California Cherry Research Review

Wednesday, January 16, 2019

CALIFORNIA CHERRY RESEARCH REVIEW
Wednesday, January 16, 2019

Evelyn Costa Assembly Room
San Joaquin County – Office of the Agricultural Commissioner
2101 E. Earhart Avenue, #100, Stockton, California 95206
Sponsored by the University of California and California Cherry Board

9:00	**Welcome** Nick Matteis, California Cherry Board
9:05	**Measuring cherry evapotranspiration and deriving crop coefficient (Kc) values for use in irrigation scheduling** Dr. Daniele Zaccaria, Dept. of Land, Air & Water Resources, UC Davis
9:30	**Develop nutrient budget and early spring nutrient prediction model for nutrient management in sweet cherry** Dr. Douglas Amaral, Dept. of Plant Sciences, UC Davis
9:55	**Investigating biological controls to suppress spotted wing drosophila populations** Dr. Kent Daane, Dept. of Environmental Science, Policy, & Management, UC Berkeley
10:20	**Management and epidemiology of pre- and postharvest diseases of sweet cherry** Dr. James Adaskaveg, Dept. of Plant Pathology, UC Riverside
10:45	**Break**
11:10	**Improved management of fungal canker diseases of sweet cherry** Dr. Florent Trouillas, Dept. of Plant Pathology, UC Davis, Kearney Agricultural Research & Extension Center, Parlier, CA
11:35	**Engineered transgenic *Drosophila suzukii* for wild population suppression & eradication: production, performance assessment and effective wild releases** Dr. Bruce Hay, Div. of Biology & Biological Engineering, Caltech
12:00	**Electronic sensors to capture spatiotemporal population density of SWD** Dr. Joanna Chiu, Dept. of Entomology, UC Davis
12:25	**Lunch** (Courtesy of California Cherry Board)
1:30	**Adjourn**

CDPR CE Credits: 1.50 Hours

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CALIFORNIA CHERRY BOARD - RESEARCH COMMITTEE BUDGET

Wednesday, January 16, 2019: California Cherry Research Review

	Project Leader	Institution	Project Title	Status	Additional Information	Approved Funding	Comments
1	Adaskaveg	UC Riverside	Management and epidemiology of pre- and postharvest diseases of sweet cherry	On-going	Continuing annual work on bacterial blast and canker, powdery mildew and brown rot blossom blight, and pre- and postharvest management of fruit decay	$ 51,000.00	Kasugamycin (Kasumin) approved by CDPR for control of walnut blight in walnuts and bacterial blast and canker in cherry in January of 2018
2	Trouillas	UC Davis	Investigating the cause of sudden decline of sweet cherry in California	On-going	Determining the pathogenicity of various fungi that were isolated from diseased sweet cherry rootstock in commercial orchards in California	$ 20,662.00	Trouillas has requested a no-cost extension to allow additional time for disease development in trial rootstock that were innoculated with fungi isolated from commercial orchards experiencing sudden decline disease symptoms
3	Daane	UC Berkeley	Biological control of spotted wing drosophila: classical and augmentation	On-going	Continuing to both evaluate non-native parasitoids - to obtain USDA APHIS release permits for field release - and develop mass pupae rearing and storage methods	$ 18,870.00	Has submitted a petition for review to USDA-NAPPO for release of foreign parasitoids
4	Akbari/Hay	UC San Diego/Caltech	Engineered transgenic *Drosophila suzukii* for wild population suppression & eradication: Production, performance assessment and effective wild releases	On-going	Engineering gene drives for the wild population suppression and eradication of spotted wind drosophila (SWD)	$ 100,000.00	The Washington Tree Fruit Research Commission (WTFRC) and Oregon Sweet Cherry Commission (OSCC) are stakeholders on this project
5	Walse	USDA ARS	Postharvest residue remediation: Breaking MRL trade barriers for the specialty crop industry	On-going	Development of postharvest techniques to diminish pesticide/agrochemical residues on sweet cherries destined for export	$ 5,000.00	Funds provide Spencer Walse (USDA ARS) California sweet cherries for postharvest residue remidiation trials
6	FNRIC	UC Davis	Online chill portion estimates and error checking for the California Cherry Board	On-going	Error checking of CIMIS station data and the UC ANR online chill portion calculator	$ 5,000.00	A minimum service given the majority of growers maintain their own stations
7	Trouillas	UC Davis	Improved management of fungal canker diseases of sweet cherry	New	Determine latent infection of canker pathogens in California nursery stock and improve management and understanding of fungal canker diseases	$ 39,325.00	In 2019,Trouillas would like to employ a full-time postdoctoral scholar to thoroughly explore the biology, epidemiology and control of fungal canker pathogens
8	Chiu	UC Davis	Electronic sensors to capture spatiotemporal population density of SWD	New	Refinement of insect classification algorithm - to improve accuracy of identification between related *Drosophila* species - and deployment of sensors in field to evaluate performance	$ 11,769.00	Chiu has requested a no-cost extension to further refine the identifier algorithm and to test sensors in field. WTFRC and OSCC are stakeholders on this project.
					Total(s):	**$ 251,626.00**	

2018 FINAL RESEARCH REPORTS

2018 Annual Crop Report, California Cherry Board

Investigating Biological Controls to Suppress Spotted Wing Drosophila Populations

Kent M. Daane[1], Xin-Geng Wang[1,3], Evelyne Hougardy[1,2], Brian N. Hogg[2], Kim A. Hoelmer[3],

[1]Department of Environmental Science, Policy and Management, UC Berkeley, CA; [2]USDA ARS, Beneficial Insects Introduction Research Unit, Newark, DE; [3]USDA ARS, Invasive Species and Pollinator Health: Albany, CA.

Summary. The spotted wing drosophila (SWD), *Drosophila suzukii* has become a major cherry pest in California. To develop sustainable management options for this highly mobile pest, we have been working to get imported parasitoids released from Quarantine through a USDA evaluation process. Here, we report on our progress to improve SWD control using imported parasitoids collected during our explorations in Asia. We focused 2017-2018 work on evaluations of two larval parasitoids (*Ganaspis brasiliensis* and *Leptopilina japonica*) that have been selected based on previous evaluations of their efficiency and host specificity. Our goal is to develop data to answer specific concerns that USDA reviewers had with an earlier petition to release material from Quarantine. Additionally, while we are preparing the release permit, we also evaluated the potential of two indigenous pupal parasitoids for SWD control, developed mass-rearing methods, and an initiated "augmentative" field release trial. Much of what is presented here is the detailed biology studies – the nuts and bolts needed to build the USDA petition. The ultimate goal is to suppress SWD outside of the cherry orchard to reduce the numbers of adults flying into the orchard and damaging the crop.

Introduction

Spotted wing drosophila (SWD), *Drosophila suzukii*, is native to East Asia, but has invaded North America and Europe where it has become a key pest of soft and thin skin fruits such as blueberries, cherries, raspberries, and strawberries. Currently, control efforts rely on the use of insecticides that target adult SWD. However, insecticide-based programs can be limited by the fact that many host fruits in non-crop habitats act as reservoirs for SWD and support its reinvasion into commercial fields.

Our work focuses on improving biological controls. Our surveys in California showed a lack of specialized parasitoids that can specifically attack SWD larvae. Only two generalist indigenous pupal parasitoids *Trichopria drosophilae* (Diapriidae) and *Pachycrepoideus vindemiae* (Pteromalidae) were found to attack SWD in California. The lack of effective biological controls in California (and other invaded regions) 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 SWD. This will be accomplished through foreign exploration to find novel parasitoids and then quarantine evaluations of the parasitoids' efficiency, specificity (non-target impacts), and temperature tolerances (where can they be released and establish).

During explorations in South Korea and China, we discovered three key larval parasitoids, *Asobara japonica* (Braconidae), *Ganaspis brasiliensis* and *Leptopilina japonica* (Figitidae) (there are no 'common names") (Fig. 1). These parasitoids have been imported into our quarantine at UC Berkeley, and in 2016 we completed evaluations of their biology and efficiency (e.g. egg maturation dynamics, functional responses, host age preference and suitability and life-time fecundity) as well as potential non-target impacts (host range test). This information is needed to develop effective rearing methods for these parasitoids, and more importantly for obtaining the USDA APHIS release permits to get material out of quarantine and into the field. The two dominant parasitoids (*G.*

brasiliensis and *L. japonica*) both in South Korea and China, were shown to be the most promising agents in terms of their low potential risk to non-target species and their effectiveness against SWD. Petitions to release both parasitoids were submitted and reviewed, and while 5 of 8 reviewers approved the petition, the USDA APHIS regulators have requested more studies on a) temperature development and potential range in the US if released, b) non-target impact, c) parasitoid taxonomy, and d) efficacy in their origin (China and/or South Korea. Here, we provide a summary of our efforts to answer these last four concerns.

