Y-chromosome and systematically testing all of our components to build the the X-shredding system we have proposed.

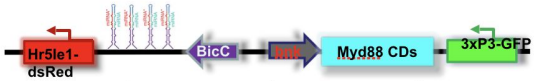
(B) Development of a D. *Suzukii Medea* based drive system

Background

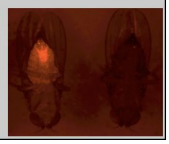
We have developed our first *Drosophila suzukii* functional replacement system termed *Medea*. Over the next year we will continue rigorously testing this system in caged laboratory populations to determine its effectiveness. We will also perform several pairwise crosses with different genetic backgrounds to determine fecundity. We plan to apply for a permit with the USDA-Aphis to begin field testing this approach.

Medea was first discovered in the flour beetle (Wade and Beeman 1994), and multiple versions were later reverse engineered from scratch and shown to act as robust gene drives in the laboratory fruit fly, *Drosophila melanogaster (Akbari et al. 2014; Chen et al. 2007)*. Such engineered *Medea* systems rely on a *Medea* element consisting of a toxin-antidote combination. The toxin consists of a miRNA that is expressed during oogenesis in *Medea*-bearing females, disrupting an embryonic essential gene. A linked antidote is expressed early during embryogenesis and consists of a recoded version of the target gene that is resistant to the miRNA. This combination results in the survival of half of the embryos originating from a *Medea*-bearing heterozygote female, as those that do not inherit the *Medea* element perish. If a heterozygous *Medea* female has mated with a heterozygous *Medea* male, the antidote from the male will also take effect in the embryo, resulting in 3/4 of the embryos surviving. Therefore, *Medea* will rapidly spread through a population, carrying any linked genes with it.

In the case of *D. suzukii*, since elimination of the pest population is ultimately the goal, an engineered *Medea* system could spread a gene proffering susceptibility to a particular pesticide, or a conditional lethal gene that would be activated by some substance or environmental cue such as diapause - a state that allows insects survive periods of adverse conditions such as cold (Shearer et al. 2016). For example, a *Medea* element can be used to spread a gene conferring sensitivity to a particular chemical that is normally innocuous, rendering such a chemical capable of being used as an environmentally-friendly, species-specific pesticide. Trigger-inducible transcription control elements – ones that turn on expression in the presence of a chemical such as tetracycline or vanillic acid (Urlinger et al. 2000; Gitzinger et al. 2012)– can be engineered to drive expression of an insect-specific toxin (e.g., (Fu et al. 2007)). A *Medea* element can also be used to spread a gene under the control of a diapause-induced promoter that will splice to produce a toxin in females only, so that, upon the onset of the diapause-inducing environmental cue, all of the females will perish, causing a population crash (Akbari et al. 2013). Further, if a *Medea* element is inserted into a fertility gene, it could cause a population crash by spreading through a population and making it infertile as it does. However, although transgenesis of *D. suzukii* has been established (Schetelig and Handler 2013), no gene drive systems in this major pest have yet been engineered.



Generation	Sex (# crossed out)	Number of Progeny	% Medea
G2	Female (9)	126	100%
	Male (3)	45	50%
G3	Female (32)	299	100%
	Male (9)	130	50%
G4	Female (50)	603	100%
G5	Female (31)	785	97%
Total Medea+ from females n=122		1788/1813	99%



99% Medea inheritance

Current Progress (2016) and future work (2017)

Generation and testing of *D. suzukii Medea*

To create a *Medea*-like maternal-effect selfish genetic element in *D. suzukii*, we engineered synthetic *Medea* elements based on the same architecture used to generate the *Medea*^{myd88} system previously built in *D. melanogaster (Akbari et al. 2014; Chen et al. 2007)*. In *D.*

melanogaster, maternal Myd88 is required for dorsal-ventral patterning in early embryo development, and germ-line loss-of-function myd88 mutant females produce embryos that fail to hatch(Kambris et al. 2003). Myd88 is highly conserved in *D. suzukii* (and in many other Drosophilids), and we reasoned that it would likely be essential in this species, as well.

Briefly, we generated a *piggybac* transposable element vector in which the predicted *D. suzukii* female germ-line–specific bicoid (bic) promoter drives the expression of a "toxin" consisting of three synthetic microRNAs (miRNAs) designed to target the 5' untranslated region (UTR) of *D. suzukii* myd88. The synthetic miRNAs were generated using the mir6.1 backbone, as used previously (Akbari et al. 2014; Chen et al. 2007). This vector also contains an "antidote" transgene consisting of *D. suzukii* myd88, recoded to be insensitive to the miRNAs, expressed under control of the early embryo–specific bottleneck (bnk) promoter (Schejter and Wieschaus 1993). The vector also contained two separate transformation markers – GFP under control of the eye-specific 3xP3 promoter (Berghammer, Klingler, and Wimmer 1999), and dsRed under control of the ubiquitous opie2 promoter (Theilmann and Stewart 1992).

The vector, along with a source of transposase, was injected into *D. suzukii* embryos using standard injection procedures, and the surviving G0 adults were individually outcrossed to wild type (WT) individuals. G1 progeny were screened for the presence of the *Medea* element (as evidenced by ubiquitous dsRed expression), and one G1 transformant male was recovered. When outcrossed to WT virgin females, the male produced 50% *Medea*+ and 50% WT individuals (as would be expected with Mendelian transmission ratios). The *Medea*+ G2 progeny were further individually outcrossed to WT individuals of the opposite sex. From these crosses, the males (n=3) gave rise to ~50% *Medea*+ progeny, as expected; the females (n=9) gave rise to 100% *Medea*+ progeny, which would be expected if the *Medea* element was functioning as predicted. Of the G3 *Medea*+ progeny, nine males and 32 virgin females were further individually outcrossed to WT. The males have rise to 50% *Medea*+:50% WT individuals, and male outcrosses were discontinued at this stage. The females all gave rise to 100% *Medea*+ offspring (with a mean=9.34 G4 progeny recovered). Fifty G4 heterozygous *Medea*+ virgin females were further individually outcrossed to WT males, and all of their progeny (n=603) were *Medea*+ . Thirty-one resulting G5 heterozygous *Medea*+ virgin females were then outcrossed further. In this outcross, a small number of progeny that were negative for the *Medea* element were recovered for the first time, indicating that the system did not function at 100%. Of the 31 G4 crosses that gave rise to scorable progeny, eight G4 females produced a small number of *Medea*– offspring (ranging from one to seven per female), while 23 gave rise to 100% *Medea*+ progeny. Although a *Medea* system that works perfectly would be ideal, one that gives rise to mostly *Medea*+ progeny would still be expected to spread through a population. In this case, of the total G5 progeny, almost 97% (n=785) were *Medea*+, demonstrating that the system functions very efficiently. Overall, when all generations were summed together, the percentage of *Medea*+ progeny arising from single heterozygous female outcrosses was nearly 99%, with 1788 *Medea*+ progeny out of 1813 total.

Population cage experiments

To determine whether the generated *D. suzukii Medea* is capable of spreading through populations, population cage experiments were set up as follows. Heterozygous *Medea*

(*Medea*/+) males and WT (+/+) males were allowed to mate with WT (+/+) females in proportions of 25 *Medea*/+ males: 25 +/+ males: 50 +/+ females (for an allele frequency of ~12.5%) and 30 *Medea*/+ males: 20 +/+ males: 50 +/+ females (for an allele frequency of ~15%). Additionally, heterozygous *Medea* (*Medea*/+) males were allowed to mate with WT (+/+) females in proportions of 50 *Medea*/+ males: 50 +/+ females (for an allele frequency of ~25%), and homozygous *Medea* males (*Medea*/ *Medea*) were allowed to mate with WT (+/+) females in proportions of 50 *Medea*/ *Medea* males: 50 +/+ females (for an allele frequency of ~50%). The total number of flies for each starting population was 100. After being placed together, adult flies were removed after exactly seven days. After another seven days, progeny were collected and separated in half arbitrarily. One half was counted, while the other half was placed in a new bottle to continue the simulation, and this process continued throughout the duration of the experiment. All experiments were conducted in triplicate. All fly experiments were carried out at ~20°C with ambient humidity in 250 ml bottles containing a fly medium prepared based on a recipe from USDS. These experiments are ongoing; however, given the observed genetic behaviour of the present *Medea* system, we anticipate that the *Medea* element will spread through the experimental populations in the predicted manner.

Strain origin	# of progeny	% *Medea* +
Clayton, WA	152	88%
Watsonville, CA	62	100%
Brentwood, CA	251	94%
Enime, Japan	133	100%
Oahu, HI	167	99%
Beltsville, MD	100	98%
Oregon	155	100%
Tracy, CA	283	88%
Maryland	16	100%
Total	**1319**	**96%**

To determine the effectiveness of the *Medea* system in different genetic backgrounds collected from various locations around the world we performed pairwise crosses with strains collected from 9 distinct locations (see table). From these crosses we confirm that the *Medea* system was quite effective, with an overall transmission rate of 96%, suggesting that this system would be highly efficient at quickly modifying a wild population of *D. suzukii* present in any of these locations

To safeguard, reduce risk, and mitigate the spread of the *D. suzukii Medea* system into wild populations it will be important to develop a reversal *Medea* (RM) system and demonstrate that it can function as advertised. Reversing the drive of a *Medea* system has been theorized, however it has never been experimentally demonstrated. Therefore, this should be of high impact and relevance when it comes to regulators assessing the risk associated with gene drives. Therefore, in the coming year we will develop a system that can be used to reverse the spread of the Medea system.

gRNA	Sequence	Target sites on X Chromosome
1	ATTTTCGCATTTTTTGTAAGGGG	63
2	TCATCAAAATTTGCAAAAAATGG	62
3	AAATTTTCGCATTTTTTGTAAGG	43
Double	ATTTTCGCATTTTTTGTAAGGGG TCATCAAAATTTGCAAAAAATGG	125
Triple	ATTTTCGCATTTTTTGTAAGGGG TCATCAAAATTTGCAAAAAATGG AAATTTTCGCATTTTTTGTAAGG	168

Overall Progress Summary:
We have a number of tools needed to get Y drive
1. Guide RNAs that target the X
2. Evidence that expression of these can cause killing of the X
3. We know we can home into a locus, albeit an autosomal locus.
4. We also have a number of male-specific promoters.
5. What we have been unable to get is Y insertions of our gene cassettes

Going forward with Y-Drive
What we are going to do is to focus on:

A. Trying promoters from Y-based genes

Figure 1: gRNA target sites for CRISPR mediated cleavage. *gRNA sequences and frequency of target sites on the X chromosome are listed.*

B. Inserting insulator sequences to protect genes from negative influence of the Y.

Summary
In 2016, we have made considerable progress, we developed a functional gene drive system in D. suzukii, we developed/tested a technique Y-docking strains (our major rate limiting step), we developed functional germline Cas9 expressing lines that can be used to develop Y-drive and other systems. In 2017, we will keep up this progress and if we are successful in these key lab-based experiments, our plan is still to continue with cage trials and ultimately wild releases, as outlined in our initial proposal.

References

Akbari, Omar S., Chun-Hong Chen, John M. Marshall, Haixia Huang, Igor Antoshechkin, and Bruce A. Hay. 2014. "Novel Synthetic Medea Selfish Genetic Elements Drive Population Replacement in Drosophila; a Theoretical Exploration of Medea-Dependent Population Suppression." *ACS Synthetic Biology* 3 (12): 915–28.

Akbari, Omar S., Kelly D. Matzen, John M. Marshall, Haixia Huang, Catherine M. Ward, and Bruce A. Hay. 2013. "A Synthetic Gene Drive System for Local, Reversible Modification and Suppression of Insect Populations." *Current Biology: CB* 23 (8): 671–77.

Bachtrog, Doris. 2013. "Y-Chromosome Evolution: Emerging Insights into Processes of Y-Chromosome Degeneration." *Nature Reviews. Genetics* 14 (2): 113–24.

Berghammer, Andreas J., Martin Klingler, and Ernst A. Wimmer. 1999. "Genetic Techniques: A Universal Marker for Transgenic Insects." *Nature* 402 (6760). Nature Publishing Group: 370–71.

Chen, Chun-Hong, Haixia Huang, Catherine M. Ward, Jessica T. Su, Lorian V. Schaeffer, Ming Guo, and Bruce A. Hay. 2007. "A Synthetic Maternal-Effect Selfish Genetic Element Drives Population Replacement in Drosophila." *Science* 316 (5824): 597–600.

Fu, Guoliang, Kirsty C. Condon, Matthew J. Epton, Peng Gong, Li Jin, George C. Condon, Neil I. Morrison, Tarig H. Dafa'alla, and Luke Alphey. 2007. "Female-Specific Insect Lethality Engineered Using Alternative Splicing." *Nature Biotechnology* 25 (3): 353–57.

Gitzinger, Marc, Christian Kemmer, David A. Fluri, Marie Daoud El-Baba, Wilfried Weber, and Martin Fussenegger. 2012. "The Food Additive Vanillic Acid Controls Transgene Expression in Mammalian Cells and Mice." *Nucleic Acids Research* 40 (5): e37.

Gratz, Scott J., Jill Wildonger, Melissa M. Harrison, and Kate M. O'Connor-Giles. 2013. "CRISPR/Cas9-Mediated Genome Engineering and the Promise of Designer Flies on Demand." *Fly* 7 (4): 249–55.

Kambris, Zakaria, Hana Bilak, Rosalba D'Alessandro, Marcia Belvin, Jean-Luc Imler, and Maria Capovilla. 2003. "DmMyD88 Controls Dorsoventral Patterning of the Drosophila Embryo." *EMBO Reports* 4 (1): 64–69.

Schejter, E. D., and E. Wieschaus. 1993. "Bottleneck Acts as a Regulator of the Microfilament Network Governing Cellularization of the Drosophila Embryo." *Cell* 75 (2): 373–85.

Schetelig, Marc F., and Alfred M. Handler. 2013. "Germline Transformation of the Spotted Wing Drosophilid, Drosophila Suzukii, with a piggyBac Transposon Vector." *Genetica* 141 (4-6): 189–93.

Shearer, Peter W., Jessica D. West, Vaughn M. Walton, Preston H. Brown, Nicolas Svetec, and Joanna C. Chiu. 2016. "Seasonal Cues Induce Phenotypic Plasticity of Drosophila Suzukii to Enhance Winter Survival." *BMC Ecology* 16 (March): 11.

Theilmann, D. A., and S. Stewart. 1992. "Molecular Analysis of the Trans-Activating IE-2 Gene of Orgyia Pseudotsugata Multicapsid Nuclear Polyhedrosis Virus." *Virology* 187 (1): 84–96.

Urlinger, S., U. Baron, M. Thellmann, M. T. Hasan, H. Bujard, and W. Hillen. 2000. "Exploring the Sequence Space for Tetracycline-Dependent Transcriptional Activators: Novel Mutations Yield Expanded Range and Sensitivity." *Proceedings of the National Academy of Sciences of the United States of America* 97 (14): 7963–68.

Wade, M. J., and R. W. Beeman. 1994. "The Population Dynamics of Maternal-Effect Selfish

Genes." *Genetics* 138 (4): 1309–14.
West, Adam G., Miklos Gaszner, and Gary Felsenfeld. 2002. "Insulators: Many Functions, Many Mechanisms." *Genes & Development* 16 (3): 271–88.

University of California

Agriculture and Natural Resources

PROJECT REPORT/RESEARCH PROPOSAL

Project Year 2015-16 Anticipated Duration of Project2 year

Project Leader Jhalendra Rijal Location UCCE-Stanislaus

Cooperating Personnel Joseph Grant, UCCE San Joaquin

Project Title Oviposition Deterrents and Insecticides for Spotted Wing Drosophila Control in Cherry

Problem and Previous Research Accomplishments:

Out of ~1500 *Drosophila* species worldwide, Spotted Wing Drosophila (SWD) is one of the two species capable of depositing eggs on healthy and ripening fruits because female SWD is equipped with a serrated (i.e., saw-like) ovipositor (i.e., egg laying apparatus) for depositing eggs inside fruits. One female is capable of laying more than 300 eggs during her lifetime and, in most instances, one fruit is infested with multiple larvae. The ovipositor is capable of incising the intact fruit skin rendering the cherry fruit with typical oviposition scars. Direct damage on fruits by internal-feeding larvae of SWD leads to fruit tissue damage and ultimately the fruit collapse. Fruits injured by oviposition and feeding become an easy target for several other pests such as vinegar flies and other secondary infections, which are otherwise not a threat to intact fruits. In addition to indirect damage associated with secondary pest and disease incidence, there is a high risk of fruit lots being rejected during the processing and/or exporting of fruits if SWD infestation is found on fruits. Thus the economic threshold for this pest in cherry is 'zero' in practical terms.