Ganaspis brasiliensis (KM Daane) *Leptopilina japonica* (KM Daane) *Asobara japonica* (Tim Haye)

Figure 1. Major Asian larval parasitoids of spotted wing drosophila

1. Evaluations of Asian parasitoids temperature range

1.1. Laboratory studies

The parasitoids developmental rate (how fast they grow at different temperatures), offspring survival, and reproductive success were assessed for *G. brasiliensis* and *L. japonica* populations from South Korea and China.

For developmental rate and offspring survival, 20 host larvae were exposed to a single female parasitoid at room temperature (23 °C) for 24 h. After which, the exposed larvae were assigned to one of seven constant temperatures, ranging from about 55 to 86 °F (12, 15, 18, 20, 25, 28 and 30 °C). The numbers of emerged flies and parasitoids were recorded daily. There were 30 replicates for each parasitoid population at each temperature and 15 controls (where host larvae were not exposed to any parasitoids). Data of development time from egg to adult were pooled from all replicates for each temperature treatment, and linear models were used to describe the relationship between developmental rate and temperature.

To determine reproductive success, 20 host larvae were exposed to a single female parasitoid at one of six constant temperatures from about 59 to 86 °F (15, 18, 20, 25, 28 and 30 °C). After 24 h, the female parasitoid was removed, and exposed hosts were kept at room temperature and held for fly or parasitoid emergence. The number and sex of emerging wasps were recorded. There were 15 replicates for each parasitoid population at each temperature treatment and 10 controls at each temperature treatment. After insect emergence has stopped for at least a month, all unemerged pupae were dissected to determine the presence of fly or parasitoid cadavers.

Parasitoid offspring survival from egg to adult was estimated for each replicate as the number of emerged parasitoid adults divided by the total number of parasitized hosts. The total number of parasitized hosts, or initial parasitism, was calculated by dividing the sum of emerged and dead unemerged parasitoids (as revealed by the dissection) by the total number of hosts. Data were analyzed using two-way ANOVA, looking at the effect of temperature and parasitoid populations, as well as the interaction between these two factors, on mean percentages of offspring survival and mean numbers of offspring produced respectively. Significant differences were subsequently sorted out using Tukey's honest significance difference (HSD).

The results were very interesting as no parasitoids emerged at 53 and 58 °F (all parasitoid populations) and sometimes 63 °C (*G. brasiliensis* South Korea only, see Table 1). In fact, we stopped the experiments after 113, 112 and 74 days for 53, 58, and 63 °F , respectively. Similarly, no parasitoid emergence was observed at the higher temperature of 85 °F (Table 1).

Table 1. Mean development time (from egg to adult) in days (± SE) of *D. suzukii* (SWD), *G. brasiliensis* South Korea (GBSK), *G. brasiliensis* China (GBC), *L. japonica* South Korea (LJSK) and *L. japonica* China (LJC) at different temperatures (T)

T (°C)	SWD	GBSK	GBC	LJSK	LJC
11.8	39.3 ± 0.16	-	-	-	-
14.4	28.2 ± 0.38	-	-	-	-
17.2	19.9 ± 0.08	-	49.8 ± 0.69	49 (1)	50.8 ± 1.78
19.4	15.6 ± 0.07	34.5 ± 0.17	35.3 ± 0.23	30.4 ± 0.16	30.5 ± 0.21
24.8	12.1 ± 0.02	22.9 ± 0.10	22.5 ± 0.16	19.6 ± 0.07	19.6 ± 0.16
27.5	11.6 ± 0.07	22.0 ± 0.09	22.9 ± 0.14	17.3 ± 0.09	18.2 ± 0.17
29.3	13	-	-	-	-

The mid-range temperature data 64 – 82 °F (18 - 28°C) for each parasitoid population were fit to a linear regression (Fig. 1), and the extension of this line often indicates the low temperature threshold, which appears to be 41-52 °F (the figure is in Celsius), depending on the populations.

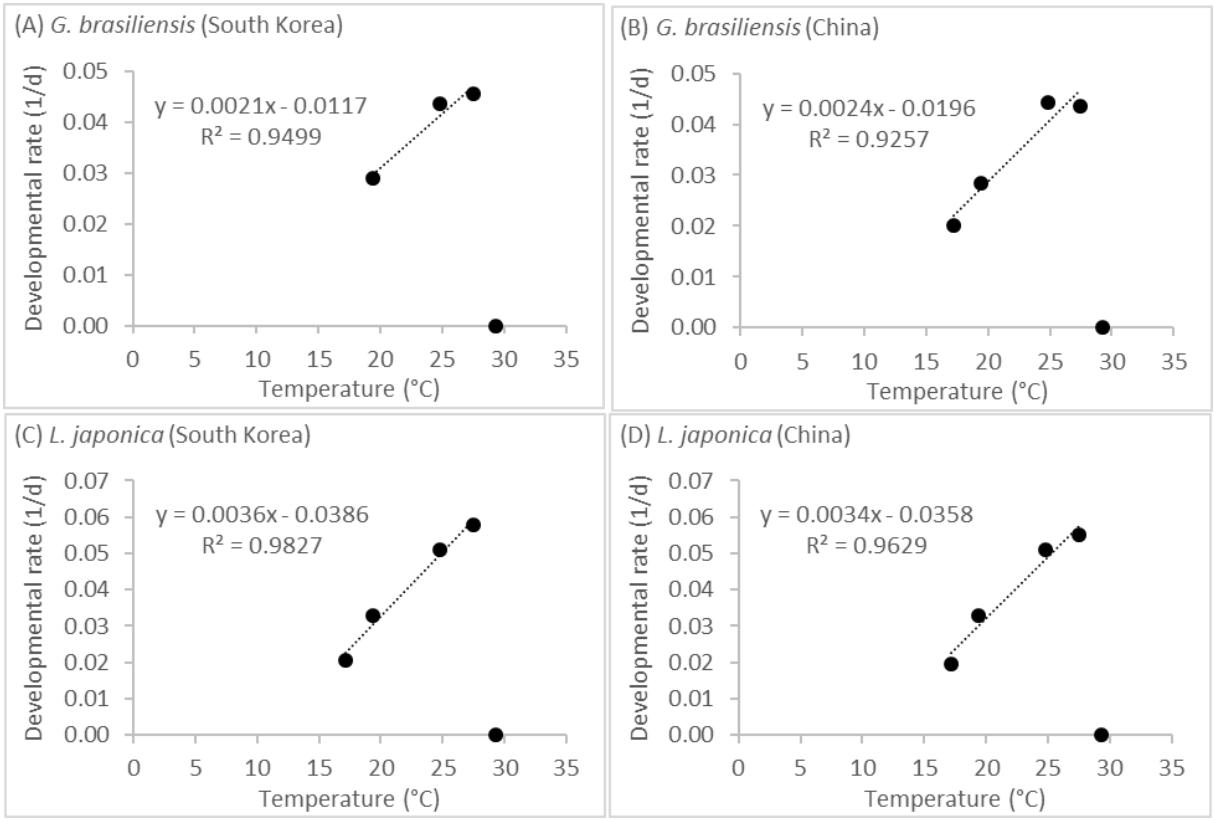

Figure 1. Relationship between temperature and developmental rate (1/d) for *G. brasiliensis* South Korea, *G. brasiliensis* China, *L. japonica* South Korea and *L. japonica* China. Lower to mid-range data (18–28°C) were fitted to a linear model.

These data are very interesting as dissections of unemerged pupae revealed the presence of healthy 'prepupae' suggesting that the parasitoid offspring may have entered a dormant stage at these lower temperatures. **This may be a mechanism for the parasitoid to survive cold periods, which is especially interesting as SWD is not known to enter diapause at cool temperature, and we observed SWD emerging from these lower temperature treatments. The results also suggest that the Chinese populations had better cold hardiness.**

Because dissections of unemerged pupae revealed that immature parasitoids may have entered a dormancy state between 53 – 59 °F (12 – 15 °C), offspring survival could not be estimated for these temperatures and were excluded from the analysis. For the remaining range of temperatures (18 – 30 °C), parasitoid offspring survival was affected by temperature ($F = 232.6$, $df = 4$, $P < 0.001$) and parasitoid populations ($F = 13.6$, $df = 3$, $P < 0.001$), and there was a significant interaction between the two factors ($F = 4.6$, $df = 12$, $P < 0.001$). No significant differences between parasitoid populations were found in the 20 – 28 °C range. No parasitoids survived at a constant 29.3 °C (Fig. 2). Some significant differences between populations were found at 17.2 °C: populations from China had a significant better survival than populations from South Korea within each species (Fig. 2).