Because of wide host range, unique egg-laying behavior, high fecundity, and large number of generations per season, damage by SWD in susceptible fruits such as cherry becomes severe very quickly. Current management practices for SWD in California cherry production rely heavily on a limited number of insecticides, particularly of pyrethroid and spinosyn products. Frequent use of these insecticides can lead to pest resistance, adversely affect natural enemy populations, and lead to outbreak of secondary pests such as scale insects. Also, use of insecticides close to harvest can lead to unacceptably high residue levels in fruits.Given this situation, exploring alternative measure(s) that can reduce the fruit damage by

SWD, while minimizing insecticide related problems is crucial. One option worth exploring to achieve this is to use oviposition deterrents activity of the commercial neem-based products. There are reports in pest management literature that this approach is working against some fruit fly and some *Drosophila* species in several crops. Several concentrations (0.2-4%) of neem seed kernel (NSK) extracts (in acetone)have reducedOriental fruit fly, *Bactroceradorsalis* egg deposition by 87.5-99.2% in guava fruit in choice tests (Chen et al. 1996).Similarly, acetone-based extract of deoiled NSK powder has significantly deterred oviposition by some tephritid fruit flies (*B. dorsalis* and *B. cucurbitae*) (Singh and Singh 1998). Somecompounds derived from plant and microbes have shown repellent effects on vinegar fly (*D. melanogaster*) in laboratory bioassays (Devaud 2003, Inamdar et al. 2010).In addition of oviposition activity by neem-products, we have also looked at the efficacy of some commercial and/orexperimental products against SWD mortality under laboratory condition.

Objectives and Anticipated Outcomes:

1. To evaluate oviposition deterrent activities of neem-based commercial products (containing either Azadirachtin or Clarified Hydrophobic Neem Oil Extract or both) in the laboratory using various combinations of choice and no-choice bioassays.
2. To test reduced-risk insecticides against SWD under laboratory condition.

Anticipated Outcomes. This project will ultimately helpful in improving current pest management practices targeting SWD in California cherry production by incorporating oviposition deterrent products in combination with other control methods. This will help to reduce the issues related to insecticide-focused pest management program in cherries.

Plans and Procedures:

SWD rearing. SWD stock population was obtained from Dr. Chiu lab at UC Davis and establish colony at UCCE-Stanislaus using the Jazzmix-based fly diet. For diet preparation, 10 g Jazzmix was added to 70 ml of distilled water, boil it for about 2 min. The mix was poured into the Drosophila rearing vials and allow to cool overnight. Place roughly 10 flies (both males and females) were released into vials egg laying. The newly emerged adults were collected from the vials and used in studies.

Cherry fruit source. Cherry fruits with similar level of ripeness visually were collected from stores. Fruits were washed thoroughly to remove any external dirt, and other potential contamination, and

allowed to air dry for ~20 min. under room temperature.Cherries with with intact petiole were used for several no-choice and choice bioassays.

Effect of neem products on oviposition using no-choice study. Cherry fruits treated singly with each of individual treatments were hung on the lid insidethe small plastic cups (12 oz.) with screened lids were used. The other sets were treated with distilled water (control) and use as the control. Several neem-based products were evaluated. 3 female and 2 male SWD flies (age: 7-10 days old) were released into the container to allow egg laying on fruits. Each set of trial had 10 replicates for each of treatment and control. The fruits will be inspected for oviposition stings (oviposition scars on the fruit) at 24 hours. Two sets of trials were conducted for each product tested.

Effect of neem products on oviposition using choice study. Single fruit treated with one of the neem products was hung in one corner inside the ventilated container (36 oz.) while an untreated fruit (i.e. control) was hung to the opposite corner of the container. 5 female and 3 male flies (age: 7-10 days old) were released into the containers to allow egg laying. Fruits were inspected for the oviposition stings after 24 hours.

Effect of cyclaniliprole on SWD adult mortality. Laboratory bioassays were conducted by exposing SWD adults to a diamide insecticide, cyclaniliprole (rates: 12 oz/acre and 16 oz/acre). Cherries were treated with the insecticide and hung inside a ventilated cup (12 oz.). 10 adults of the same age (7-10 days old) were released into the container and closed the lid. Bioassay was conducted at room temperature condition. The mortality of the flies was recorded at 1, 4, 7 days after treatment (DAT).

Table 1. Neem products used to conduct SWD oviposition deterrent studies

Treatments	Active Ingradient	Rate
Bonide Neem Oil	Neem oil extract 0.97%	0.97%
Trilogy	Neem oil extract 70%	1%
Debug Turbo	67% (Neem oil extract + Azadirachtin)	1 quarts/100 gallon water
Triple Action Neem	Neem oil extract 70%	1 fl oz/gallon water
Neemix 4.5	Azadirachtin 4.5%	4 oz/acre
Neemix 4.5	Azadirachtin 4.5%	8 oz/acre
Azamax	Azadirachtin 1.2%	32 oz./gallon water

Results

Effect of neem products on oviposition using choice and no-choice studies:

Bonide neem oil. In choice tests, significantly reduced number of oviposition stings was recorded in Bonide neem oil treatment compared to the control (Fig 1).

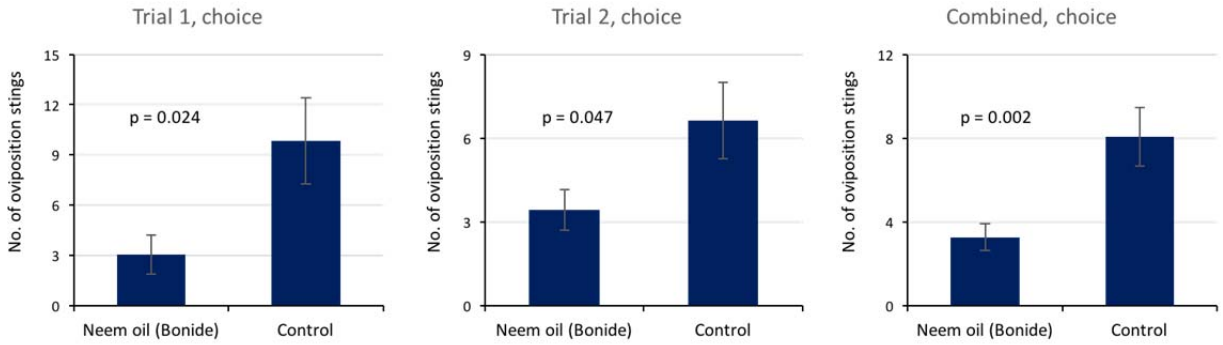


Fig. 1. Effect of Bonide neem oil on SWD oviposition in choice tests

In no-choice tests, significant oviposition activity was observed in Bonide neem oil treated cherries compared to the control (Fig 2).

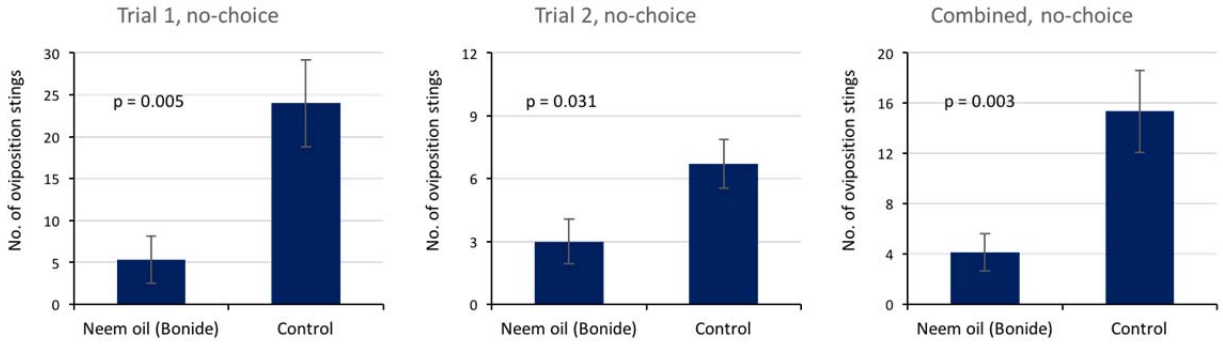


Fig. 2. Effect of Bonide neem oil on SWD oviposition in no-choice tests

Trilogy. Although oviposition sting counts were numerically higher in control compared to the Trilogy treatment in two sets of the choice tests conducted, no statistical difference was observed in no-choice studies. However, oviposition activity was significantly reduced in Trilogy when combined data from the two trials (Fig 3).

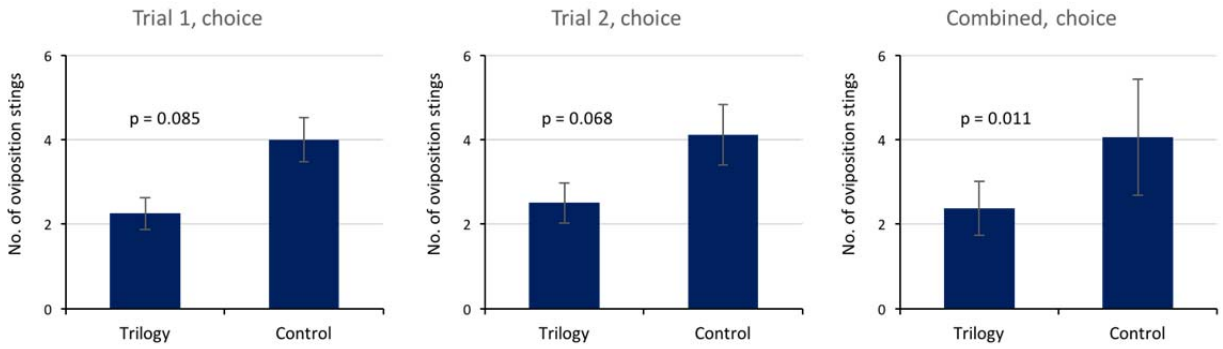


Fig. 3. Effect of Trilogy on SWD oviposition in choice tests

In no-choice tests, Trilogy did not perform well in reducing the oviposition activities (Fig 4)

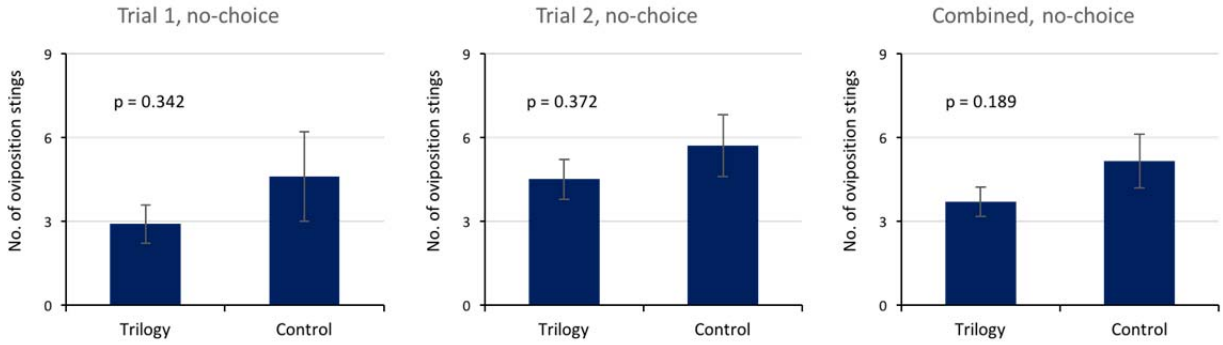


Fig. 4. Effect of Trilogy on SWD oviposition in no-choice tests

Debug Turbo. No significant effect of Debug Turbo was observed in no-choice test and two separate sets of choice bioassays. The effect was statistically significant when combined data from two sets of choice bioassays (Table 2).

Table 2. Effect of Debug Turbo on SWD oviposition

		Average no. of oviposition stings	SE	N	P-value	t	df
No-choice test							
Trial 1	Treated	6.7	0.790569	10	0.325006	2.100922	18
	Control	9.3	1.498888				
Choice test							
Trial 1	Treated	2.53	0.567926	15	0.192368	2.048407	28
	Control	3.93	0.880837				
Trial 2	Treated	3.125	0.790569	16	0.123185	2.042272	30
	Control	5.25	1.498888				
Combined	Treated	2.84	0.420181	31	0.041938	2.000298	60
	Control	4.614	0.742963				

SE=standard error, t = t-test value, df = degree of freedom

Triple Action Neem. We were able to conduct one set of choice test using this product, and there was a significant statistically difference between treated and control (Table 3).

Table 3. Effect of Triple Action Neem on SWD oviposition						
Choice test	**Average no. of oviposition stings**	**SE**	**N**	**P-value**	**t**	**df**
Treated	8.07	1.64	15	0.003394	2.048407	28
Control	17.47	2.43				

Azamax. Azamax did not effectively reduced the oviposition activities of the flies (Table 4).

Table 4. Effect of Azamax on SWD oviposition						
	Average no. of oviposition stings	**SE**	**N**	**P-value**	**t**	**df**
No-choice test						
Treated	1.5	0.428174	10	0.073471	2.100922	18
Control	2.8	0.533333				
Choice test						
Treated	0.6875	0.222673	10	0.512312	2.042272	18
Control	0.875	0.279881				

Neemix. There was no statistical difference between Neemix and control treatments when used at the rate of 4 oz/acre. However, increased rate Neemix (8 oz/acre) showed significant reduction in oviposition activities (Fig. 5). Only single set of choice bioassays was conducted for the Neemix evaluation due to time constraint.