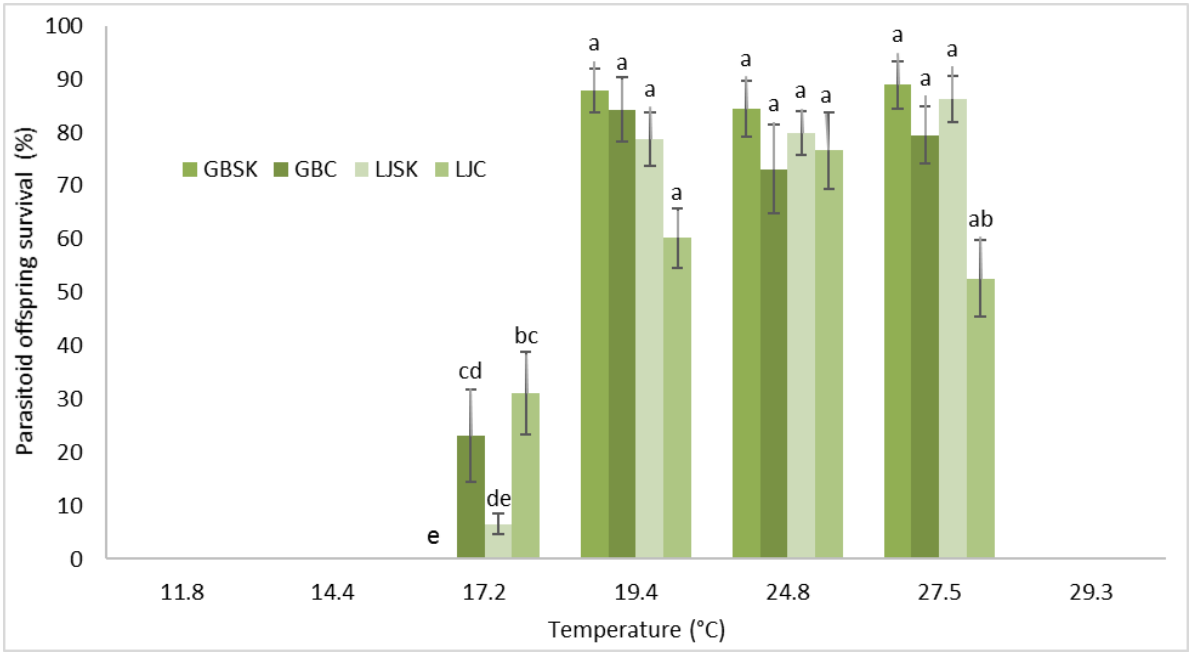

Figure 2. Mean parasitoid offspring survival (± SE) of *G. brasiliensis* from South Korea (GBSK) and China (GBC), and of *L. japonica* from South Korea (LJSK) and China (LJC) at different constant temperatures. Bars with different letters indicate significant differences ($P < 0.05$).

Temperature also affected parasitoid offspring production ($F = 11.5$, $df = 5$, $P < 0.001$). Offspring production was the highest at 28.2 °C for all parasitoid populations, and the lowest at 14.4 °C for all parasitoid populations except *G. brasiliensis* South Korea that showed the lowest offspring production at 17.5 °C a (Fig 3). Within each species, the South Korean population usually produced more offspring than the Chinese population, although this difference was only significant at 15.9 °C for *G. brasiliensis* (Fig 3). Parasitoids were able to successfully parasitize their hosts at the highest temperature tested. The short 24 h exposure for egg-laying did not affect the parasitoid nor the

hosts, showing that the adverse effect of a high temperatures depends on how long the insect are exposed to it.

What all this means is that temperature is important for these parasitoids in terms of their geographic range. We are pleased that they seem to enter a cold temperature diapause as this may allow them to survive in cherry production areas as far north as Michigan. We are concerned that their offspring production was lowered even at relatively mild temperatures in the low 60's (°F), although these tests were conducted at constant temperatures, which can impact parasitoid behavior.

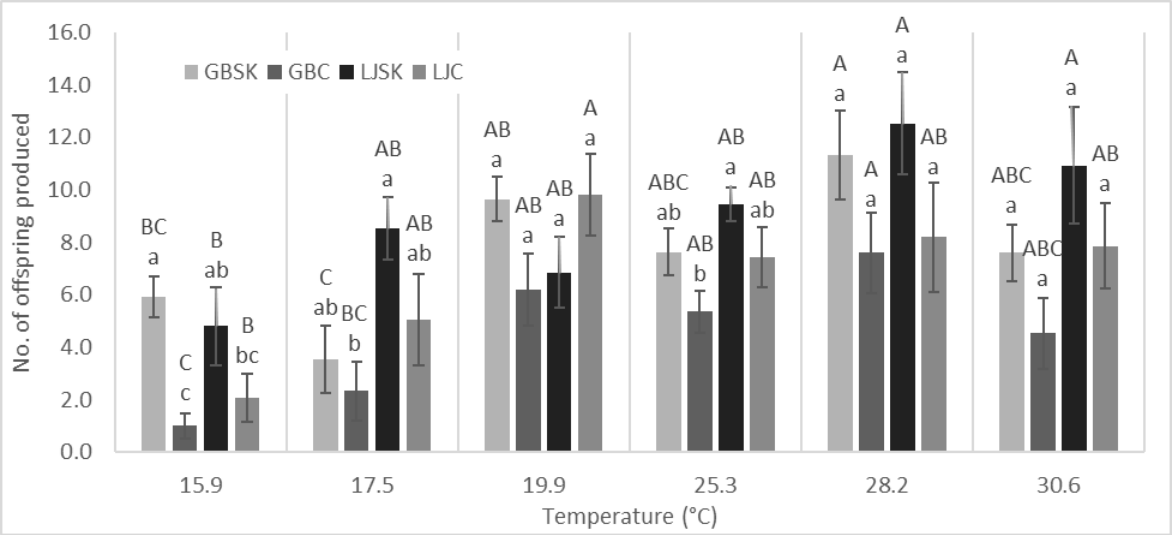

Figure 3. Mean number of offspring produced (± SE) by *G. brasiliensis* from South Korea (GBSK) and China (GBC) and *L. japonica* from South Korea (LJSK) and China (LJC) during a 24-hour exposure at six constant temperatures. Bars with different letters indicate a significant difference ($P < 0.05$). Multiple comparisons were performed between parasitoid populations for each temperature separately (lower case) and between temperatures for each parasitoid population separately (upper case).

1.2. Natural geographic range.

The laboratory studies described above are an important part of any biological description of a natural enemy's temperature development, but information can also be gathered from simply looking at where the insect naturally occurs. All lineages of *G. brasiliensis* occur in Asia, but the 'specialist lineage' (later in this report we discuss parasitoid taxonomy) was recorded only from East Asia (China, Japan and South Korea). The early historical specimens of *G. brasiliensis* found in the Smithsonian Institution (Washington DC) and in the Natural History Museum (Paris, France) were all collected in the Caribbean and in Panama, whereas the newly collected specimens were all collected in Japan, South Korea and China. Recent reexamination of the morphology confirmed some specimens previously reported as *G. xanthopoda* or *Ganaspis* sp. in the literature in Indonesia, Malaysia, Thailand, the Philippines, Hawaii, Uganda, Benin and Brazil are *G. brasiliensis*. We can use this information to develop a 'CLIMEX model' to predict the potential geographical range of *G. brasiliensis* (Fig. 4).

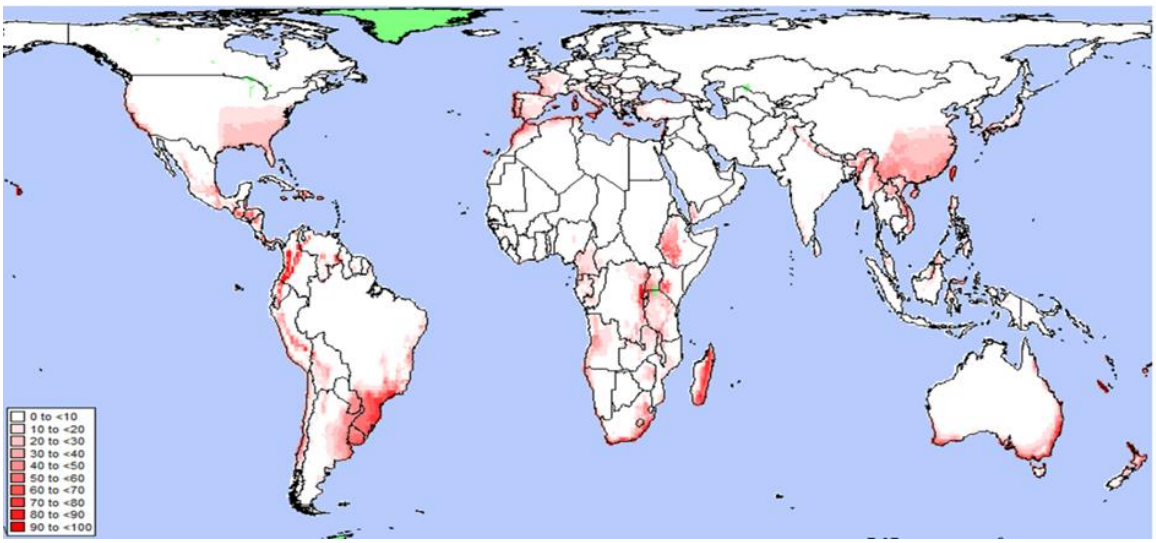

Figure. 4. Predicted distribution of *G. brasiliensis* worldwide based on CLIMEX climatic suitability indices. EI: < 10 is not suitable; 10-50 moderate level of suitability; and > 50 highly suitable for survival.