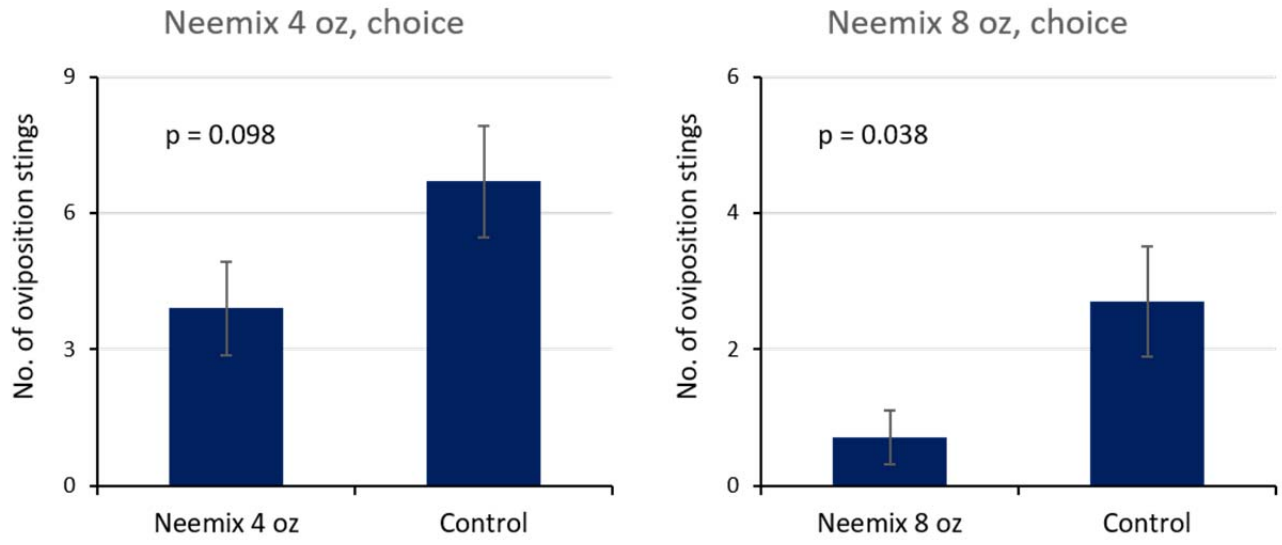
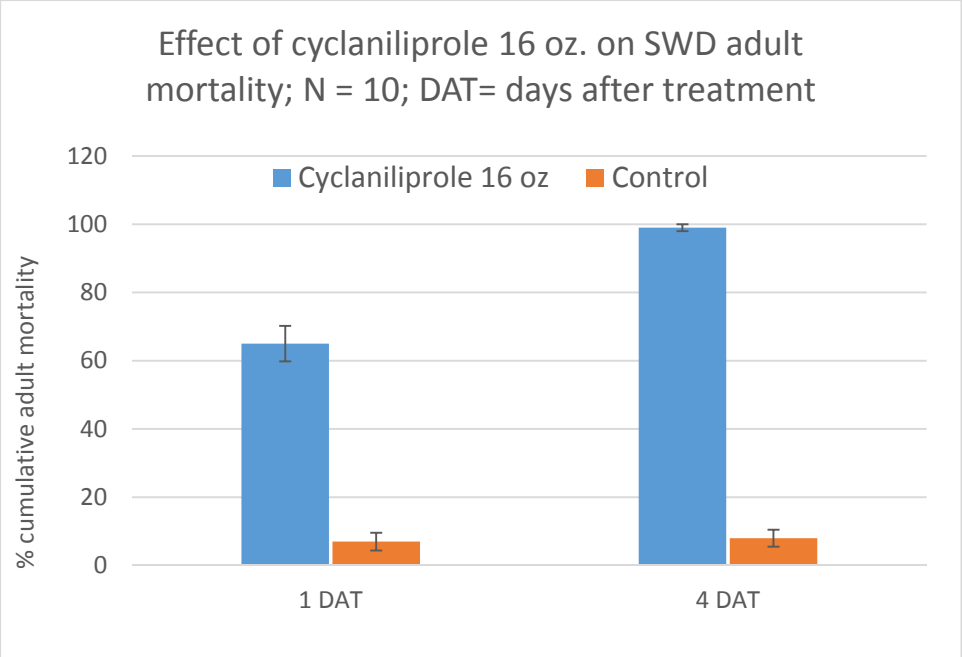
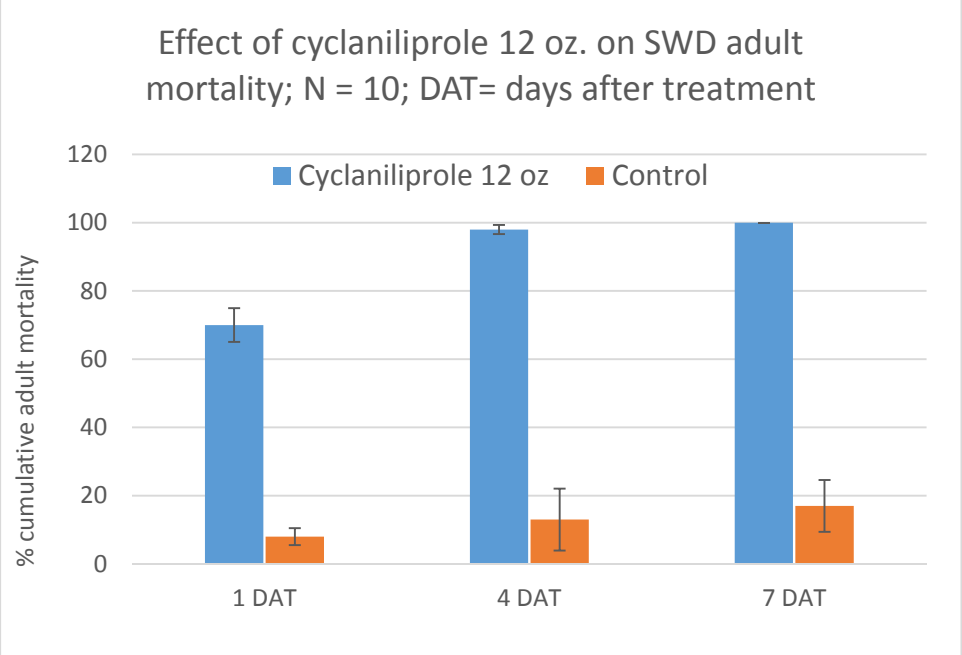


Fig. 5. Effect of Neemix on SWD oviposition in choice tests

Effect of cyclaniliprole on SWD adult mortality. Results showed that IKI-3106 (cyclaniliprole) at the rate of 12 fl oz/acre caused 70% mortality after 24 hours. The mortality was increased to 98% after 4 days and 100% at 7 DAT. Increasing the rate to 16 fl oz/acre did not improve the mortality significantly (i.e. 65% at 1 DAT; 99% at 4 DAT) compared to the 12 oz/acre rate used. Control mortalities were under 8% and 13% at 4 DAT for 16 oz. and 12 oz. rates, respectively.





Conclusion and plan for further studies

Neem products used in the bioassay showed effectiveness in reducing spotted wing drosophila oviposition activity. The most effective product was Bonide Neem Oil which contains 0.90% Clarified Hydrophobic Extract of Neem Oil. When neem oil is mixed with alcohol, azadirachtin is separated from the oil, and the remaining oil without azadirachtin is called clarified hydrophobic extract of neem oil. Another promising product is Neemix 4.5 that contains 4.5% azadirachtin. Triple Action Neem, which is, also containing hydrophobic extract of the neem oil showed a very good promise for future potential use in oviposition reduction. Currenlty, we are conducting further in-depth evaluation of some of these products particularly focusing on behavioral response of SWD females to few of these products in collaboration with Dr. Zain Syed, University of Notre Dame. This study is underway, thus results are not included in this report.

In 2017 season, our interest is to evaluate promising oviposition deterrent products using field cage studies in a commercial cherry orchards. One or two most promising treatments based on the lab study results will be selected for the field study. Five clusters with about 20 cherry fruits in each will be selected and and treated with the selected products. The other set of 5 clusters will be considered as control treatment. Fruits will be evaluated for the infestation of SWD on those clusters. Another sets of clusters will be sampled after 10 days of the original set up, and do similar evaluation. SWD activities on the orchard will be monitored by deploying SWD traps as well.

Based on this year results, what we found was neem products may be helpful in reducing the oviposition activities. The further research question is, can we combine currently used insecticides with the neem products improve the effectiveness in reducing the oviposition in cherries? In addition to continuation of the screening of more potential neem products, we will conduct studies by combining the currently used insecticides with the neem-based insecticides in laboratory and/or in the field.

Since we did not spend all the grant money allocated in 2016 for this project, we would like to request for the no-cost extension of this project for the funding cycle 2017/18.

BUDGET REQUEST	Budget Year -2017	
Funding Source		
Salaries and Benefits		
Postdocs/RA's		$_____
SRA's		$_____
Lab/Field Assistance		$0
Subtotal	Sub 2	$_Employee benefits

Supplies and Expenses Sub 3 $0_____

Equipment Sub 4

_____ $_____ Travel Sub 5

Department account number _____

_____

Date _____

Originator's Signature

COOPERATIVE EXTENSION County Director _____ Date _____

AGRICULTURAL EXPERIMENT STATION Department Chair_____ Date _____

Liaison Officer _____ Date _____

D2454-2(1/84)

(Rev. 9/96, 10/12)

University of California
Agriculture and Natural Resources

PROJECT REPORT/RESEARCH PROPOSAL

Project Year 2015-16 Anticipated Duration of Project 2 years

Project Leader Jhalendra Rijal Location UCCE-Stanislaus

Cooperating Personnel Janet Caprile, UCCE Contra Costa; Joseph Grant, UCCE San Joaquin

Project Title San Jose Scale Control in Cherries

Keywords San Jose scale, die-back, insecticide, integrated pest management, cherry

Commodity(s) Cherry Relevant AES/CE Project No._____

Problem and Previous Research Accomplishments:

San Jose Scale (SJS), *Diaspidiotus perniciosus* (Comstock), is a 'hard' type of scale insect with a very wide host range including major fruit crops (apple, pear), many nut crops, and stone fruits (peach, plum, nectarine, cherry). It has rarely been a problem in cherry production in the Northern San Joaquin Valley (NSJV) in the past. However, there has been a dramatic increase in the incidence of SJS occurring in cherries in recent years. Many infestations have been quite severe, resulting in rapid limb dieback, gumming, and tree death within a year or two (Caprile 2015). The increase in the incidence of this pest has coincided with the onset of the regular use of broad spectrum insecticides (pyrethroids and organophosphates) early in the season to control spotted wing drosophila. As we need to use these sprays to control spotted wing drosophila, we expect to continue to see problems with SJS outbreaks. It is presumed that these broad spectrum sprays have reduced the populations of the naturally occurring biological control which kept SJS in check in these orchards. At least three SJS parasitoids, *Encarsia perniciosi*, *Aphytis aonidiae*, and *Aphytis vandenboschi*, were reported as abundant in several tree, nut and stone fruit crops including cherry in San Joaquin Valley (UC IPM guidelines, Daane et al. 2002). Given the changing scenario of pest management in cherry, it is very important to assess occurrence and abundance of major biocontrol agents of SJS, and to reevaluate their role in today's California cherry production. Thus, we need to develop an effective management program appropriate for cherries.

Objectives and Anticipated Outcomes (2-year project)

1. Develop (or verify) a phenology and degree day model for San Jose Scale in cherry in the NSJV
2. Develop an effective management program for San Jose Scale in cherries in the NSJV
    a. Evaluate the role of biocontrol agents in cherry in the NSJV
    b. Evaluate control materials and timings in cherry in the NSJV
3. Develop and deliver an extension program for cherry growers and PCAs in the NSJV to help them identify and control SJS.

Plans and Procedures:

Phenology and degree day model evaluation. Phenology study was conducted in 9 commercial cherry orchards in Contra Costa, San Joaquin and Stanislaus Counties using pheromone traps (for male SJS) and sticky tapes (for crawlers). 4 pheromone traps were placed in each of four random trees within the orchard in 5 orchards located in Stanislaus and San Joaquin Counties while one trap and 2 tapes were deployed in a single tree in each of 4 orchards in Contra Costa County. Traps were read and serviced weekly from March through October.

Biocontrol activities assessment. Since major natural enemies of SJS are also attracted to SJS pheromone, the SJS pheromone traps also served to measure occurrence and abundance of the SJS biocontrol agents *Aphytis* spp. and *Encarsia* spp. Dormant spur sampling will be conducted during November-December to assess the extent of parasitism.

Results

Male SJS and biocontrol agents activity

In Stanislaus County, high male SJS activity was observed during April (4-20 April). Two parasites, *Aphytis* spp. and *Encarsia* spp. were abundant in all orchard sites, and their higher activities were coincided with the male SJS peak flight activity. In all three sites (Shiloh 1, Shiloh 2, Faithhome), peak counts of *Encarsia* spp. was significantly higher than the *Aphytis* spp. Activity (Fig. 1).

In San Joaquin County, Alpine site has three peaks of male SJS flight (23 March, 7 June, and 16 Aug). The activity of *Encarsia* spp. was higher and well coincided with the male SJS peak flights. The abundance of *Aphytis* spp. in this site was relatively low. (Fig. 2). In Buck site, male SJS activity was only observed in mid-August. Similar to Alpine, the overall activity of *Encarsia* spp. was higher than that of *Aphytis* spp (Fig. 2).

In Contra Costa County, male SJS flight activity was not observed in 1 of 4 sites. Peak activity of SJS males was observed during mid-June in Jason and Bloomfield sites, but was in mid-March in Pomeroy site. In contrast to San Joaquin site, *Aphytis* spp. was more abundant than *Encarsia* spp. (Fig. 3).

SJS crawler activity. Crawler counts were low in most of the orchard sites. Alpine site in San Joaquin County had moderate crawler activity with the highest count (1.08 crawler/cm tape length) on April 5. In Stanislaus sites, crawler activities were observed during April-May, and mid-August, although the overall crawler counts was low. Since the trapping activity is still underway, we will report the final results of this study at a later date.

Plan for 2016-17:

Continuation of the phonology study. As we have proposed in our 2015/16 proposal, this project was planned for two consecutive field seasons. Currently, we have conducted phonological studies of SJS males, crawlers, and SJS parasites, and will continue in 2017 season as well. Also, we would like to include 2-4 orchards from the southern San Joaquin Valley in 2017 season. Combining southern San Joaquin Valley, this study will provide a comprehensive data to develop a phenology model that will be applicable in major cherry production regions in California.

Insecticide evaluation. Spray trials will be conducted in commercial cherry orchards in the Northern SJV to evaluate the effectiveness of materials and timing. Materials to be evaluated include various rates of oil and/or growth regulators (Centaur or Seize). Timings to be evaluated include dormant (2016- 2017) and in-season (April/May 2017) if the spring timing does not interfere with cherry harvest. We will utilize the monitoring data that we have generated in 2016 field season to conduct in-season insecticide trials to test the effectiveness of spray materials registered in cherries, and determine effective timing options

The details of the treatments and designs (single tree vs block treatment) will be decided based on the condition and extent of SJS problem in available orchards. We will coordinate with growers in finding orchards with a moderate-to-high infestation of SJS. The degree of infestation will be determined by the dormant season fruit spur sampling prior to spraying which will consist of taking 10-20 spurs per tree from 1-2 trees from each orchard and recording the number, the stages (adult, crawler) and the conditions (i.e., live, dead, parasitized) of SJS on those spurs. The effectiveness of sprays will be evaluated with pheromone traps and crawler tapes during the 2017 season.

Extension and delivery of the results.
Scientific information derived from this project will be delivered to clientele using several delivery methods including field days/meetings, newsletters, and online blog (www.ipmcorner.com).

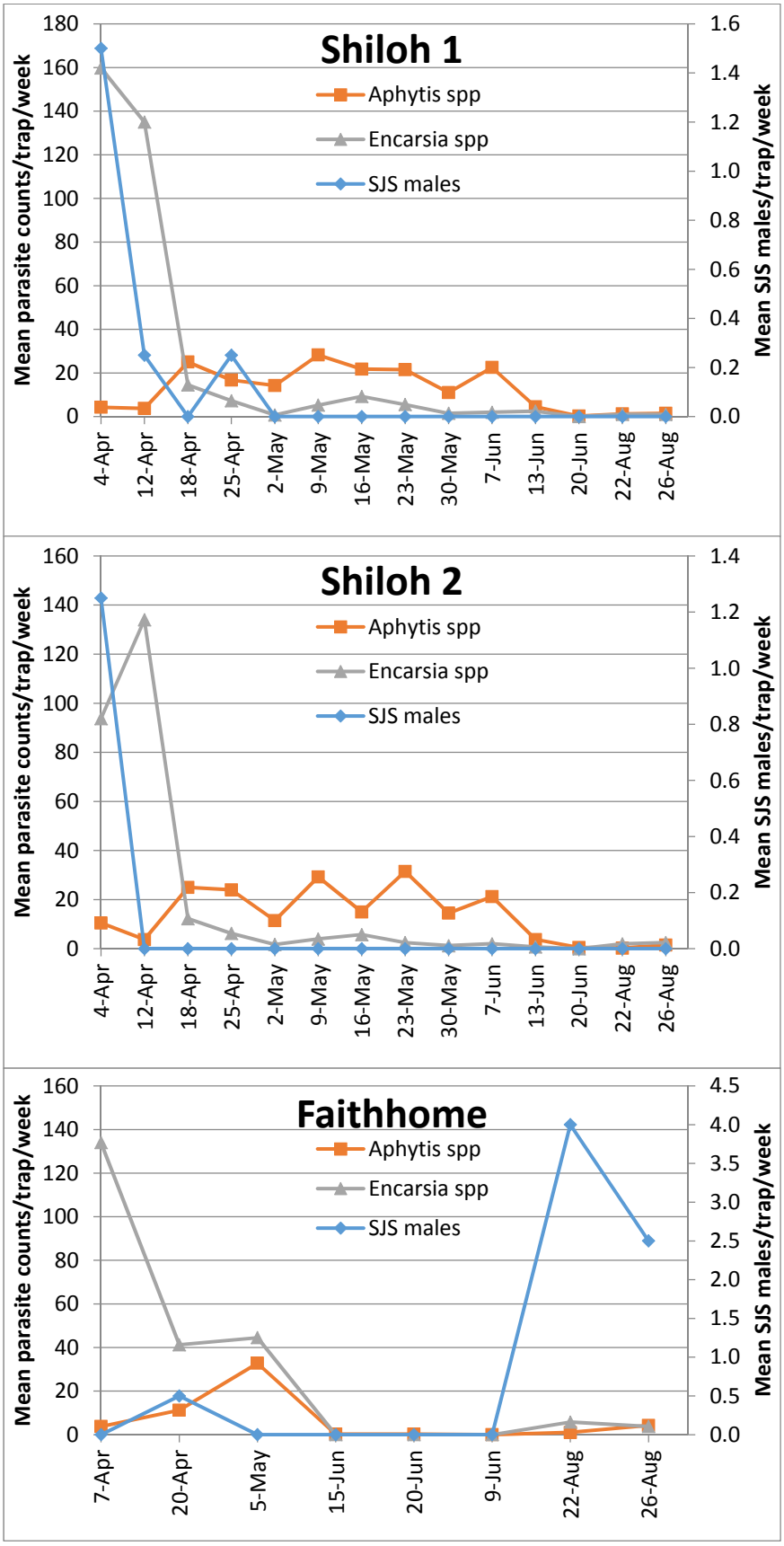


Fig. 1. Activity of male SJS and SJS parasites in three cherry orchard sites in Stanislaus County

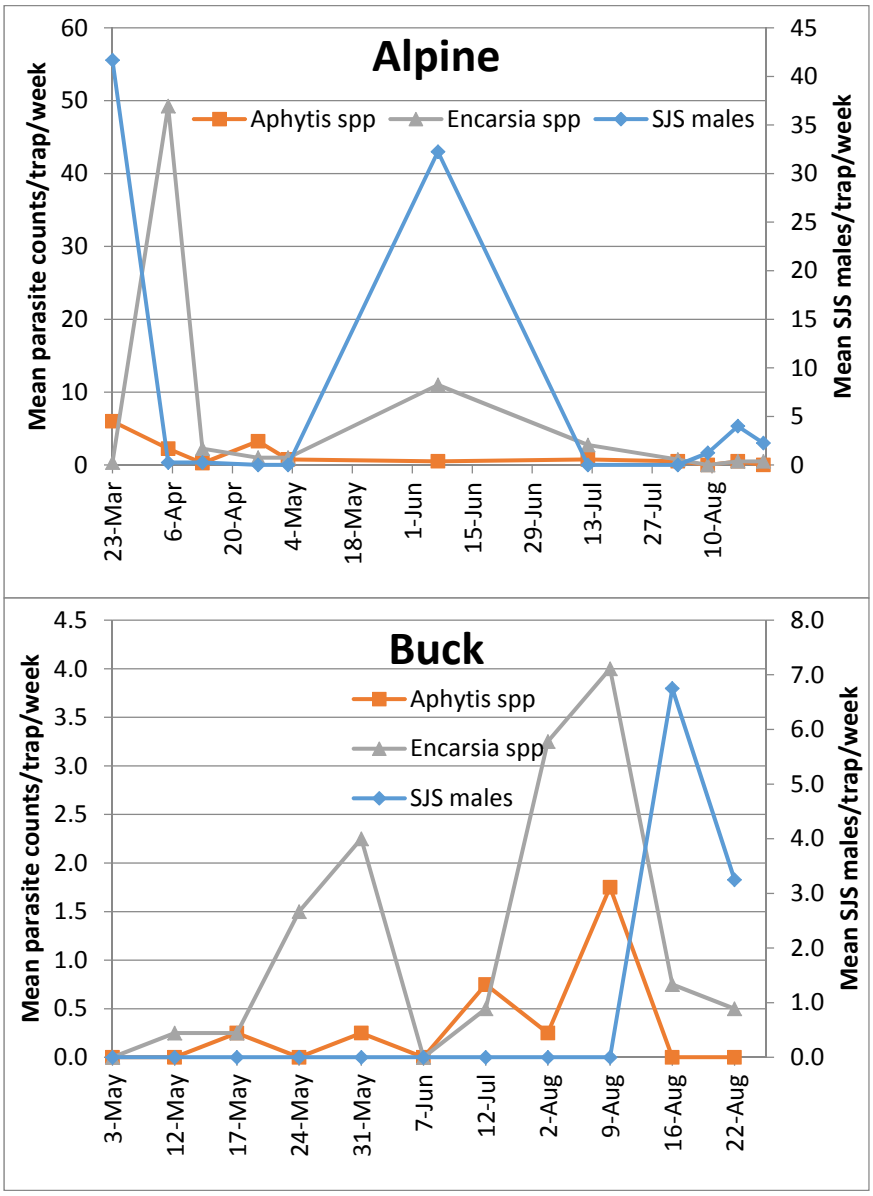


Fig. 2. Activity of male SJS and SJS parasites in three cherry orchard sites in San Joaquin County

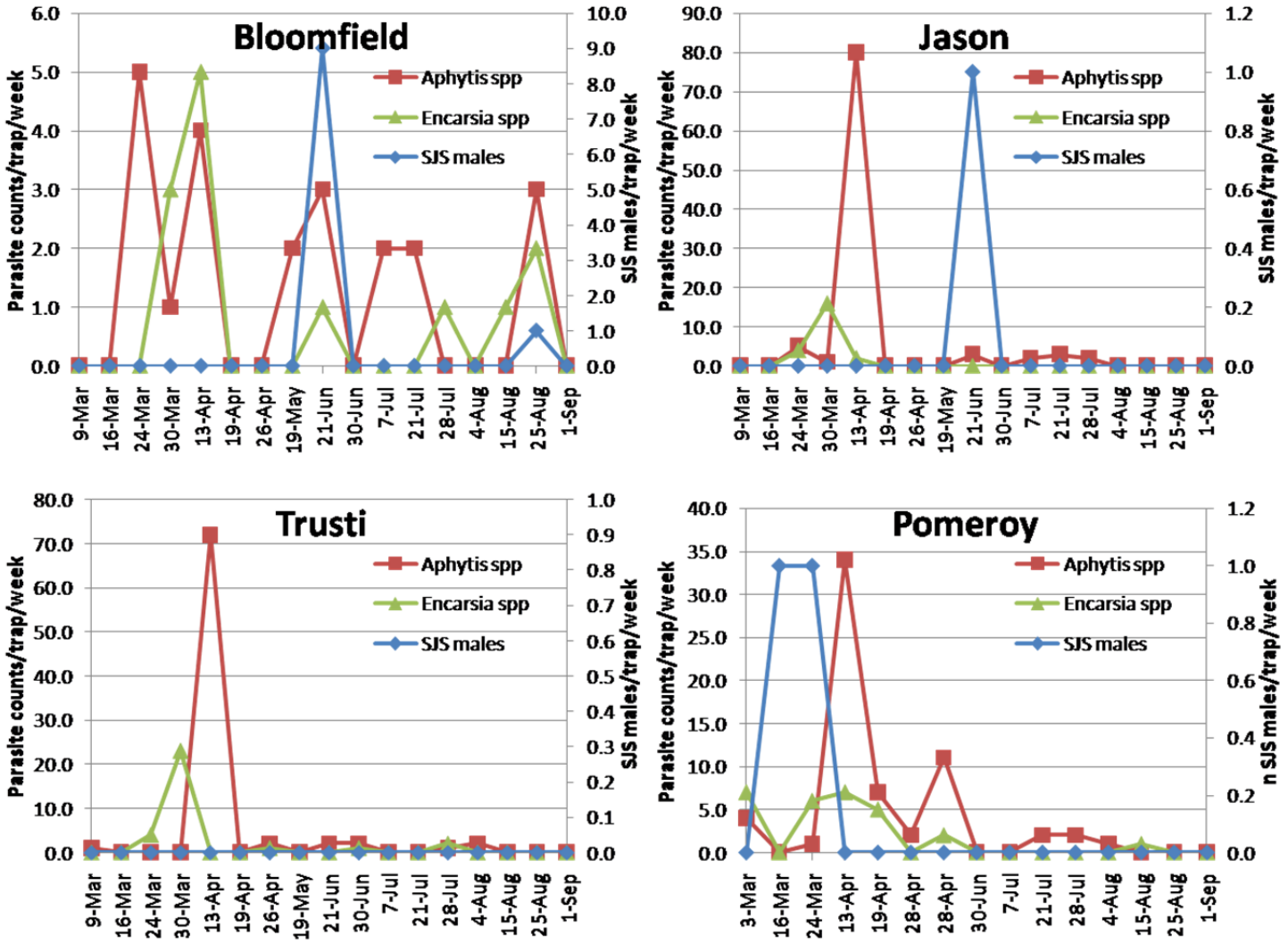


Fig. 3. Activity of male SJS and SJS parasites in three cherry orchard sites in Contra Costa County

BUDGET REQUEST Budget Year

Funding Source

Salaries and Benefits

Postdocs/RA's $_____

SRA's $_____

Lab/Field Assistance $__7500_____

Subtotal Sub 2

$_____Employee benefits Sub 6

$_____ SUBTOTAL

$_____

Supplies and Expenses Sub 3 $__500_____

Equipment Sub 4

_____$_____Travel Sub 5

$__2000_____ TOTAL

$__10000_____

Department account number _____

Date _____

Originator's Signature

COOPERATIVE EXTENSION County Director _____ Date _____

AGRICULTURAL EXPERIMENT STATION Department Chair_____ Date _____

Liaison Officer _____ Date _____

D2454-2(1/84)
(Rev. 9/96, 10/12)

References:

Daane, K. M., G. Y. Yokota, W. J. Bentley, K. Sime, and B. Hogg. 2002. San Jose scale and its natural enemies: investigating natural or augmented controls.California Tree Fruit Agreement, Research report-2002. p29.

Rice, R.E., and R.A. Jones. 1982. Collections of Prospaltellaperniciosi Tower (Hymenoptera: Aphelinidae) on San Jose scale (Homoptera: Diaspididae) pheromone. Environmental Entomology, 11: 876-880.

McClain, D.C., G.C. Rock, and J.B. Whoolley. 1990. Influence of trap color and San Jose scale (Homoptera: Diaspididae) pheromone on sticky trap catches of 10 aphelinid parasitoids (Hymenoptera). Environmental Entomology, 19: 926-931.

UC IPM guidelines for San Jose scale in cherry. http://www.ipm.ucdavis.edu/PMG/r105301111.html
UC IPM guidelines for San Jose scale in almond. http://www.ipm.ucdavis.edu/PMG/r3300811.html

Caprile, J. San Jose scale alert. Crop current newsletter.
http://cecontracosta.ucanr.edu/newsletters/Crop_Currents58856.pdf

PROJECT PERFORMANCE REPORT
University of California - Agriculture and Natural Resources

- **Name of Grantee:** California Cherry Board.

- **Name of Grantee Point of Contact:** Nick Matteis, Research coordinator
  Address: 1521 "I" St., Sacramento, CA. 95814
  Phone: 916-441-1063
  Fax: 916-446-1063
  Email: nmatteis@agamsi.com
  Employer Identification Number: 38-2740166

- **Type of Report:** Performance Report.

- **Submission date**: December 8, 2016

- **Project Title:** Better understanding of sweet cherry postharvest cracking in California and potential strategies to reduce its incidence.

- **Project summary:**

This document represents a performance report (April 2016 – December 2016) for the findings of the project involving the better understanding of sweet cherry postharvest cracking in California and potential strategies to reduce its incidence. Examples of cherry cracking, commonly found in California Cherry varieties, can be seen in Fig. 1.

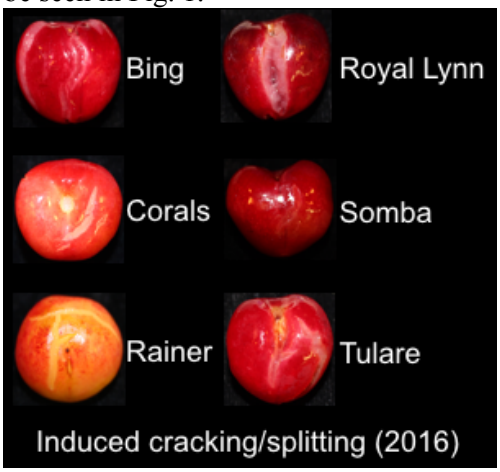


Fig. 1. Examples of different types of undesirable sweet cherry cracking/splitting

Cherry cracking or splitting is undesirable and difficult to detect and/or reduce, regardless of the type of splitting. Much work has gone into reducing the incidence of cherry cracking in before harvest, as preharvest-sprayed application of compounds that reduce the osmotic potential of rainwater (e.g. 0.5% calcium chlorine – CaCl2) or hydrophobic compounds (e.g. RainGard®) to reduce water intake. While these efforts have reduced the incidence of rain-induced fruit cracking, they have not kept pace with the stricter consume, especially when it is hypothesis that the processing line might increase cherry cracking.

The long-term goal of our work is to provide the California sweet cherry industry with advancements and information that will allow them to market a high quality product. Subsequently this will assist the cherry industry in sustainability through accessing continued and new technology utilizations. The aim of this first-year effort is to study concept development; which will potentially

allow for application of technologies in the cherry industry. It is hypothesized that better understandings of the postharvest cherry cracking phenomena will enable us to predict the incidence of cracking in relationship to cherry properties as well as pre-cooling and postharvest managements. Therefore, this study provides preliminary information (initial survey) as outcomes in the form of basic concepts toward the better understanding of postharvest cherry cracking in California. Preliminary information includes the effect of rapid hydro-cooling, physical characteristics of cherry tissue leading to differences in cherry cracking, and investigation into the use of non-corrosive salts or sugars to reduce hydro-cooling water osmotic potential, as is similarly utilized at the pre-harvest stage.

In this project, the following short-term (1-year) objectives will be pursued:

1. Investigate if the packaging line and the presence of surface moisture induces cherry cracking.
2. Assess if different postharvest cherry properties can be used to predict cracking, including: Firmness, color, weight, size, ºBrix, Near-infrared (NIR) absorbance, NIR reflectance, and Dry matter content.
3. Evaluate if changes in the osmotic potential (calcium and sucrose) and temperature (32, 41, and 50 ºF) of hydro-cooling water will reduce sweet cherry cracking.

- **Project approach:**

Included is a description of performed activities for the project objectives.

- **Objective 1: Investigate if the packaging line and the presence of surface moisture induces cherry cracking.**

An overview of the experimental design for Objective 1 is included in Fig. 2.

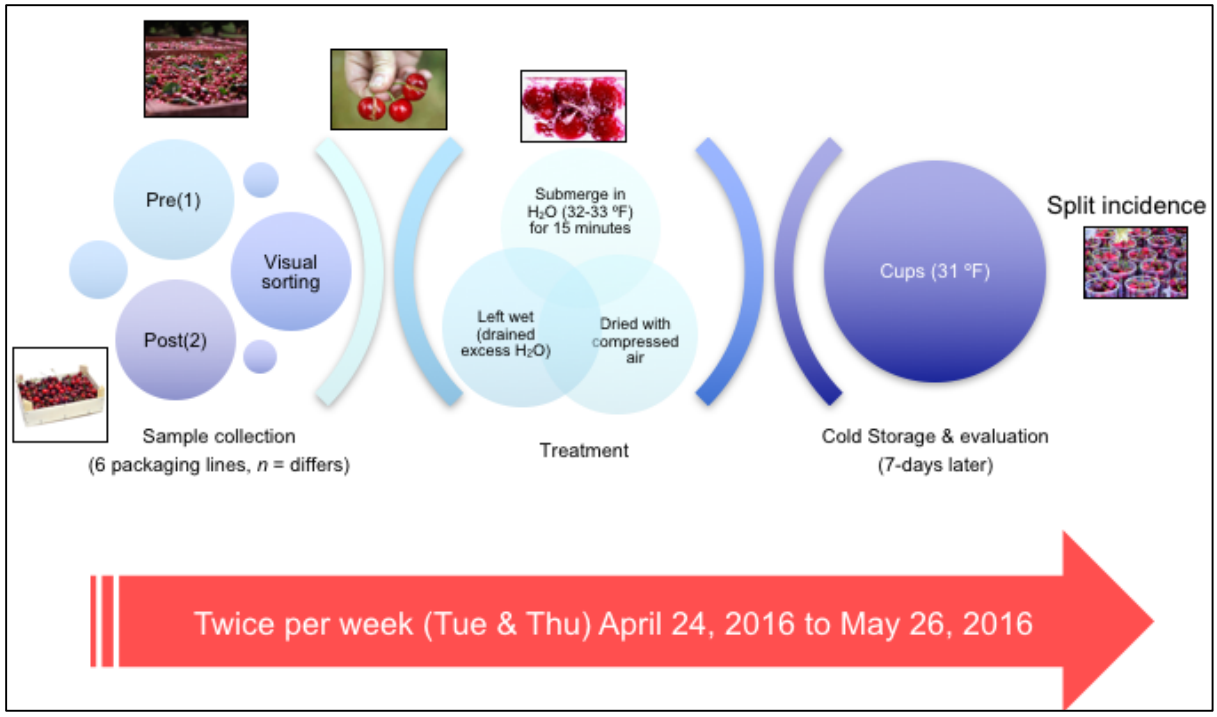


Fig. 2. Objective 1 experimental design

- **Tasks and outcomes 1 (Objective 1): Pre-packaging (1) and post-packaging sample collection and preparation.**

A total of 4,706 physiologically mature none-cracked fresh cherry samples (8-varieties) were picked and evaluated for Objective 1, from April 2016 – October 2016. Number of cherries per variety differs, but it was a minimum of 120 pre-packaged (1) and 120 post-packaged cherries (2) per variety. Original pre-packaged and post-packaged cherry samples were collected twice a week from 6-different processing lines across California (Stockton, Lodi, and Linden), and were subsequently processed at UC Davis at the Department of Biological and Agricultural Engineering, and the Postharvest technology laboratory.