We conclude that if released in North America, *G. brasiliensis* would likely establish along western coastal zones and much of south-eastern and east coastal states where SWD is a major concern of small fruit crops.

2. Non-target impact

2.1. *Ganaspis brasiliensis* from China

In previous reports we showed results from non-target tests using 24 different fly species. These tests are done to determine if a released natural enemy will have any negative impacts by attacking a 'non-target' species. All of our previous studies were completing using the *G. brasiliensis* from South Korea. Because *G. brasiliensis* from China performed better in quarantine (e.g., greater temperature tolerance) we were requested to conduct non-target work with this 'strain' as well. Rather than repeat the entire test, we selected those species most similar to SWD. As before, the test using Yunnan-collected *G. brasiliensis* was conducted under controlled conditions (22 ± 2°C, 14L: 10D, 40–60% RH) in a quarantine at UC Berkeley. To expose the fly larvae to adult *G. brasiliensis*, 40 larvae were transferred from the Petri dish colony to a small plastic vial filled with diet and immediately exposed to two mated, experienced (3–4 day old) female *G. brasiliensis* for a 2-day period. Vials of exposed larvae were then held for the emergence of adult flies or parasitoids, with emerged adult parasitoids transferred to plastic vials supplied with 50% honey water streaked on the vial plug; the adults were later used for colony maintenance or quarantine trials.

Colonies of native non-target species tested were initiated from specimens purchased from the UC's *Drosophila* Stock Center in San Diego, California, where these species were originally collected from different locations in the USA. Nine non-target species were tested: *Drosophila simulans*, *D. melanogaster*, *D. persimilis, D. pseudoobscura*, *D. busckii*, *D. montana, D. robusta*, *D. funebris* and *Hirtodrosophila duncani*. Non-target species selection was based on their phylogenetical relationship to *D. suzukii*: *D. simulans* and *D. melanogaster* are closely related to *D. suzukii* (all belong to the *D. melanogaster* species group) whereas the other species are more distantly related.

The non-target test consisted of no-choice exposures to determine if *G. brasiliensis* could attack and develop from the drosophilid species tested. For each replicate, 20 host larvae were transferred from the Petri dish colonies to a plastic vial filled with 1 cm cornmeal diet and then exposed to a single mated, experienced (3–4 day old) female wasp for a 24 h period. Exposed fly larvae were held in these vials until the emergence of flies and wasps. For each fly species, there were 20-30 replicates

with five positive control replicates (each consisting of 20 *D. suzukii* larvae similarly exposed to *G. brasiliensis*) and five negative control replicates (unexposed fly larvae held under the same conditions). After insect emergence ceased, all unemerged dead pupae were reconstituted in water for 1 day and then dissected under a microscope to determine the presence or absence of recognizable fly or parasitoid cadavers (e.g. pharate adults).

Results found that *G. brasiliensis* successfully developed from *D. suzukii, D. simulans* and *D. melanogaster* (the same closely related fly species as we previously found) and the number of offspring that developed were not significantly different among these three species (Fig. 5). Host species affected the number of offspring developed ($F_{9,236}$ = 18.6, P < 0.001), SP ($F_{9,236}$ = 27.9, P < 0.001) or DI ($F_{9,236}$ = 10.5, P < 0.001) (Fig. 5). Additionally, a few adults developed from *D. montana* or *D. persimilis*. There was no significant difference in the number of offspring developed or in the SP among all these seven non-target species (Fig. 5). In total, only three out of 292 *D. simulans* adults and one out of 323 *D. suzukii* adults contained black capsules, whilst all emerged flies of other species (totally 2370) did not contain them.

We conclude that the China *G. brasiliensis* has the same host range as the South Korean *G. brasiliensis* – which includes a group of closely related species – all of which are not native to North America. We believe that there is no or minimal risk to non-target species if *G. brasiliensis* is released in North America.

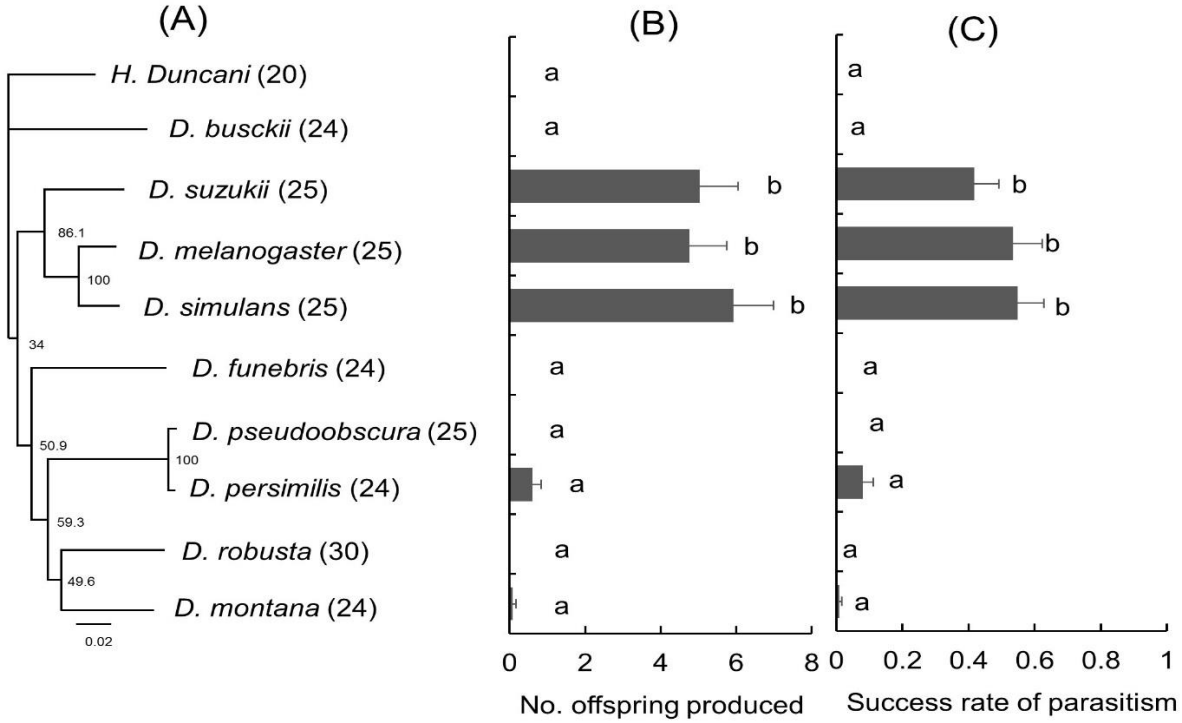

Figure 5. (A) Phylogenetic tree of the host species tested in the quarantine experiment. The phylogenetic relationship was re-structured based on available COI gene sequences from NCBI database. (B) Number of offspring produced, (C) success rate of parasitism and (D) degree of infestation by *Ganaspis brasiliensis* when tested with different *Drosophila* host species. Values are mean ± SE and bars bearing different letters are significantly different (Tukey's HSD, P < 0.05).

3. Parasitoid taxonomy

At this point our focus is on *G. brasiliensis* from China as this parasitoid is more of a 'specialist' than *L. japonica* and has better performance in terms of killing and reproducing on SWD than other

strains (or geographic populations). A large stumbling block is that other researchers have suggested that *G. brasiliensis* can be categorized as a specialist or generalist based on a molecular description, and that there may be different species within this group. This hypothesis was raised by the USDA APHIS reviewers.