- **Tasks and outcomes 2 (Objective 1): Treatment.**

After sample preparation, all cherries were subsequently submerged in deionized water at around 0.5 °C (33 °F) for 15-minutes to simulate the procedure during packaging. Afterwards, water was drained from samples and 50% were left superficially wet, while water was superficially removed from the additional 50% using compressed air (Pressure = 40 in of water, 1.4 psi). Immediately, cherries were transferred to plastic cups in groups of 20 cherries.

- **Tasks and outcomes 3 (Objective 1): Cold storage & Evaluation.**

Cups containing cherry samples were covered with plastic bags and stored in a walk-in a cooler at around -0.5°C (31 °F). After 7-days, the incidence of splitted cherries was visually determined and expressed as the ratio between the total numbers of cherries and the number of spitted cherries. Higher incidence values indicate that cherries in each sample split at a higher rate.

Fig. 3 offers an outline of the variation of splitting incidence from different cherry varieties collected before the packaging line or pre-packaged (Type 1) and after the packaging line or post-packaged cherries (Type 2). In every variety, cherries collected before the packaging line (Type 1) contain lower splitting incidence in comparison to cherries collected after the processing line (Type 2). Brooks, Corals, Garnet, Royal Lynn, and Somba cherry varieties collected before the packaging line (Type 1) contain statistically lower splitting incidence (P = 0.05) in comparison to cherries collected after the processing line (Type 2). Findings in Fig. 3 indicates that the processing line plays an important role in increasing cherry splitting. It is hypothesized, that the increase in cherry splitting incidence might be caused by the water intake, temperature fluctuations, and/or skin damage while cherries are moving through the packaging line. Other unknown factors, like the pre-harvest foliar application of gibberellins might also induce cracking.

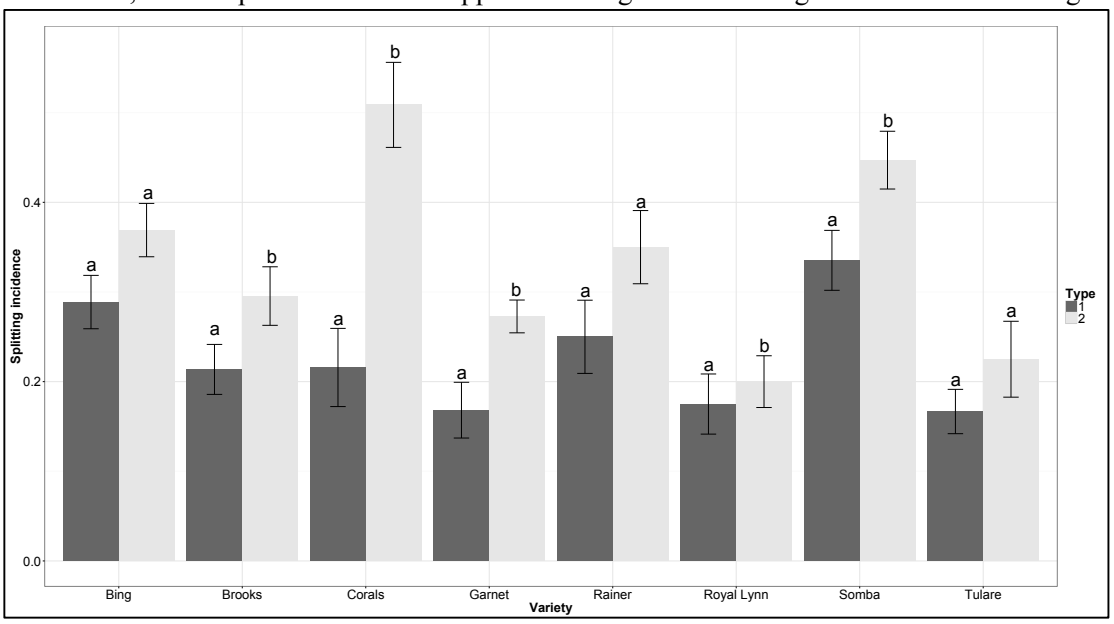


Fig. 3. Splitting incidence from different cherry varieties collected before the packaging line or pre-packaged (Type 1) and after the packaging line or post-packaged cherries (Type 2). Bars followed by the same letter, within a specific variety, are not significantly different between each other at P = 0.05 (t-test). Black vertical lines (error bars) reflect the sample standard deviation.

Fig. 4 shows the variation of splitting incidence from different cherry varieties either dry (compressed air) or wet before storage, regardless if samples were collected before the packaging line or pre-packaged (Type 1) and after the packaging line or post-packaged cherries (Type 2). In every variety, cherries that were dried before storage contain lower splitting incidence in comparison to cherries that were left wet before storage. Dried Bing, Brooks, Corals, Royal Lynn, and Somba cherry contained statistically lower splitting incidence (P = 0.05) in comparison to their respective wet group. Results in Fig. 4 denote that drying cherries before storage might significantly reduce the incidence of post-harvest splitting. Further evaluation is required to infer if large scale drying systems (*e.g.* air knife system) might significantly reduce cherry splitting.

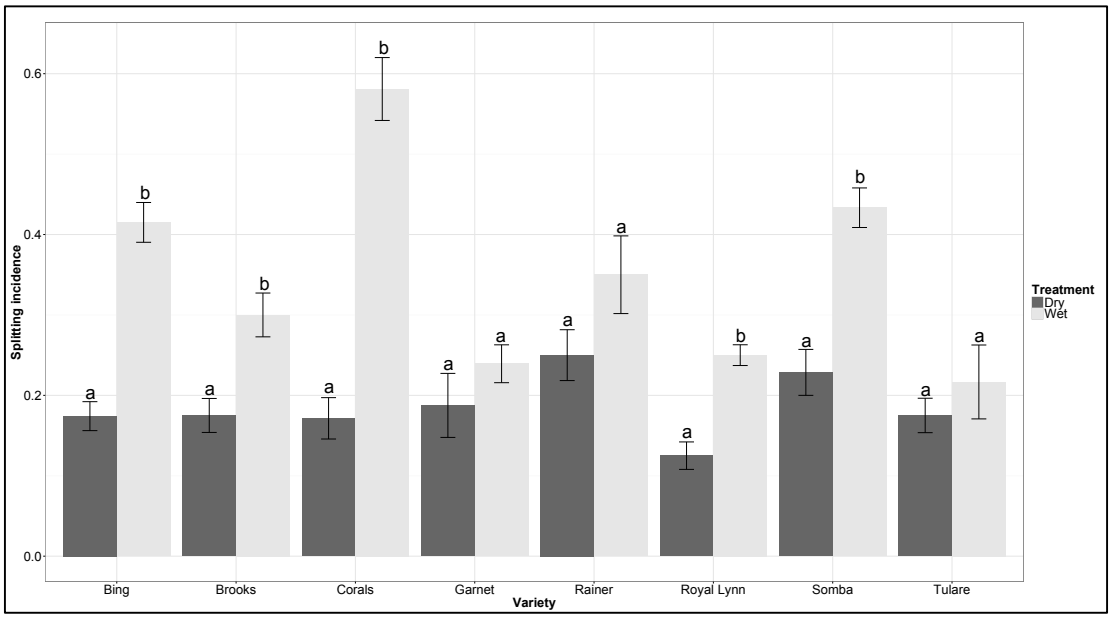


Fig. 4. Splitting incidence from 8 cherry varieties. Bars followed by the same letter, within a specific variety, are not significantly different between each other at P = 0.05 (t-test). Black vertical lines (error bars) reflect the sample standard deviation.

- **Objective 2: Assess if different postharvest cherry properties can be used to predict cracking, including: Firmness, color, weight, size, ºBrix, Near-infrared (NIR) absorbance, NIR reflectance, and Dry matter content.**

An overview of the experimental design for Objective 2 is included in Fig. 5.

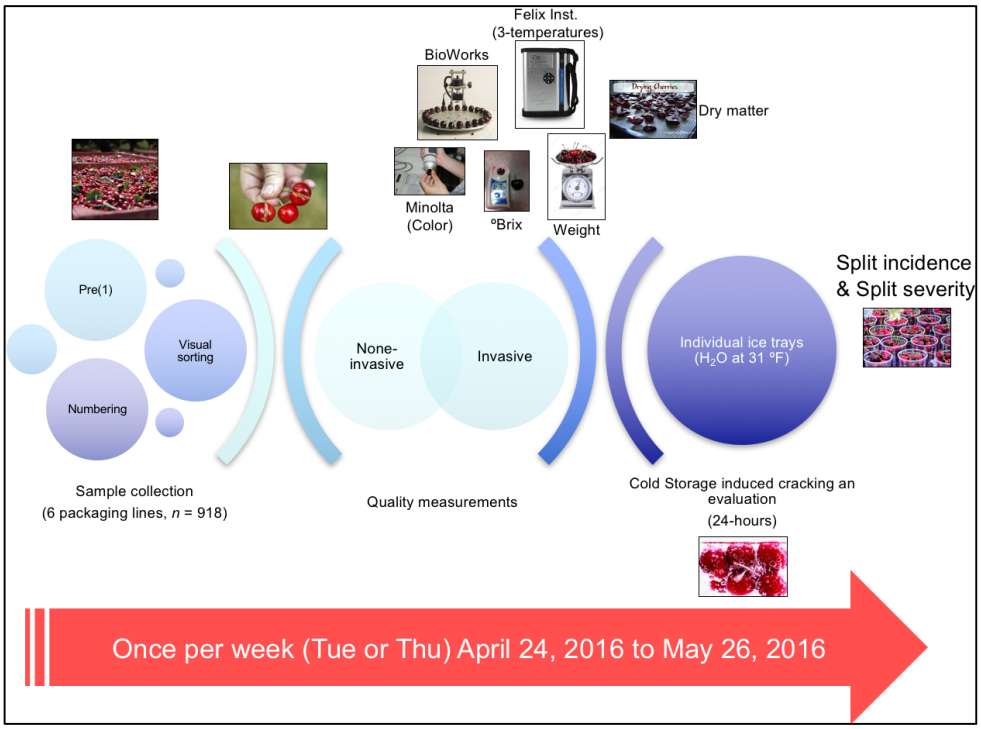


Fig. 5. Objective 2 experimental design

- **Tasks and outcomes 1 (Objective 2): Sample collection and preparation.**

A total of 918 physiologically mature none-cracked fresh cherry samples (7-varieties) were picked and evaluated for Objective 2, from April 2016 – October 2016. Number of cherries per variety differs, but it was a minimum of 75 pre-packaged (1) cherries per variety. Samples were collected twice a week from 6-different processing lines across California (Stockton, Lodi, and Linden), and were subsequently processed at UC Davis at the Department of Biological and Agricultural Engineering, and the Postharvest technology laboratory.

- **Tasks and outcomes 2 (Objective 2): Quality measurements.**

After sample preparation, all cherries were individually analyzed for the following quality properties/parameters: Firmness and size (BioWorks Firmness test equipment), color (Handheld Minolta), weight, ºBrix (Atago handheld analyzer), Near-infrared (NIR) absorbance and NIR reflectance at three different temperatures – 1°C (33.8 °F)/10 °C (50 °F)/25 °C (77 °F) (Felix instruments), and Dry matter content (weight after over drying).

- **Tasks and outcomes 3 (Objective 2): Cold storage induced cracking & Evaluation.**

After quality measurements, each cherry was submerged in a tray containing deionized water at around 0.5 °C (33 °F) for 24-hours to induce cracking. Afterward, the severity of cracked cherries was measured and expressed the mean size (mm) of the cracks (MeanSplit). Higher severity values indicate cherries with higher degrees of cracking. This method is broadly applied across multiple produce including table grapes, as it is a good indicator if the produce will crack/split.

Fig. 6 shows the relationship between quality measurements and the severity of cracked cherries (MeanSplit) (linear models) with their corresponding coefficients of determination (R^2), and histogram distribution. R^2 is a handy, seemingly intuitive measure of how well the linear model fits a set of observations. R^2 provides an estimate of the strength of the relationship between the distribution of the different measurements. In other words, high R^2 values (> 0.8) indicate if a specific cherry property can be used to predict cracking. Unfortunately, none of the measured properties can accurately predict cherry splitting, therefore we are unable to forecast cracking by measuring postharvest cherry properties. The

only high relationship is between the initial weight (wet) and cherry size, which is expected, as larger cherries tend to be heavier. The same scenario was observed by evaluating each variety separately, and by applying this to NIR absorbance and reflectance (Data not included), Nontheless, other methodologies and analysis are currently being performed and evaluated to better infer if cracking can be predicted by NIR spectroscopy.

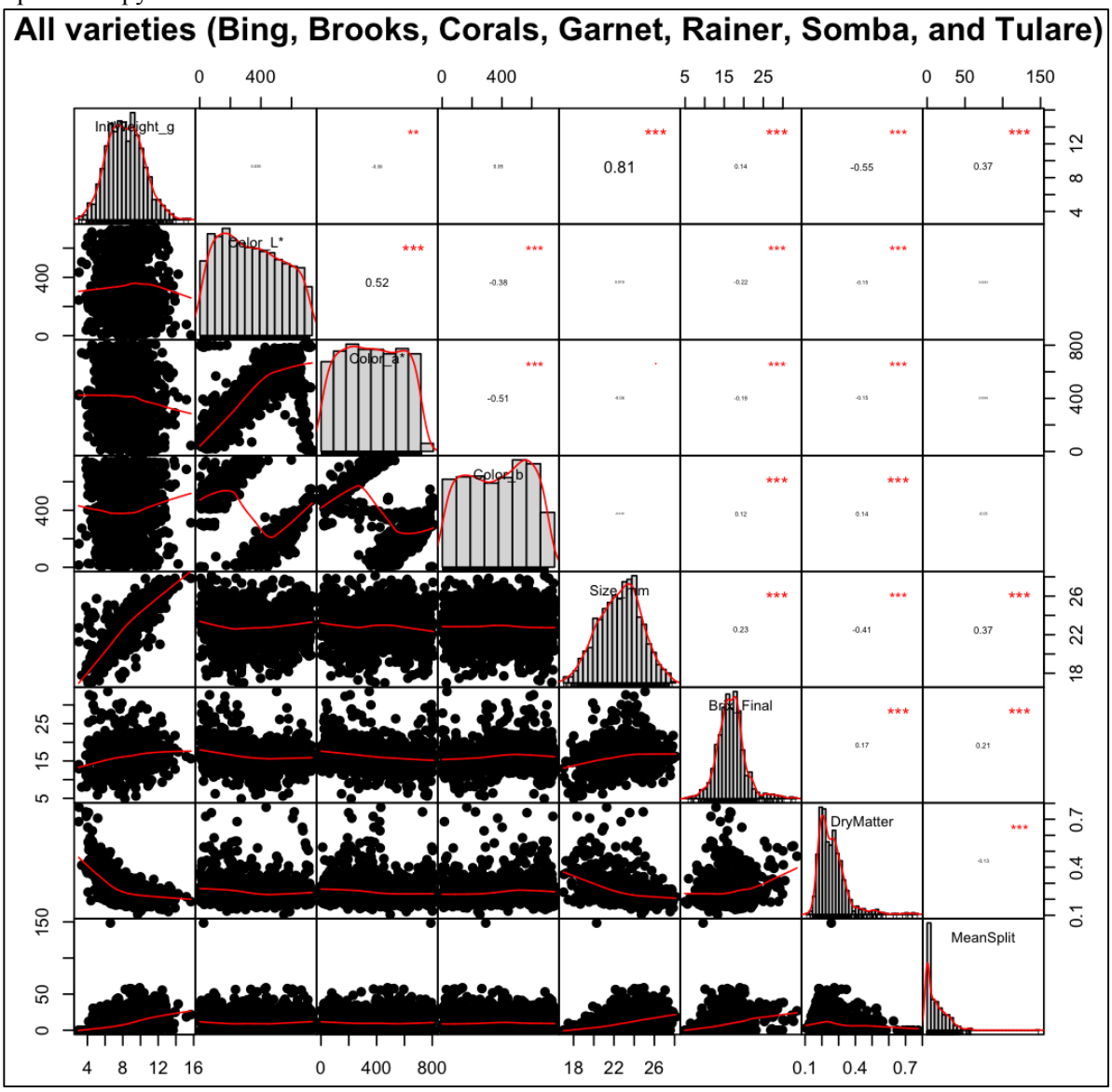


Fig. 6. Relationship between quality measurements and the severity of cracked cherries (MeanSplit) (linear models) with their corresponding coefficients of determination (R2), and histogram distribution

- **<u>Objective 3: Evaluate if changes in the osmotic potential (calcium and sucrose) and temperature (32, 41, and 50 ºF) of hydro-cooling water will reduce sweet cherry cracking.</u>**

An overview of the experimental design for Objective 3 is included in Fig. 7.