As a response to this suggestion, we have teamed with world taxonomic experts to better classify *G. brasiliensis* populations. The 'taxonomic' issues are whether we can separate specialist (narrow host range) vs generalist (wide host range) based on a molecular description, and whether the Chinese or Japanese populations of *G. brasiliensis* are similar. Clearly, we are dealing with the tiny nuts and bolts to build the USDA APHIS petition, but a positive outcome of this effort is an agreement with Canadian and European researchers to work with the same material all towards the goal of getting material out of quarantine. Below is a summary description of the 2017-2018 molecular work, recently published by Giorgini et al. (I list our recent publications at the end of this report).

Molecular analyses focused exclusively on *G. brasiliensis*; the *cytochrome oxidase subunit I* (COI) gene was sequenced to clarify our Chinese specimens' genetic identity Molecular COI results produced a phylogenetic 'tree' (figure is in Giorgini et al. in press) showing that *G. brasiliensis* from Yunnan, China clustered in two groups corresponding to the G1 lineage (suspected specialist on *D. suzukii*) and the G3 lineage (suspected generalist on drosophilids). Previous researchers recognized five lineages of *G. brasiliensis*, which differed in geographic distribution and host range: G1, including individuals from Japan parasitizing only *D. suzukii*; G2, including individuals from a subtropical Japanese isle parasitizing *Drosophila ficusphila*; G3, including individuals from temperate regions of Japan and high mountains of Southeast Asia (Indonesia, Malaysia) parasitizing different species of *Drosophila*, except *D. suzukii*; G4, including individuals from Indonesia parasitizing *Drosophila eugracilis*; G5, including individuals from Japan, Taiwan, Hawaii and Uganda, from unknown host(s).

Our phylogenetic analysis of COI sequences revealed that our China *G. brasiliensis* samples were grouped in two lineages: 77% in the G1 lineage and the remaining 23% in the G3 lineage. Although morphologically indistinguishable from each other, theses lineages could be a complex of cryptic species. Our more recent whole genome sequencing supports the idea that the level of reproductive isolation between the G1 lineage and the other lineages is not entirely clear, and that host specificity may be based more on geographic populations and it may be better to accept the existence of a complex of species under the name *G. brasiliensis*.

Our results suggest that in China we collected two strains, including the specialist stain also reported from Japan. The result of this work will be that Europe, Canada and the US will seek to release similar material to help control SWD in all of the invaded areas.

4. Parasitoid ecology in China

For the original USDA petition, a Reviewer suggested that more information about the parasitoids in the countries of origin (Asia) should be included. We have worked with Chinese collaborators to gather this information, and we (Giorgini et al., in press) have used our collection data to provide some of this information. For the China population of *G. brasiliensis*, we report the following in a recent publication: surveys for SWD parasitoids were conducted in summers from 2013-2016 in different locations of Yunnan Province, China, using either banana-baited traps placed near natural vegetation or cultivated fields, or collections of fruits from natural vegetation including known or presumed host plants. This region in southern China is part of the presumptive native range of SWD and the closely-related species *Drosophila pulchrella*, both characterized by a serrated ovipositor that allows them to penetrate the intact skin of fruits.

A total of 458 adult parasitoids or flies were collected from fruit-baited traps from 2013-2015, with majority being Braconidae (49.56%), followed by Figitidae (37.55%), Diapriidae (7.42%), and Pteromalidae (5.46%) (Table S2). Seven *Asobara* species were collected, among which *Asobara*

mesocauda van Achterberg and Guerrieri (36.46%) and *A. brevicauda* (9.17%) were the most abundant. Four genera of Figitidae were collected; the majority of specimens reared belonged to *Leptopilina*, with *L. j. japonica* resulting the most abundant (14.41%), followed by *L. decemflagella* Lue & Buffington (5.46%). From these fruit-baited traps, *Ganaspis* species were the least represented figitids (0.22%) with only a single individual of *G. xanthopoda* collected. The Diapriidae and Pteromalidae collected were *T. drosophilae* and *P. vindemiae*, respectively, which are already present in the US. Identification of a sub-sample of 1,707 drosophilid flies emerged from the fruit-baited traps found only 18 *D. suzukii* (1.05%) and four *D. pulchrella* (0.23%).

From fruit collections, across all sample dates and sites, 48.9% of the live puparia were parasitized. Percentage parasitism varied among puparia collected from berries of *R. foliosus* (22.35%), *R. niveus* (18.81%), and *F. moupinensis* (19.75%), whereas puparia collected from *S. adnata* had the highest percentage parasitism at 63.46% (Fig. 6). The majority of parasitoids were figitids (*G. brasiliensis* at 65.4%, *L. j. japonica* at 32.9%), with braconids (*Asobara* spp. at 0.6%) and diapriids (*T. drosophilae* at 0.6%) occurring in only a few samples. Host fruit species influenced the ratio between *G. brasiliensis* and *L. japonica* (Fig. 6). *Ganaspis brasiliensis* was the most abundant species collected, reaching a parasitism rate of 31.54% on average, ranging from 4.31% (*R. niveus*) to 40.42% (*S. adnata*). Parasitism rate by *L. j. japonica* was 16.18% on average, ranging from 4.47% (*R. foliosus*) to 21.52% (*S. adnata*) (Fig. 6).

From these fruit surveys in China, we showed that *G. brasiliensis* reached parasitism levels in some locations as high as 60% and average parasitism rates of about 30%. If these same levels of parasitism can be reached in the US it would significantly reduce levels of SWD near the cherry orchards.

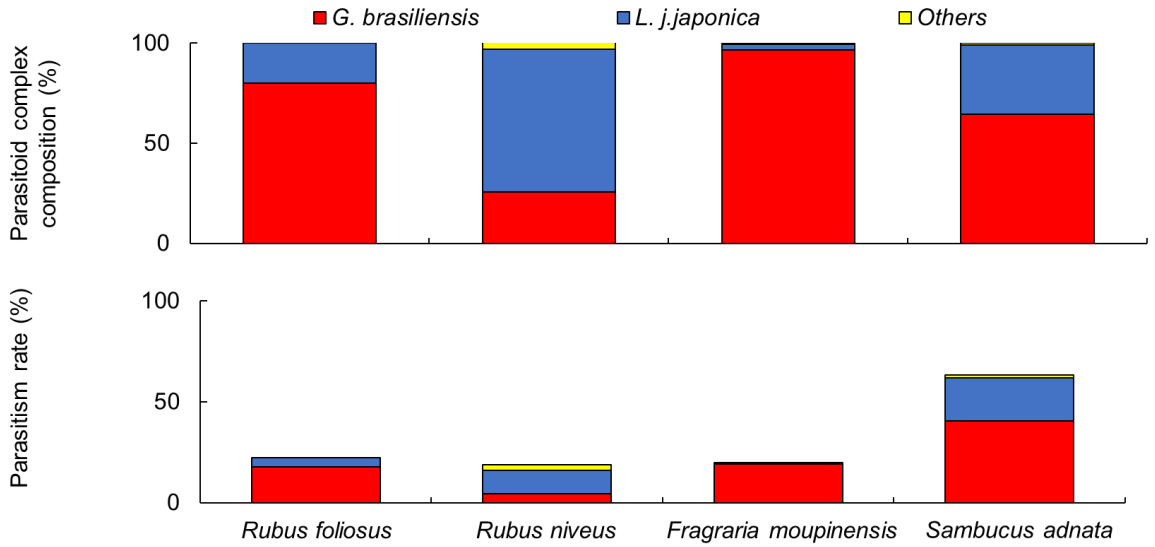

Figure 6. (A) Composition of the parasitoid complex and (B) parasitism rate of hosts (*Drosophila suzukii* and *D. pulchrella*) on four different host fruits. Others include the parasitoids *Asobara mesocauda, A. unicolorata, A. leveri* and *Trichopria drosophilae.* s

4. Augmentative release of indigenous pupal parasitoids

4.1. Surveys of crop fields and non-crop source habitats

Since many non-crop habitats could sever as refuges or overwintering sites for SWD in later seasons, providing source population for early generations, it is crucial to suppress source

populations on non-crop hosts and post-harvest cash crops to reduce pest pressure in susceptible crops. We are mass-producing the two pupal parasitoids (*P. vindemiae* and *T. drosophilae*) and we conduct augmentative release in the habitats surrounding crops in fall-winter 2017 and 2018 (i.e. targeting overwintering populations but could potentially move into early fruit crops such as cherry). To monitor seasonal population dynamics and dispersal of SWD and its natural enemies in crop fields and non-crop source habitats, SWD and its parasitoids were sampled at 10 organic cane berry (raspberry or blackberry) fields from June 2017 to December 2018. Fields were adjacent to non-crop habitats infested with wild blackberry (*Rubus* spp.), a key non-crop host of SWD. To assess whether non-crop habitats are a source for SWD and its natural enemies, SWD numbers and parasitism rates were monitored along transects extending 160 m into berry fields and 30 m into non-crop habitats, with traps placed at 30 m intervals along transects in crop fields and 0-10 m (i.e., at the edge) and 30 m into non-crop habitats. Three traps were placed 20 m apart at each transect distance. Traps remained in the field for one-week periods. Sampling occurred every 4-6 weeks; all transect distances were sampled from June to October 2017 and in May 2018, and from December 2017 to December 2018 three traps per distance were placed at three distances, 100 m and 10 m into crop fields and 50 m into the non-crop habitats.

Standard SWD traps were baited with apple cider vinegar; waterproof waxed cardboard covers were placed over the traps to block sunlight and rain. Each trap was hung on low trellis wires in cane berry fields or on low-hanging tree branches in non-crop habitats at a height of 1 to 1.5 m above the ground. Parasitism rates were monitored using raspberries or banana slices infested with sentinel SWD larvae and pupae. The infested fruit was placed in traps consisting of clear plastic containers with holes punctured in their sides to allow flies and parasitoids to enter. Sentinel traps were placed in the same locations as vinegar-baited SWD traps.

Samples collected from June to December 2017 were processed in spring and summer 2018; data from subsequent dates are still being processed and analyzed. Numbers of SWD were consistently higher in riparian habitats at all times from June to December 2017 and were lowest in spring in both habitats (Fig. 7). The generalist pupal parasitoids *Trichopria drosophilae* and *Pachycrepoideus vindemiae* parasitized 15.9% and 0.05% of SWD pupae, respectively. Parasitism rates of *D. suzukii* did not differ consistently between habitats (Fig. 2). These results suggest that riparian habitats act as refuges for *D. suzukii*, and that parasitoids are equally successful in both habitats. Control strategies for SWD should focus on suppressing populations in non-crop habitats in the spring.

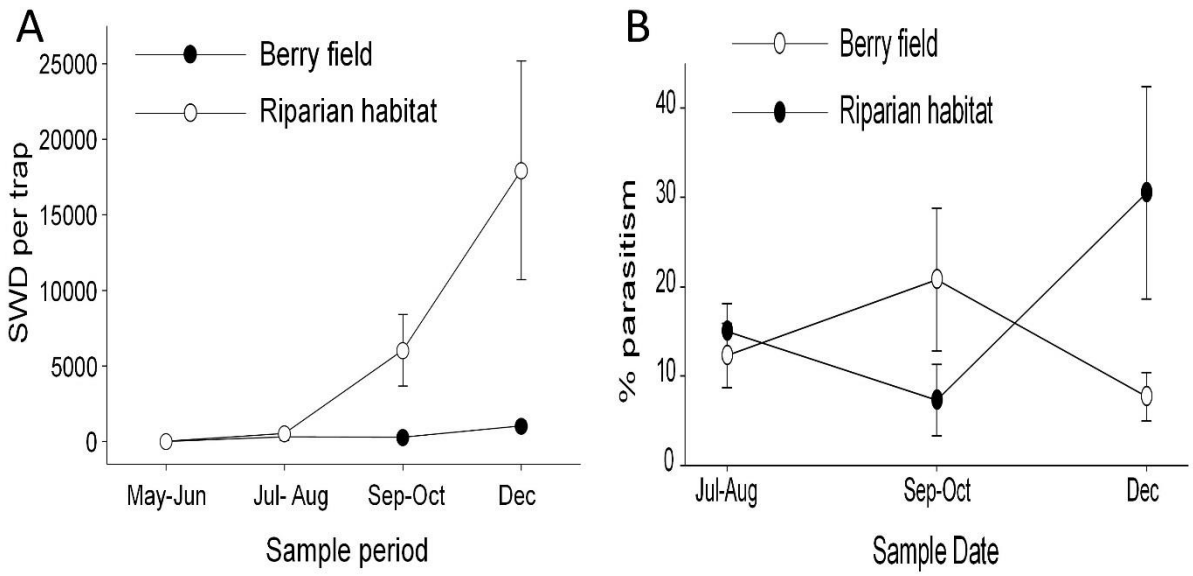

Figure 7. (A) Numbers of SWD per trap in cane berry fields and adjacent non-crop habitats from June to December 2017, (B) Percent parasitism of SWD by the pupal parasitoid *Trichopria vindemiae* in cane berry fields and adjacent non-crop habitats from July to December 2017.

4.2. Mass releases of SWD parasitoids

Mass releases of pupal SWD parasitoids occurred in October 2017, June 2018 and October 2018 to test the feasibility of rearing and releasing large numbers of these parasitoids. In October 2017 Approximately 16,000 *P. vindemiae* were reared at the UC Berkeley Kearney Agricultural Center and released into three crop fields and one non-crop habitat on 25 October 2017. Each release site was paired with a control site >400 m away where parasitoids were not released. Parasitoids were released at a central release point at each site, and sentinel traps containing raspberries infested with SWD larvae and pupae were placed along four transects extending away from the release point in all four cardinal directions. Three sentinel traps were placed along each transect at distances of 10m, 40m and 70m from the release point. Ten raspberries were also collected at each transect distance and were taken back to the laboratory and searched for SWD pupae. The pupae were then placed in emergence containers for parasitoid emergence.

Although this test demonstrated that mass rearing this parasitoid is possible, only one SWD pupae in the sentinel traps at all the release sites was parasitized by *P. vindemiae*. In contrast, 209 SWD pupae in sentinel traps were parasitized by *T. drosophilae*, which was not released. Although *P. vindemiae* is the dominant parasitoid in all other areas of western North America, perhaps is unsuited to coastal conditions in this area, where it is generally far less common than *T. drosophilae*.

Releases in June and October were conducted in single cane berry fields, to more closely examine whether it was possible to elevate parasitism of SWD by releasing large numbers of parasitoids. In June 2018 approximately 10,000 *P. vindemiae* and 10,000 *T. drosophilae* were released into one blackberry field, and in October 2018 approximately 7,000 *P. vindemiae* and 5,000 *T. drosophilae* were released into one raspberry field. Data from these releases are still being processed.

5. Ongoing studies

5.1. Interactions between imported parasitoids

We are investigating the interspecific actions between the two selected larval parasitoids (*G. brasiliensis* and *L. japonica*). These ongoing studies suggest that *L. japonica* may interfere with *G.*

brasiliensis, which helped our decision to petition only for *G. brasiliensis* – the better of the two natural enemies. The information will help us to predict the potential geographical ranges of these introduced parasitoids if released in North America.

5.2. Interactions between *G. brasiliensis* and resident pupal parasitoids

We are also investigating the interspecific actions among *G. brasiliensis* and the two pupal parasitoids (*P. vindemiae* and *T. drosophilae*) to determine if augmentative releases of the pupal parasitoids will interfere with the establishment or effectiveness of *G. brasiliensis*.

5.3. Shipment of resident pupal parasitoids

We can cheaply rear large numbers of *P. vindemiae* and *T. drosophilae*; however, we have found it difficult to store and ship material, observing as much as 75% mortality of adult parasitoids collected, stored and shipped in just a 7-day period. We are investigating better methods to collect, store and ship these beneficial insects.

5.4. Molecular description of *G. brasiliensis*

Dr. Buffington and Dr. Hooper (USDA) are teaming with our group and researchers in Europe and Asia to produce a complete molecular description of the different *G. brasiliensis* populations, which will be tied to our studies on host attack rates.

6. Publication produced during the reporting period

Kaçar, G., Wang, X.-G., Biondi, A., and Daane, K. M. 2017. Linear functional response by two pupal *Drosophila* parasitoids foraging within single or multiple patch environments. *PloS ONE* 12(8): e0183525. https://doi.org/10.1371/journal

Biondi, A., Wang, X-G., Miller, J. C., Miller, B., Shearer, P. W., Zappalà, L., Siscaro, G., Walton, V. W., Hoelmer, K. A., and Daane. K. M. 2017. Innate olfactory responses of *Asobara japonica* toward fruits infested by the invasive spotted wing drosophila. *Journal of Insect Behavior* 30: 495-506. DOI 10.1007/s10905-017-9636-y

Wang, X.–G., Nance, A., Jones, J. M. L., Hoelmer, K. A., and Daane, K. M. 2018. Aspects of the biology and developmental strategy of two Asian larval parasitoids evaluated for classical biological control of *Drosophila suzukii. Biological Control* 121: 58-65. doi.org/10.1016/j.biocontrol.2018.02.010

Wang, X.–G., Serrato, M. A., Son, Y., Walton, V. M., and Daane, K. M. 2018. Thermal performance of two indigenous pupal parasitoids attacking the invasive *Drosophila suzukii* (Diptera: Drosophilidae). *Environmental Entomology* 47(3):764-772. doi: 10.1093/ee/nvy053

Giorgini, M., Wang, X.-G., Wang, Y., Chen, F.-S., Hougardy, E., Zhang, H.-M., Chen, Z.-Q., Chen, H.-Y., Liu, C.-X., Casconea, P., Formisano, G., Carvalho, G. A., Biondi, A., Buffington, M., Daane, K. M., Hoelmer, K. A. & Guerrieri, E. 2019. Exploration for native parasitoids of *Drosophila suzukii* in China reveals a diversity of parasitoid species and narrow host range of the dominant parasitoid. *Journal of Pest Science* doi.org/10.1007/s10340-018-01068-3.