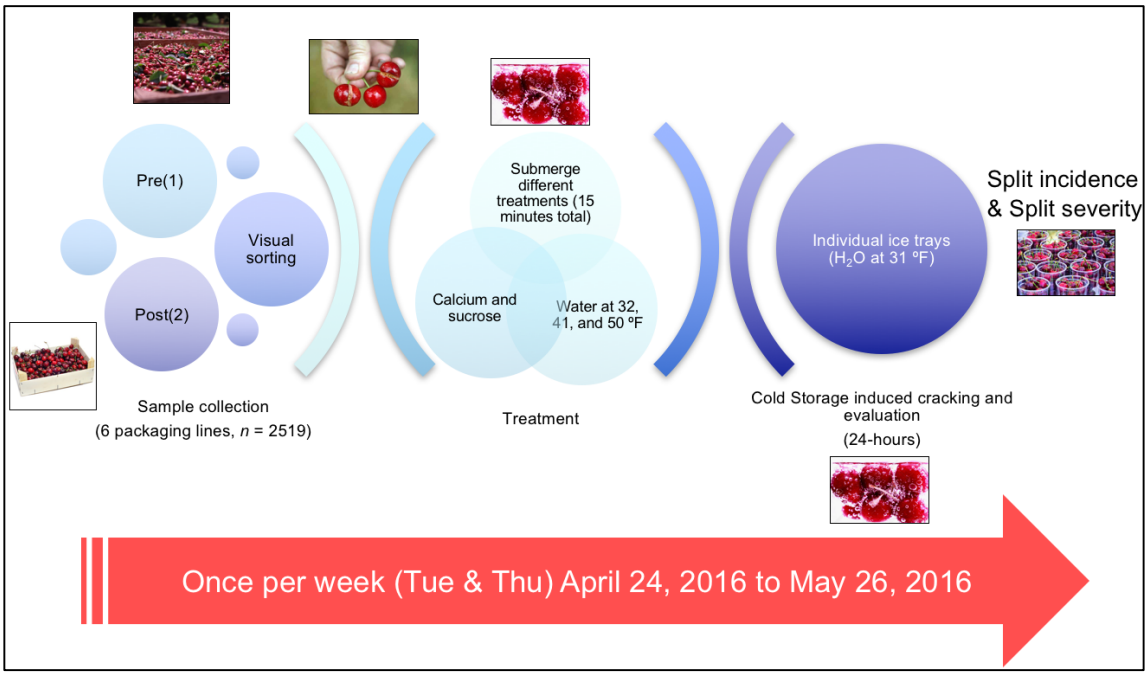


Fig. 7. Objective 3 experimental design

- **Tasks and outcomes 1 (Objective 3): Pre-packaging (1) and post-packaging sample collection and preparation.**

A total of 2,519 physiologically mature none-cracked fresh cherry samples (8-varieties) were picked and evaluated for Objective 1, from April 2016 – October 2016. Number of cherries per variety differs, but it was a minimum of 150 pre-packaged (1) and 150 post-packaged cherries (2) per variety. Original pre-packaged and post-packaged cherry samples were collected twice a week from 6-different processing lines across California (Stockton, Lodi, and Linden), and were subsequently processed at UC Davis at the Department of Biological and Agricultural Engineering, and the Postharvest technology laboratory.

- **Tasks and outcomes 2 (Objective 3): Treatment.**

After sample preparation, all cherries (25°C/77 °F) were subsequently submerged in treatments (Table 1). Immediately after, each cherry was submerged in a tray containing deionized water at around 0.5 °C (33 °F) for 24-hours to induce cracking. Afterward, the severity of cracked cherries was measured and expressed the mean size (mm) of the cracks (MeanSplit). As in objective 2, higher severity values indicate cherries with higher degrees of cracking.

Table 1. Factorial design - Treatments for objective 3.

Treatment	Calcium (%)	Calcium (g)	Sucrose (%)	Sucrose (g)	Temp. (°C)
1 (1-20)	0	0	1	30	5
2 (21-40),3 (41-60) ,12 (221-240)	0.5	45	0.5	45	5
4 (61-80)	1	30	0.5	15	10
5 (81-100)	0.5	15	0	0	10
6 (101-120)	0	0	0.5	15	0
7 (121-140)	0	0	0.5	15	10
8 (141-180)	0	0	0	0	5
9 (161-180)	1	30	0	0	5
10 (181-200)	0.5	15	1	30	0
11 (201-220)	0.5	15	1	30	10
13 (241-260)	1	30	1	30	5
14 (261-280)	1	30	0.5	15	0
15 (281-300)	0.5	15	0	0	0
16 (301-320)	1	30	1	30	10
17 (321-340)	0	0	0	0	0

- **Tasks and outcomes 3 (Objective 2): Cold storage induced cracking & Evaluation.**

Figs. 8, 9, and 10 show a set of contour plots that reflect the degree of change in cherry splitting severity in relationship to the different treatments. A contour plot is a graphical technique for representing a 3-dimensional surface by plotting constant cherry split severity lines, called contours, on a 2-dimensional format. That is, given a value for split severity, lines are drawn for connecting the (*x,y*) coordinates (treatments) where that split severity value occurs. In the contour plots, a low split severity is expressed and colored in green, while a larger split severity is conveyed as yellow, brown and white. As it can be seen, the steepest descent in split incidence occurs when Sucrose (%) and $CaCl_2$ (%) concentrations increase (Fig. 8). But, also when cherry temperature is gradually reduced from initial (25°C/77 °F) to final temperature (0.5°C/33 °F) – Figs. 9, and 10. These findings confirm that higher concentration of both $CaCl_2$ (%) and Sucrose (%), as well as the gradual reduction in cherry temperature will significantly reduce splitting.

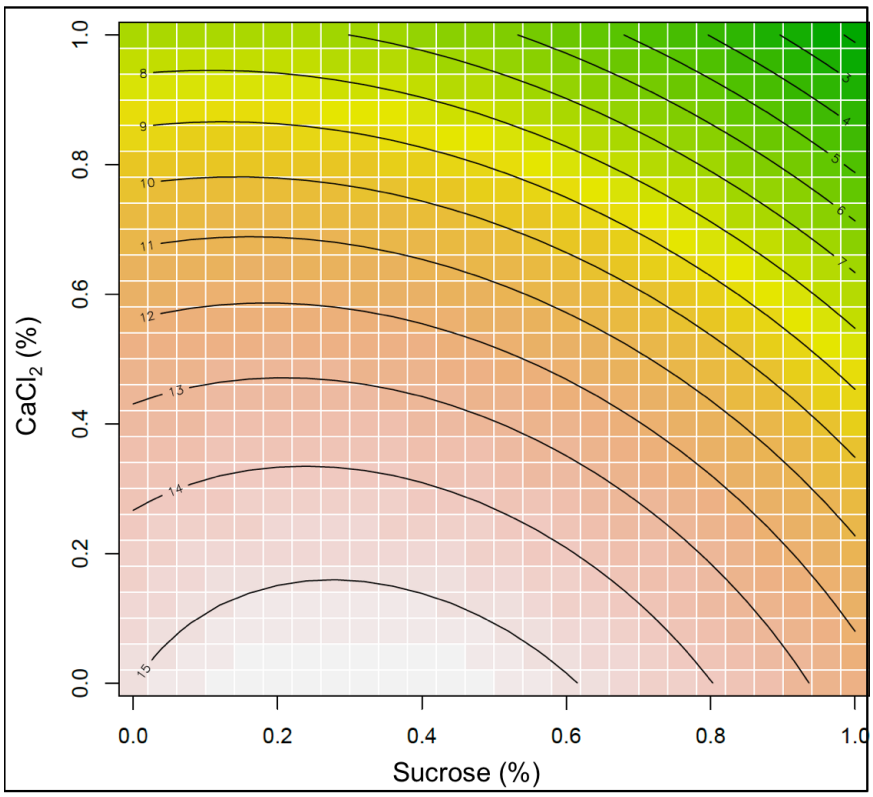


Fig. 8. Split severity in relationship to changes on Sucrose (%) and $CaCl_2$ concentration

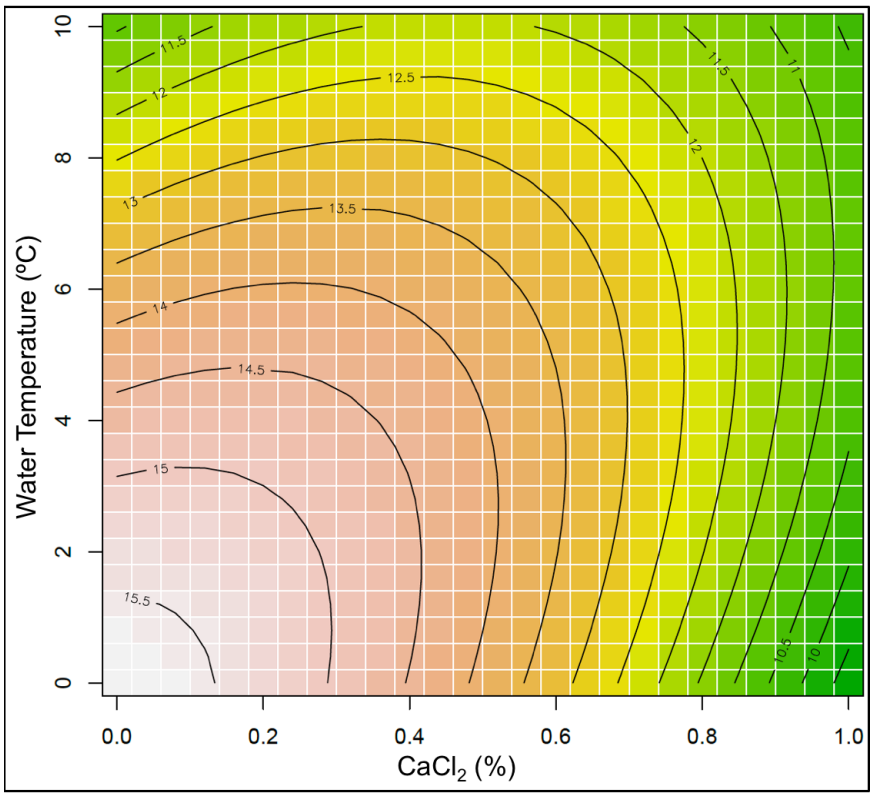


Fig. 9. Split severity in relationship to changes on $CaCl_2$ conc. (%) and water temperature (°C)

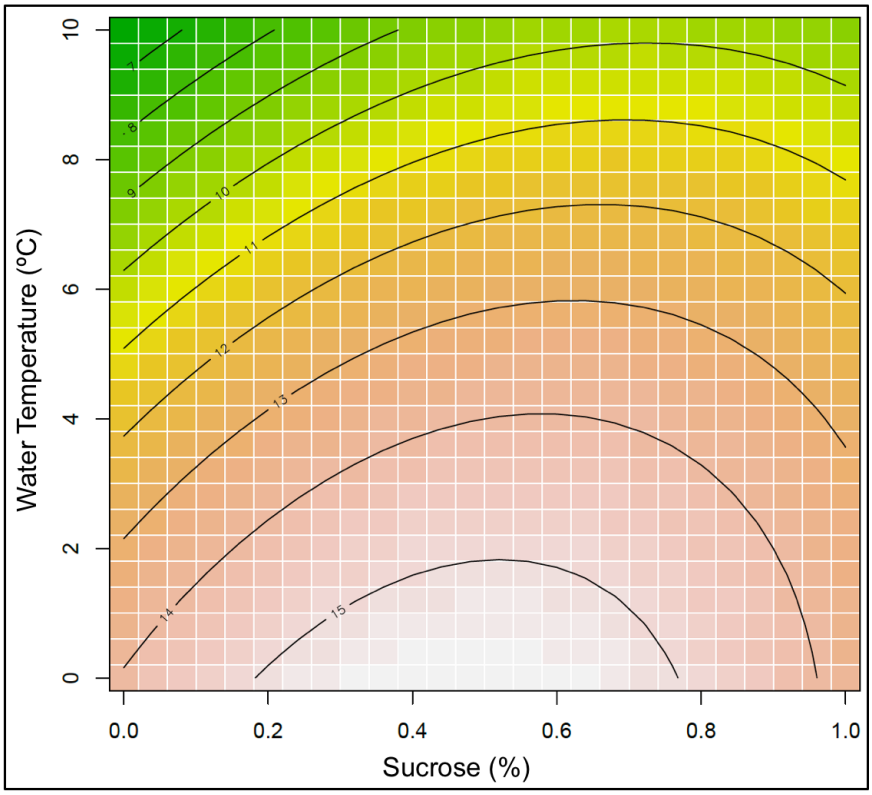


Fig. 10. Split severity in relationship to changes on Sucrose conc. (%) and water temperature (°C)

- **<u>Beneficiaries and conclusions:</u>**

Better understanding of the effect of the packaging line in the incidence of cherry splitting will potentially enable the California cherry industry as a whole (producers and processors) to offer a better quality product, therefore increasing consumer satisfaction and decreasing industry liability issues. Generated information will contribute toward the evaluation of technologies, which are practical and suitable to apply for current packaging lines to significantly reduce cherry splitting. Unfortunately, there is no evidence that cherry property measurements can effectively predict the severity of cherry splitting.

Currently, based on our studies, there is a risk of introducing approximately 35 lb. of undesirable splitted cherries per 100 lb. of package product, if cherries are not dried before storage. This is unacceptable by the cherry industry, as it represents a high quality concern. By removing water from the cherries surface (drying), before storage, by using commercially available techniques (*e.g.* air knife systems) there is a potential that the cherry industry might reduce the risk of introducing splitted cherries down to around 19 lb. per 100 lb. of packaged product. In addition, it can be seen that cherry split severity can be significantly reduced by adding Sucrose and $CaCl_2$ to the water, and by gradually reducing the initial (25°C/77 °F) temperature of the cherries to their final storage temperature (0.5°C/33 °F).

GENETIC SOLUTIONS FOR BIOLOGICAL PEST CONTROL: A SYSTEMIC APPROACH TO SUSTAINABLE AGRICULTURE PRODUCTION WITHOUT PESTICIDES

A REPORT TO THE CALIFORNIA CHERRY BOARD

Submitted by

Technology Innovation Group

December 9, 2016

INTRODUCTION

An invasive insect, the Spotted Wing Drosophila (SWD), *Drosophila suzukii*, was first identified in California cherry orchards in 2008. Although native to Asia, the species quickly spread to most agricultural areas in the State and to other states in the U.S., and is now resident across the country. Researchers at North Carolina State University have formed a SWD working group to monitor the effects of this insect, and their report for 2014 estimates that the potential national crop loss due to SWD, based on 2014 average damage surveys, is $1.3 billion to cherry, berry and other soft fruit in the U.S.[1]

California cherry growers, through the California Cherry Board, have been active in addressing the challenge of mitigating the threat posed by SWD. For example, the Cherry Board has funded researchers at Cal Tech and UC Riverside for developing SWD that have the trait of expressing desired genes with the intent to dramatically reduce or eliminate populations of SWD in the U.S. To facilitate the introduction of these new SWD, however, requires a systemic and transparent approach to gaining regulatory and public acceptance. Key to this acceptance is the clear understanding of the risks and benefits of these technologies, first by being properly understood by the scientific community and then communicated to the general public, government regulatory agencies, and political leaders in a form to enable informed decisions about their adoption and use. The introduction of a new intervention must also be compared to existing risks and benefits of the status quo. Without proper introduction to the public and regulatory agencies, these technologies are likely to be met with skepticism expressed through public resistance in the political process.

New mechanisms and business models could also be put in place to coordinate efforts to align stakeholder interests and secure needed funding to introduce new technologies for sustainable agricultural production. To investigate alternative models for advancing new SWD to widespread release, the California

[1] https://swd.ces.ncsu.edu/swd-impacts-2014/

Cherry Board engaged Technology Innovation Group (TIG) to review alternative strategies and prepare an outline of a business model for gaining regulatory approval and funding the introduction of these SWD into affected areas.

The team at TIG was specifically asked to:

1) Facilitate an initial story for public dissemination on technology choices for sustainable agriculture production.
2) Participate in a regulatory information exchange meeting on these technology choices and scopes the requirements for demonstrating safety and efficacy of a new biological approach to pest control.
3) Prepare an outline of a business plan for a B-Corporation that would coordinate efforts to introduce new technologies for sustainable agriculture production.
4) Investigate the feasibility of "Crowd-Funding" to finance the initial operating budget for the B-Corporation to address solutions for mitigating damage caused by the Spotted Wing Drosophila.

This report presents our findings and recommendations.

STORY FOR PUBLIC DISSEMINATION

The current standard of control for SWD combines diligent surveillance with the use of certain pesticides. Unlike native fruit flies, female SWD have a serrated ovipositor, or egg-laying device, to cut a slit into the skin of intact fruit to lay their eggs. This makes SWD a more significant pest than the native flies that require damaged fruit to lay eggs. Soft skinned fruit such as cherries, blueberries, raspberries, and blackberries are at the greatest risk. Larval feeding by SWD causes fruit to collapse and increases the risk of larvae being found at harvest time.

Effective management of SWD consists of several components:

1. Monitor fields with traps and check them regularly.

2. Check trapped flies to determine presence and number of male and female SWD.

3. If SWD are detected in traps, apply effective insecticides registered to protect the fruit.

4. Continue monitoring to evaluate the orchard's management program, and respond quickly if needed.

5. If possible, remove leftover fruit to reduce SWD breeding and food resources.

There are several challenges associated with this guidance to cherry growers. First, frequent monitoring and pesticide applications are added costs to production. Furthermore, the effectiveness of pesticides decreases over time as the insects develop resistance to the chemicals, thus requiring more of its use to reduce populations. Also, when pesticides are sprayed, they could possibly travel outside their intended area of use by air, soil or water.

Employing new techniques that promote the inheritance of a particular gene to increase its prevalence in a population of SWD could offer a better alternative for controlling SWD populations. This approach offers a targeted method for reducing populations of SWD and therefore mitigating the damage caused by SWD without the use of pesticides and their associated costs. Furthermore, the reduction or elimination of an invasive species would support conservation efforts in agricultural regions.

There are many solid reasons to predict that these techniques would be successful in field releases. For example, another drosophila, *D. Melanogaster,* is arguably the most scientifically studied insect, and as such there is a good deal of understanding of its genetics and biology. In addition, the *D. suzukii* (SWD) genome has been sequenced, and so is available for studying a number of possible modifications. However, there is very little probability of transfer of the modified genes from *D. suzukii* to *D. melanogaster* after a release of new SWD in the wild.

Since SWD is a recent invasive insect, it may be likely that there is less genetic diversity in the U.S. population than there would be if this was a long time invasive pest. This makes potential control strategies easier, as there is less room for genetic diversity to defeat the control mechanisms.

Also, since SWD is invasive, recently introduced, and reproduces by causing fruit damage for egg deposition, there should be no downside to removal. No native species are known to have come to depend on SWD for food or other ecological purposes. In fact, with more study it could be that SWD damage to certain rare native plant fruits results in population decline and potential extinction of those plant fruits. In summary, there is an unknown, but real, ecological cost to allowing SWD to remain here because it damages fruit. This damage need not be just to commercial fruit, but also those of other native plants.

REGULATORY ISSUES

New non-pesticide approaches for SWD control that involve genetic manipulation of the insects have been developed from Cal Tech and UC Riverside research and offer promise of controlling SWD populations. As a class, emerging technologies for gene drive and gene editing are so new, however, that regulatory guidelines have yet to be fully codified. The FDA, USDA APHIS and EPA are currently in the midst of discussions of how to manage the approval process for a growing number of plants and animals that have their genes modified for commercial use, and, to date, there are only a few recent regulatory decisions that provide guidance as to the direction the regulatory agencies will take, (e.g. Oxitec's approval by the FDA of genetically engineered "self-limiting males", and the USDA APHIS's approval of genetically engineered pink bollworms).

In general, the policy of the United States Government is to seek regulatory approaches that protect health and the environment while reducing regulatory burdens and avoiding unjustifiably inhibiting innovation, stigmatizing new

technologies, or creating trade barriers.[2,3,4] These principles also apply to updates of the regulatory framework and systems that regulate the products of biotechnology put forward in the National Strategy for Modernizing the Regulatory Framework for such products.

While the current regulatory system is designed to effectively protect health and the environment, new processes that employ emerging biotechnologies have created uncertainty about agency jurisdiction, enhanced the lack of predictability of timeframes for review, and imposed uncertainty for companies and academic research. These concerns have filtered up through the regulatory system, and as a result in July 2015, the Executive Office of the President issued a memorandum[5] directing the primary agencies that regulate the products of biotechnology—the U.S. Environmental Protection Agency (EPA), the U.S. Food and Drug Administration (FDA), and the U.S. Department of Agriculture (USDA)—to accomplish three tasks:

- Update the Coordinated Framework for the Regulation of Biotechnology (Coordinated Framework) by clarifying current roles and responsibilities;

- Develop a long-term strategy to ensure that the Federal regulatory system is equipped to efficiently assess the risks, if any, of the future products of biotechnology; and

- Commission an expert analysis of the future landscape of biotechnology products to support this effort.

In directing the agencies to accomplish these tasks, the Administration's goal is to enhance public confidence in the regulatory system and improve the

---

[2] "Improving Regulation and Regulatory Review", Executive Order 13563, January 18, 2011.

[3] "Identifying and Reducing Regulatory Barriers", Executive Order 13610, May 10, 2012.

[4] "Principles for Regulation and Oversight of Emerging Technologies", Memorandum for the Heads of Departments and Agencies, March 11, 2011.

[5] Memorandum for Heads of Food and Drug Administration, Environmental Protection Agency, and Department of Agriculture, "Modernizing the Regulatory System for Biotechnology Products", Executive Office of the President, July 2, 2015. The memorandum can be found at https://www.whitehouse.gov/sites/default/files/microsites/ostp/modernizing_the_reg_system_for_biotech_products_ memo_final.pdf

transparency, predictability, coordination, and, ultimately, efficiency of the biotechnology regulatory system. This National Strategy for Modernizing the Regulatory System for Biotechnology Products was developed in order to satisfy the second of the three tasks identified in the July 2015 Presidential memorandum and the accompanying proposed Update to the Coordinated Framework was developed to satisfy the first of the three tasks. EPA, FDA, and USDA have commissioned an independent study by the National Academy of Sciences (NAS) to satisfy the third of the three tasks.

In responding to the update to the Coordinated Framework, the Agencies have publically recognized that advances in science and technology have "dramatically altered" the biotechnology landscape in recent years and, in that context, seek to clarify through the newly proposed update which biotechnology product areas are within the authority and responsibility of each Agency.

The update is intended to clarify the role each Agency plays in biotechnology regulation and the different regulatory paths applicable to various product types. Specifically, the directive outlines:

- EPA's regulatory authority over chemical pesticides, microorganisms, biochemicals, and plant-incorporated protectants (PIPs) under the Federal Insecticide, Fungicide and Rodenticide Act (FIFRA) and Federal Food, Drug and Cosmetic Act (FFDCA), and EPA's oversight responsibilities for a wide range of microbial biotechnology applications under the Toxic Substances Control Act (TSCA);

- FDA's regulation of human and animal food derived from (i) genetically engineered plants; (ii) genetically engineered animals; and (iii) human drugs, biologicals, and medical devices derived from genetically engineered sources under the FFDCA; and

- Regulation by USDA's Animal and Plant Health Inspection Service (APHIS) of biotechnology products that may (i) introduce pests or cause disease to livestock under the Animal Health Protection Act (AHPA), (ii) be deemed plant pests

or noxious weeds under the Plant Protection Act (PPA), or (iii) be used in veterinary biologics under the Virus-Serum-Toxin Act (VSTA); along with the role played by USDA's Food Safety and Inspection Service (FSIS) in reviewing the safety of meat, poultry, eggs, or fish from genetically engineered animals intended for human consumption.

On September 18, 2016, the Obama Administration issued two major documents in connection with its ongoing efforts to modernize the federal Coordinated Framework for the Regulation of Biotechnology. The first document,[6] a proposed update to the 1986 framework, intends to clarify the current roles of the U.S. Environmental Protection Agency (EPA), U.S. Food and Drug Administration (FDA), and the U.S. Department of Agriculture (USDA) – the three primary agencies involved in the regulation of biotechnology products. Members of the regulated industry, other stakeholders, and members of the broader public had until November 1, 2016 to submit comments on the proposed update. The second document[7] presents a national strategy that outlines future steps that the Agencies intend to take to ensure that the regulatory system addresses novel biotechnology product types going forward. As directed by the Executive Office of the President in July of 2015, the Agencies have also commissioned development of a third document by the NAS to address future biotechnology products and opportunities to enhance capabilities of the regulatory system. The NAS expects to release its report early in 2017.

The NAS report is designed to answer the questions "What will the likely future products of biotechnology be over the next 5-10 years? What scientific capabilities, tools, and/or expertise may be needed by the regulatory agencies to ensure they make efficient and sound evaluations of the likely future products of biotechnology?"

[6] www.whitehouse.gov/sites/default/files/microsites/ostp/biotech_coordinated_framework.pdf
[7] www.whitehouse.gov/sites/default/files/microsites/ostp/biotech_national_strategy_final.pdf

The committee's report should:

- Describe the major advances and the potential new types of biotechnology products likely to emerge over the next 5-10 years.
- Describe the existing risk analysis system for biotechnology products including, but perhaps not limited to, risk analyses developed and used by EPA, USDA, and FDA, and describe each agency's authorities as they pertain to the products of biotechnology.
- Determine whether potential future products could pose different types of risks relative to existing products and organisms. Where appropriate, identify areas in which the risks or lack of risks relating to the products of biotechnology are well understood.
- Indicate what scientific capabilities, tools, and expertise may be useful to the regulatory agencies to support oversight of potential future products of biotechnology.

A TIG principal, Tom Turpen, has recently participated in meetings with regulatory agencies and provided professional opinion as to a new regulatory dispensation. In addition, Dr. Turpen and another TIG principal, Dan Hanson, met with Dr. Greg Simmons, an Entomologist with the USDA APHIS's Center for Plant Health Science and Technology (CPHST) in Salinas, California. Dr. Simmons participated in the research of a genetically engineered strain of pink bollworm and co-authored the environmental impact assessment of this technology that led to approval of its application in the field. The regulatory approval process for this case can provide valuable insights into the data and analytics required for regulatory approval for modified SWD.

The Salinas CPHST site coordinates and conducts scientific support activities for light brown apple moth and European grapevine moth. The station develops control and detection methods for these pests and provides technical analysis of program data to assist our stakeholders to maintain export markets of affected commodities. The station also supports development of mass-rearing strategies for bio-control agents of the Asian citrus psyllid.

In the meeting with Dr. Simmons, the possibility arose as to the opportunity to partner with the USDA site in Salinas for the raising and testing of modified SWD. This would involve creating a Cooperative Research and Development Agreement (CRADA) between the USDA and a private company to govern the conditions for working together on research and development. If the gene drive technology is eventually approved by the USDA APHIS, the Salinas site could also produce commercial quantities for field release.

THE APPROPRIATE TYPE OF ORGANIZATION

Researchers funded by the Cherry Board have demonstrated the ability to artificially bias certain inheritable genes in SWD, and these successes in the laboratory provide hope for ultimately reducing wild populations of SWD. In addition, some of these technologies will soon be ready to take forward to APHIS for regulatory approval, in particular with the Biotechnology Regulatory Services unit of APHIS for field trial permits.

In determining the most appropriate organizational entity to advance the technology to adoption in the orchards, there are several guiding principles to consider from the outset that provide insight as to the best legal structure to carry forward the mission and business model. Below is framework to help triage a for-profit or non-profit choice of entity:

1. How Does The Organization Plan To Fund Itself: Earned Income

If the primary way the organization plans to fund itself is through an earned-income strategy, the organization should normally be a for-profit entity. First, income derived from commercial operations that are not themselves directly integrated into the pursuit of the organization's charitable mission is likely to be considered "unrelated business income," and subject to the traditional corporate income tax. So, they would be taxed on the income anyway.

Second, if such income comprises a substantial portion of the organization's overall income (typically more than 20 percent), it is likely or at least very possible that the IRS will consider the organization as having a substantial non-exempt purpose. In that instance, the IRS would typically revoke the charity's tax-exempt status. This means the entity would return to traditional taxable-entity status, and might have to pay back taxes and fines to the IRS. As a result, the organization would risk losing the entire organization's tax-exempt status and risk paying fines to the IRS. The important caveat to this guideline, however, is that products or service delivery which is *fully integrated* into the pursuit of the organization's charitable mission are not subject to the unrelated business income tax.

2. How Does The Organization Plan To Fund Itself: Grants Or Donations

If the primary way in which the venture plans to fund itself is through donations or grant-based revenue streams, the entity should almost certainly be non-profit and tax-exempt. The rationale is that most grant and donation-based money available in a region is available only to non-profit, tax-exempt entities. This has changed somewhat with the launch of crowdfunding, where smaller donors can fund a project regardless of its exempt status, purely because it has a mission, cause, or focus that the donor supports.

But the general rule still stands: organizations who are providing a product or service to a stakeholder group that cannot afford to pay for the product/service, or who cannot otherwise be billed for the product/service provided, should likely be tax-exempt, non-profit entities in order to reap the benefits of grants and donative support for this mission which is difficult to monetize.

3. Who Do The Products and Services Of The Organization Primarily Benefit?

This perspective is particularly useful if the organization does not yet know exactly how it will fund itself – this question is a back door into the question 1 framework. If the venture's products or services primarily benefit the needy or the

environment, with no clear stakeholders who are willing to pay for the product or service, then the venture likely ought to be a non-profit, tax-exempt organization.

On the other hand, if the venture's products and services benefit primarily a target audience that can afford to pay for the products and services, the venture is likely commercial in nature and should be structured as a for-profit.

4. How Mission-Central is the Venture?

This question is crucial to consider, even apart from the legal structure. This is an issue of values and character, vision and purpose, end goals, and initial intention. Some important questions to consider include:

- How will the organization's mission be weighed against the profit interest, should they ever become opposed?
- How will the organization measure impacts of its goods or services provided?
- What impacts will the organization seek to prioritize as part of its brand and values?
- When will the organization report its impacts and how will it engage stakeholders?
- And perhaps most importantly, how will the organization s*trategically integrate* impact creation with its business model?

When these considerations are applied to the appropriate form of organization to control SWD, it becomes apparent that neither a conventional corporation nor a non-profit organization is the best legal vehicle. It is unlikely that financing for the business operations of the organization can be maintained through grant or philanthropic donations. In addition, reliance on this one category of revenue could be politically challenged and thus subject to much uncertainty. Operations of the organization would also be constrained strictly by Federal regulations on tax-exempt non-profits.