Wang, X-G., Nance, A. H., Hougardy, E., Hogg, B. N., Hoelmer, K. A., and Daane, K. M. Potential competitive outcomes among three solitary larval endoparasitoids as candidate agents for classical biological control of *Drosophila suzukii. Biological Control* (accepted)

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Annual Report - 2018

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, and L. Wade

SUMMARY

We continued our efforts in developing new management strategies for major foliar and fruit diseases of sweet cherry in California including preharvest diseases such as bacterial canker caused by *Pseudomonas syringae* pv. *syringae*, powdery mildew caused by *Podosphaera clandestina*, blossom blights and fruit rots caused by *Monilinia* and *Botrytis* spp., as well as postharvest decays such as brown rot, gray mold, and Rhizopus rot.

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

   a. Kasumin (active ingredient kasugamycin) obtained full registration on cherry in California and the United States in the spring of 2018. Oxytetracycline is still at the EPA for registration on cherry with support from the registrants, the California Cherry Board, and other researchers in the North Central and Northeastern regions of the United States.

   b. In 2017/2018 inoculation studies failed to develop the disease due to warm, dry conditions in the dormant period. Kasugamycin and zinc thiadiazole were effective in previous trials in preventing or reducing the severity of branch cankers on cv. Coral Champagne.

2) In powdery mildew studies, the disease developed at high incidence on leaves of water sprouts (inside canopy) and on leaves of new shoots (outside canopy) on terminal branches. The incidence of fruit infections was low in 2018.

   a. In a trial in San Joaquin Co., the most effective treatments under high disease pressure (e.g., inside shoots) included SDHI (FRAC 7)-containing fungicides including Fontelis, pyraziflumid, Luna Sensation, and Merivon, selected DMI (FRAC 3)-containing fungicides such as Procure, as well as the experimental fungicides UC-2, EXP-AD, and EXP-AF.
   b. Mixtures of Quintec with Rally or Fontelis in rotational programs were effective; whereas rotations of Quintec with other single active ingredients continued to show reduced performance under high disease pressure. The new product Gatten (flutianil) was moderately to highly effective in these studies.

3) For brown rot blossom blight, Rhyme, pyraziflumid, Fontelis, Quadris Top, Luna Experience, UC-1, UC-2, EXP-AF and EXP-AD were highly effective as post-infection treatments in laboratory studies. For gray mold blossom blight, pyraziflumid, Fontelis, Quadris Top, Luna Experience, UC-2, EXP-AF, and EXP-AD were highly effective, whereas Rhyme and UC-1 were only moderately effective.

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

   a. Brown rot: In studies with 1- or 6-day PHI applications, Quadris Top, Fontelis, Merivon, EXP-AD, and EXP-AF were most effective on wound-inoculated fruit (other effective fungicides were identified in previous years). Luna Experience, Quadris Top, UC-1 and UC-2 (all containing a DMI fungicide) were still very effective on washed fruit. When harvested fruit were non-wound drop-inoculated, most treatments including FRAC 3 and FRAC 7 compounds, and several pre-mixtures were highly effective on non-washed and washed fruit.

   b. Gray mold: On fruit harvested and non-wound-inoculated 1 day after application, Quadris Top, Fontelis, Merivon, EXP-AD and EXP-AF were most effective. In the 6-day PHI treatments,

efficacy was reduced, but fungicides with good activity against gray mold included Quadris Top, Merivon, EXP-AD, EXP-AF, and pyraziflumid.

5) We continued to establish baseline sensitivities against new SDHI fungicides. Most of the 32 isolates of *M. fructicola* evaluated were highly sensitive to pydiflumetofen and pyraziflumid over a narrow range of concentrations. For pydiflumetofen one isolate and for pyraziflumid two isolates were less sensitive than the other isolates. A wider range in sensitivities was determined for 32 isolates of *B. cinerea,* and one isolate was less sensitive to pydiflumetofen and pyraziflumid. This indicates that cross resistance is present among SDHI sub-groups and that SDHI fungicides should always be rotated with different FRAC codes.

6) Several laboratory and one commercial packingline study were done for the evaluation of postharvest fungicides with emphasis on BioSpectra (natamycin) and Chairman (a pre-mixture of fludioxonil and propiconazole).

   a. BioSpectra was mostly highly effective against the major decays brown rot, gray mold, and Rhizopus rot when fruit were wound-inoculated before treatment, but brown rot was reduced to zero levels in all studies. Efficacy was reduced against gray mold in the laboratory studies. A new formulation of natamycin showed improved efficacy as compared to BioSpectra. With increasing emphasis on food safety and consumer concerns, natamycin with 'exempt from tolerance status' will likely become an important component of postharvest decay management in the future. It will be best used in mixture with low rates of Scholar.

   b. Chairman was highly effective against the three major decays after wound-inoculation at rates between 8 and 16 fl oz.

7) Eighty isolates of *Phytophthora* from tree fruit samples (mostly *P. niederhauseri, P. syringae, P. citricola* complex, *P. megasperma, P. cactorum*) were highly sensitive to oxathiapiprolin and mandipropamid, whereas for ethaboxam and fluopicolide generally higher concentrations were needed to inhibit growth. All isolates were also inhibited by mefenoxam. Thus, no resistance was detected. This information will be used to establish baselines for future monitoring programs.

INTRODUCTION

Management of bacterial blast and canker. Pseudomonas syringae pv. *syringae* is the main pathogen causing bacterial blossom blast and canker of woody tissue of sweet cherry and other stone fruit crops in California. Another less common pathovar of the species, *P. s.* pv. *morsprunorum,* can also cause the disease. Cold, wet conditions are associated with the canker phase of the disease and symptoms develop usually weeks to months later with gumming around the infected sunken bark tissue. Blossom blast develops after cold injury, and with subsequent infection, blossoms become dark to black in color, wilt, and die. The disease is often confused with brown rot blossom blight and is more commonly found on early-blooming varieties or trees treated with rest-breaking treatments that experience cooler, wet spring environments. Bud death and spots and specks on leaves and fruit are additional symptoms of the blast phase of the disease.

Based on our efforts, advances have been made in bacterial disease management with the identification of kasugamycin (Kasumin), an antibiotic that is not used in animal or human medicine. Kasumin has been recently registered for management of fire blight of pome fruits, walnut blight, and bacterial diseases of some other agronomic crops in the United States and elsewhere. Based on our research, Kasumin was state and federally registered for use on cherry for the management of bacterial blast and canker in early 2018. Therefore, it is important to continue to evaluate its efficacy under different environmental conditions to optimize its use. In our previous inoculation studies and in commercial applications for managing natural infections, Kasumin significantly reduced bacterial blast of sweet cherry. Kasugamycin was the only compound that consistently reduced the severity of bacterial canker of inoculated branches.

We evaluated numerous other compounds for these bacterial diseases of cherry, including oxytetracycline (Fireline, Mycoshield) that we are also pursuing for registration, the biocontrol treatments Actinovate (fermentation product of *Streptomyces lydicus*) and Blossom Protect/Botector (*Aureobasidium pullulans*), and copper-enhancing compounds. In 2018, we attempted to evaluate the efficacy of inhibitors of the type III bacterial secretion system that has a major role in plant infection, as well as other novel bactericides such as a nano-particle zinc compound, Zinkicide, and Cinetis, a nutritional stress reducer.

Management of powdery mildew, blossom blight, and fruit rot. Powdery mildew of sweet cherry is an ongoing problem for growers in California. Warm conditions and low rainfall but high humidity from dews or irrigation are are highly favorable conditions for disease development. Powdery mildew is especially difficult to manage in southern production areas (e.g., Tulare and Kern Co.). Sepals of blossoms, 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, as well as new combinations and rotations of registered fungicides are being evaluated. Alternative fungicides that we evaluated over several years in our field trials on sweet cherry in California include the FRAC Code 3 (DMI) Procure (triflumizole), the FRAC Code 7 (SDHI) fungicides (e.g., fluopyram, fluxapyroxad, and penthiopyrad), and the pre-mixtures Luna Sensation (fluopyram/ trifloxystrobin), Merivon (fluxapyroxad/pyraclostrobin) (FRAC Code 7/11), and Quadris Top (azoxystrobin/ difenoconazole) (FRAC Code 3/11) and polyoxin-D (FRAC Code 19). Still, other new powdery mildew fungicides such as pyraziflumid, UC-2, pydiflumetofen, Gatten (flutianil), EXP-AD, and -AF are being developed, and we are seeking their registration on cherry in California. This will allow alternatives to be used during bloom, petal fall, and preharvest. Fungicides evaluated in 2018 for management of pre- and postharvest diseases of sweet cherry are listed in Table 1.