Mitigating the damage of SWD provides benefits to growers and consumers of cherries, berries and some soft fruits, some of which can be captured financially.

This feature would typically suggest that a corporation would be the appropriate legal vehicle. However, eliminating or greatly reducing populations of SWD in agricultural regions requires cooperation among growers, and tacit acceptance by citizens who have ornamental or non-commercial cherry trees or berry bushes as well as the general public. Then, there is the problem of "free riders" or those who fail to pay for services related to reducing the damage caused by SWD. A single grower, for example, could theoretically eliminate SWD in their orchard, but be subject to SWD infestation by wild types from surrounding, untreated, areas soon thereafter. In addition, the modified SWD will not respect property boundaries and could breed with SWD in adjacent orchards where the grower did not participate in a general release of the modified SWD. In this example, the neighboring grower would benefit without paying for the threat reduction. These challenges indicate that collective action over large geographic areas is a necessary but not a sufficient condition for success.

A final consideration arises as a result of the nature of the technology to be employed, the general lack of scientific knowledge of biotechnology by government officials and the general public, and the opaque methods and lack of transparency by a few corporations in attempts over the past two decades to introduce new products developed through new biotechnologies. The most recent high-profile example is the attempt by Oxitec to introduce modified mosquitoes in the Florida Keys. For the past five years, the Florida Keys Mosquito Control District has been working with the British company Oxitec to get federal approval for a trial release of the mosquitoes in the Keys. The trial would consist of the company releasing genetically modified male *Aedes aegypti* mosquitoes into the wild. When they mate with female *Aedes aegypti*, their offspring die.

Even though Oxitec has been working for many years on developing the genetic technology and testing releases of modified mosquitoes in smaller areas, the company's approach has been to focus on working with government officials much more than engaging the public. These efforts, though, have culminated by FDA approval in August, 2016 and by two referendums in the Florida Keys on Election

Day to approve the release of Oxitec mosquitoes. The question now occupying the Commissioners on the District's Board is where to conduct the first release. The first release is likely to occur in the Spring of 2017 and, if successful, other areas of Florida will inquire with Oxitec and begin the process of initiating trial releases in those communities.

It is very likely that if Oxitec had been more communicative early in the process and more transparent in their efforts to engage the public and regulatory agencies, it would not have taken five years to gain the approval of one mosquito control district to release its modified mosquitoes. Focused public engagement should have accelerated education and eventual approvals. Furthermore, placing the release of Oxitec's mosquitoes within the context of alternative strategies to reduce the threats of Zika and Dengue should have brought this complex issue to more clarity for decisions in favor of earlier release.

There are significant, measurable damages caused by SWD. Cherry and berry growers are keenly aware of these costs, realized not only to contain the damage but in reduced sales. The cost to consumers is higher prices for these products. This ability to identify financial impacts leads to a corporation being the right entity. However, the need for operational transparency and public engagement points to a legal entity that must balance the needs of shareholders, employees, the community, and the environment equally. Such an entity, a Benefit Corporation, was designed and first approved by the State of Maryland in April of 2010, and since then over 30 states have passed legislation allowing this legal charter. As of May, 2015, it was estimated that there are over 2,100 Benefit Corporations active in the U.S.[8]

The purpose of traditional corporations as the maximization of financial gain for its shareholders was first articulated in the State of Michigan court case of *Dodge v. Ford Motor Company* in 1919. Over time, through both law and custom, the concept of "shareholder primacy" has come to be widely accepted. This focus by the directors and executives of the corporation may prevent them from operating the

[8] Berrey, Ellen, "How Many Benefit Corporations Are There?" (May 5, 2015). Available at SSRN: http://ssrn.com/abstract=2602781

company by taking into account social and environmental factors. It is a result of the legal protection offered to Directors and Officers of a Benefit Corporation that they were created. By giving directors the secured legal protection necessary to consider the interest of all stakeholders, rather than just the shareholders who elected them, benefit corporations can now be created to help meet the needs of those interested in having their business help solve social and environmental challenges.

In effect, a Benefit Corporation is similar to a normal corporation, except language in the corporate charter outlines the purpose of the corporation and the various stakeholder groups that must be treated equally by operations of the Benefit Corporation. In addition, though, a benefit corporation, by its nature, is more credible in seeking scientists and other knowledgeable communicators to put for a social risk versus benefit argument to the public as opposed to a traditional corporation.

To help verify that Benefit Corporations act as intended in their charter, a non-profit organization called B Lab offers certification for Benefit Corporations and other types of companies. To be certified as a "B Corp," the company goes through a rigorous assessment and, once certified, must re-qualify every two years. According to B Lab, there are now over 1,600 B Corps spread throughout 40 countries. B Lab will certify companies of any size, and a few B Corps have gone public while certified. The B Corp certification is an important signal to consumers of and investors in these companies.

All Benefit Corporations are taxed and are required to have directors and officers similar to other corporations. Again, the only difference is found in the charter and operations of the corporation, where impacts on shareholders, employees, the community, and the environment must be taken equally.

BENEFIT CORPORATION FUNDING ISSUES

The key element of the proposed business model calls for the creation of a Benefit Corporation. Benefit Corporations may charge for the services they perform or products they produce, and resulting profits may be used for returns to investors and/or retained earnings for future growth. This proposed Benefit Corporation for introducing modified SWD into affected areas would operate with complete transparency and proactively engage the public and regulatory agencies to educate them regarding the SWD pest impact and to clarify misunderstandings about the risks of the modified SWD. The Benefit Corporation would then manage the operations and public engagement for releasing the modified SWD.

There are two categories of uses of funds prior to getting the Benefit Corporation to a revenue producing stage: 1) additional research and development, and 2) working capital. The California Cherry Board has funded research at Cal tech and UC Riverside that has resulted in significant progress being made on biasing certain genetic expression in SWD. More funding, however, will be needed for research and development before full field trials are authorized. This funding can be raised by the Cherry Board and other agriculture associations affected by SWD. In addition, it may be possible to obtain federal grants to finance the additional research through trails and approvals.

Funding for working capital for the Benefit Corporation, however, will necessarily need to come from private sources. A seed amount (less than $10,000) is required to pay the costs of legally organizing the Benefit Corporation and maintaining a bank account of at least $1,000. Once organized, a fund raising effort could be started with contacting multiple types of funders. Since the Benefit Corporation will charge for the costs of growing and releasing modified SWD plus a profit, there may be investors willing to provide equity capital. These investors may be cherry and berry growers (or companies in the supply chain) or others who have interests in new conservation techniques to control invasive species. In addition there are a few institutional investment funds that invest in companies which offer positive societal impacts beyond those embedded in products or services that are

privately consumed. Food System 6 (FS6), for example, is a new non-profit organization created to build financial and entrepreneurial ecosystems around the agriculture industry, similar to those support systems for the tech industry in the Silicon Valley. TIG professionals met with a co-founder of FS6 to discuss this effort to combat SWD and explore possible collaborative opportunities. Should the Cherry Board decide to move forward with the creation of a Benefit Corporation, FS6 could become a valuable strategic partner.

Finally, a crowdfunding strategy could also provide operational funds for the Benefit Corporation. Crowdfunding is a recent development enabled by the internet, and provides a method of raising capital through the collective donations of friends, family, customers, and other unrelated parties. This approach taps into the collective efforts of a large pool of individuals—primarily online via social media and crowdfunding platforms—and leverages their networks for greater reach and exposure. There are two primary types of crowdfunding platforms: equity based and project based. An equity-based crowdfunding effort would not be appropriate for the Benefit Corporation, as the mission of the Benefit Corporation is to eliminate SWD across the U.S. Any further projects are not foreseen at this time, so a perpetual return to owners of the Benefit Corporation is not expected.

Project-based crowdfunding, however, is a possibility. Most crowdfunding sites are built for creative works by individuals or companies or offer tokens of appreciation for donors in a project. Of the top ten crowdfunding sites, most are focused on creative works or specific types of projects (music and apps for mobile phones are prominent). One site, however, Crowdrise (crowdrise.com) could be a good fit for the Benefit Corporation to try fundraising for the operational costs of gaining regulatory approvals, public engagement, and trial releases of modified SWD. In our discussions with Crowdrise managers, the Benefit Corporation would be eligible to use their site to request project funding and therefore post the project description. In exchange, Crowdrise would charge:

- A per transaction platform fee of 5.9% + credit card fees of 2.9%+$0.30.

- Donors have the option to cover all the fees (including credit card fees) at checkout, and over 93% of donors do so.
- If all donors cover the fees, the Benefit Corporation would have cost-free fundraising. If the donors don't cover the fees and the Benefit Corporation is getting less than $97 out of every $100 raised, by the end of the campaign Crowdrise will provide an amount that guarantees a 97% net raise.
- The guarantee includes all credit card fees.
- The guarantee, however, excludes individual donations of $1,000 or more.

In summary, a coordinated effort to obtain research and development as well as working capital funding should commence just after the Benefit Corporation is launched. These efforts should be targeted at multiple sources of grant, investment, and project funding.

RECOMMENDED NEXT STEPS

Technology Innovation Group has detailed recommendations below for the California Cherry Board Research Committee. A combination of Cherry Board investments and funds raised for the Benefit Corporation could be used to:

1) Complete a business plan and be used by the Cherry Board to recruit additional financial resources to continue technology development.
2) Coordinate with the Working Group established by North Carolina State University (NCSU) and funded with a $6.7 million grant from the USDA National Institute for Food and Agriculture. The grant is to undertake research and grower education efforts aimed at better managing SWD.
3) Organize the Benefit Corporation and appoint the initial Board members. Management services for the Benefit Corporation could be performed initially by Ag Association Management Services, a California company that provides agricultural management and operational services for associations, foundations, commissions, and marketing orders.
4) Begin engagement activities to educate the general public and the proper agencies on the biotechnology as well as the risks and benefits associated with the full spectrum of technological and cultural approaches to mitigating the damage done by SWD.

5) Obtain the appropriate intellectual property agreements and licenses for the technologies to be employed by the Benefit Corporation.
6) Conduct initial meetings with USDA APHIS to discuss the technology development roadmap and confirm that APHIS is the appropriate and only regulatory agency, based on precedent(s) of modified Pink Bollworm and the recommendations of the Coordinated Framework for the Regulation of Biotechnology.
7) Apply for and obtain permits for field testing of a modified SWD with the trait of fluorescence, eye color, or other simple marker to illustrate the safety of the technology.
8) Establish a Cooperative Research and Development Agreement (CRADA) with USDA to grow modified SWD under appropriate conditions.
9) Release a trial of SWD with a color or fluorescent marker.
10) Secure commitment from FS6 or another trusted organization to source and provide an in-kind donation of secure data infrastructure for operations of the Benefit Corporation.
11) Work with Crowdrise to develop a platform and then pilot crowd-sourced funding for a specific project. In parallel, approach institutions, philanthropic, and social impact funders for the Benefit Corporation to prepare for scaling the Corporation's staff and operations.
12) Develop an Environmental Impact Statement for modified SWD.
13) Obtain regulatory approval from USDA APHIS.
14) Conduct first release in a designated area.

MONITOR AND ENHANCE FNRIC CUMULATIVE CHILL PORTION WEBSITE

INTRODUCTION

The mission of the Fruit and Nut Research and Information Center (FNRIC) is to aid in the coordination and dissemination of University of California (UC) accomplishments and statewide research and extension activities related to fruit and nut crops. The FNRIC has been serving as a bridge between UC researchers and the fruit and nut industry since 1995. Our main website, fruitsandnuts.ucdavis.edu, contains a wealth of information about production of over 44 fruit and nut crops and receives over 120,000 visits per year.

The existing FNRIC website serves as a valuable portal for public access to research on tree fruit and nut agriculture conducted by UC faculty, extension specialists, and farm advisors. Much of this information would be unavailable to the public, or very difficult to access, without the FNRIC serving as a central hub for new and up-to-date information.

The Fruit and Nut Research and Information Center currently hosts several weather models which use CIMIS data to calculate estimates of chill accumulation throughout the state (http://fruitsandnuts.ucdavis.edu/Weather_Services/chilling_accumulation_models/). These models are used by a wide range of people working with tree fruits and nuts in California. Specifically, California cherry growers use estimates of chill portions each winter to time spray applications. Many growers and PCAs rely upon estimates available through the FNRIC website either as a primary reference, or as a tool to verify estimates obtained from independent temperature sensors.

PROJECT BACKGROUND AND MOTIVATION

The chill calculator tools available on the FNRIC website integrate the "dynamic model" of chill accumulation with queries of the CIMIS weather station network to display daily estimates of chill portion accumulation throughout the state. Currently growers can select a CIMIS station and view estimates of cumulative chill portions beginning on Sept 1st and a plot of historic chill portion accumulation (previous five years). The website was developed by the Division of Agriculture and Natural Resources Information Technology (ANR IT) group. The initial web model programming by ANR IT includes indicators for potential sources of error in the data including:

These automatic error codes are useful in identifying potential problems in the chill portion estimates provided through the website. However, in previous years' errors in chill portion estimates were identified that were not detected by the automated ANR IT error checking system. FNRIC staff observed errors while occasionally scanning the website, or were altered to potential problems by growers who utilize the site regularly. In each case, after identifying a specific problem and communicating it clearly to ANR IT, it was resolved within a day or two. Members of the California cherry industry have expressed concern about the reliability of the chill portion estimates available through the FNRIC site. We propose to allocate FNRIC staff time to regular error checking at a subset of the CIMIS stations applicable to the California cherry industry.

PROPOSED PROJECT

In communication with California cherry industry representatives and Joe Grant (San Joaquin County Farm Advisor), the FNRIC has identified opportunities to minimize errors in cumulative chill portion estimates available from currently available CIMIS stations.

Independent error checking at a subset of CIMIS stations at ("high priority"):

The FNRIC will hire an undergraduate assistant to perform regular checks of chill portion estimates from the following CIMIS stations (Fig. 1):

- Brentwood
- Lodi West
- Arvin-Edison
- Famoso
- Shafter
- Madera 2
- Delano
- San Benito
- Porterville
- Patterson
- Modesto
- Parlier

Error checking will be conducted on a weekly basis (Monday) between October 15th and December 15th. From Dec 16th through February 28th error checking will be conducted three times per week (Monday, Wednesday and Friday). The following protocol will be used by an Undergraduate Assistant and FNRIC staff:

a. Download temperature data from the CIMIS website for each of the identified stations.
b. Verify the source of missing data (ex. interpolation from previous data within the same station or substitution with data from a replacement CIMIS station).
c. Copy complete dataset into the "Dynamic chill model" spreadsheet calculator developed by K. Glozer. Generate an independent estimate of chill accumulation.
d. Compare independent estimate to model output posted on the FNRIC website and communicate with senior FNRIC staff about potential errors.
e. FNRIC staff confirms error and works with ANR IT to resolve the problem.

To ensure that website users are notified quickly of potential errors, and their resolution, FNRIC staff will add a new e-mail list subscription feature to the current chill portion accumulation website. In the event that a problem is discovered with the chill portion accumulation estimates posted on "high priority" CIMIS stations (listed above), or the new chill site (described below), a brief e-mail will be sent out altering users that data from a specific station and day are being investigated. Once the problem is resolved a brief follow up e- mail will be sent to users to inform them of the resolution.

BUDGET JUSTIFICATION

Budget item #1: Error checking chill portion calculations at "high priority" CIMIS stations.

We have begun training an undergraduate student assistant to use the current FNRIC chill calculators and the CIMIS website. After initial training we determined that it will require approximately seven minutes per CIMIS station to download data, load it into the chill portion spreadsheet, verify corrections for missing data, and compare the results to the FNRIC website.

Currently, the budget includes weekly error checking from October 15th through Dec 15th. Beginning in mid-December we plan to check the website three times per week for errors through the end of February.

In addition to the time required to monitor and compile a spreadsheet we are also requesting senior FNRIC staff time (Janet Zalom) to assess errors identified by the undergraduate assistant, communicate with ANR IT to resolve the errors, and send out notifications. Finally, one additional FNRIC staff hour is requested to establish the new e-mail list proposed to notify users of errors and subsequent corrections.

Budget

Error checking chill portion calculations at selected essential CIMIS stations for the cherry industry	$ 4,000.00

Figure 1: Google Earth image displaying the positions of the twelve focal CIMIS stations (red) and the weather stations within the UC IPM Pest Cast San Joaquin and Fresno/Madera networks (yellow).