For management of brown rot and Botrytis blossom blight and fruit rot of sweet cherry caused by *Monilinia* species (*M. fructicola* and *M. laxa*) and *Botrytis cinerea,* respectively, compounds with different modes of action (QoIs, DMIs, anilinopyrimidines, phenylpyrroles, hydroxyanilides, SDHIs, and polyoxins) have been evaluated by us and were found to be effective. The pre-mixtures Quadris Top, Pristine, Merivon, Luna Experience, and Luna Sensation represent some of the best treatments along with tank mixtures of FRAC Code 3 and 7 fungicides. Still, more new fungicides are being developed. They generally belong to the same FRAC codes as previously registered compounds, but their activity against fungal pathogens is often different due to their different affinity to fungal target sites. Some of the newer fungicides such as pydiflumetofen and UC-1 have extremely high in vitro activities. Thus, we continued to evaluate the efficacy, spectrum of activity, and persistence of residues of new fungicides and pre-mixtures, 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, seven postharvest fungicides, Tebucon (tebuconazole, FRAC 3), Mentor (propiconazole, FRAC 3), Scholar (fludioxonil, FRAC 12), Chairman (fludioxonil/propiconazole, FRAC 3/12), Penbotec (pyrimethanil, FRAC 9), and the biofungicide BioSpectra (natamycin, FRAC 48) are registered on sweet cherry. Judge (fenhexamid) was withdrawn from postharvest use. Natamycin is the first postharvest biofungicide and is exempt from tolerance 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. Chairman has the broadest spectrum of activity with controlling four decays. Of the FRAC codes registered, Tebucon and Mentor are not ‘reduced-risk’ fungicides. Scholar, Penbotec, and recently Mentor received Food Additive

Tolerances (FAT) in Japan, and the registrant of BioSpectra has submitted for a FAT. Thus, continued studies on how to use these fungicides most efficiently for the Japanese export market are critical to the industry.

Objectives

1. Evaluate new products against bacterial blast in flower inoculation studies and against canker in twig inoculation studies.
   a. Biologicals/natural products (e.g., Actinovate, Cinetis, Blossom Protect).
   b. Conventional bactericides and experimentals (Zinkicide, Type III secretion system inhibitors,)
   c. Antibiotics – kasugamycin, oxytetracycline – large-scale trials once federally registered; improve penetration into plant tissue by using registrant-recommended adjuvants.
   d. Continue to evaluate wound susceptibility of branches and antibiotic protection over time to prevent bacterial canker.
2. Evaluate, under field conditions, bloom and preharvest applications of new compounds (e.g., Fontelis), premixtures (e.g., Luna Sensation, Merivon, Quadris Top), pydiflumetofen, as well as UC-1 and UC-2, EXP-AD, EXP-AF, pyraziflumid, IL-5412, and biologicals for control of brown rot and Botrytis blossom blight, powdery mildew, and pre- and postharvest brown rot and gray mold fruit decay.
   a. Evaluate new powdery mildew fungicides polyoxin-D, pydiflumetofen, SDHI compounds (fluopyram, fluxapyroxad, penthiopyrad, pyraziflumid), and premixtures (e.g., EXP-AD, EXP-AF, UC-2) using rates as recommended by the registrants and develop a powdery mildew fungicide program that integrates new materials with single- and multi-site fungicides.
   b. Evaluate new brown rot and gray mold materials including new DMIs, SDHIs (fluopyram, fluxapyroxad, penthiopyrad, pydiflumetofen), polyoxin-D (Ph-D, Oso), and pre-mixtures (UC-2, IL-5412).
      - Develop baseline data for pydiflumetofen, pyraziflumid, UC-1.
3. Evaluate new fungicides as postharvest treatments and develop cost-effective application methods:
   a. Continue to evaluate Scholar, Mentor, Scholar-Mentor, and BioSpectra (natamycin) mixtures with Scholar or Mentor.
   b. Continue to develop EC_{50} values, baseline sensitivities, and monitor resistance in target pathogen populations to newly developed fungicides.
4. Initiate laboratory studies on new Phytophthora root rot fungicides
   a. Develop baseline sensitivity data for oxathiapiprolin, mandipropamid, fluopicolide, and ethaboxam for *P. cambivora* and other *Phytophthora* spp. that occur on cherry.

MATERIALS AND METHODS

Evaluation of treatments for control of bacterial canker. In winter of 2017/2018, the bark of 2-year-old twigs of Coral cherry trees at UC Davis was puncture-wounded using a 12-gauge needle (3 wounds per twig). Wounds were sprayed with bactericides to run-off using a hand sprayer, allowed to air-dry, and spray-inoculated with a copper-resistant strain of *Pseudomonas syringae* pv. *syringae* (2×10^8 cfu/ml). Treatments included ChampION^{++}, the copper activity-enhancer DAS1, FireLine, a nano-preparation of zinc (Zinkicide), the natural product Cinetis, the biocontrol Blossom Protect, three type III secretion inhibitors, Kasumin, as well as selected mixtures. In May, inoculated branches were sampled and evaluated for the severity of canker formation by measuring canker length (in mm).

Evaluation of new fungicides for control of powdery mildew of sweet cherry. A field trial in San Joaquin Co. was conducted on powdery mildew control. Treatments were done on 3-29-18 (petal fall) for protection from primary inoculum (ascospores from overwintering chasmothecia) and were followed by two additional treatments on April 19 and May 17 for protection from secondary infection from conidia. Single fungicides, pre-mixtures, and three rotation programs were evaluated (Fig. 1). 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 30. 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. Data were expressed as disease intensity (the multiplication product of incidence and severity) and 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 post-infection activity of fungicides against brown rot and gray mold blossom blight. Blossoms were collected at white bud, allowed to open in the laboratory, and inoculated with a spore suspension of *M. fructicola* or *B. cinerea* (30 K/ml) until water droplets formed on anther filaments. After 20 h at 22C, blossoms were treated using 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).

Table 1: Fungicides used in 2018 studies*.

FRAC group	Trade name	Active ingredient
Single active ingredients		
3	Rally	myclobutanil
3	Tebucon/Toledo	tebuconazole
3	Rhyme	flutriafol
3	Fontelis	penthiopyrad
3	Procure	triflumizole
7	Miravis	pydiflumetofen
7	Pyraziflumid	pyraziflumid
13	Quintec	quinoxyfen
19	Ph-D	polyoxin-D
22	Intego	ethaboxam
40	Revus	mandipropamid
43	Presidio	fluopicolide
48	Orondis	oxathiapiprolin
49	BioSpectra	natamycin
U13	Gatten	flutianil
Experimentals	EXP-AD	not disclosed
	EXP-AF	not disclosed
	UC-1	DMI
	UC-2	not disclosed
Premixtures		
7 + 11	Luna Sensation	fluopyram + trifloxystrobin
7 + 3	Luna Experience	fluopyram + tebuconazole
7 + 11	Merivon	fluxapyroxad + pyraclostrobin
3 + 11	Quadris Top	difenoconazole + azoxystrobin
12 + 3	Chairman	fludioxonil + propiconazole

* - Sorted by Fungicide Resistance Action Committee (FRAC) code or mode of action. Some fungicides were used with adjuvants such as Breakthru or DyneAmic.

To evaluate preharvest fungicide applications for control of fruit decay, an experimental orchard was used at UC Davis and a commercial orchard in San Joaquin Co. Treatments were applied 1 day (UC Davis) or 6 days (commercial orchard) PHI using a back-pack sprayer calibrated to deliver 100 gal/A. Fruit (8 fruit from each of three or four 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* (30,000 conidia/ml). In non-wound inoculations, fruit were inoculated with droplets (200,000 conidia/ml) or 50 to 60 fruit were sprayed (15,000 conidia/ml) with a conidial suspension For gray mold, 16 fruit from each replication were non-wound drop-inoculated with a spore suspension (200,000 spores/ml prepared in cherry juice). All fruit were incubated for 5-7 days at 20-24 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 with *M. fructicola* or *B. cinerea* as described above.

Efficacy of new and registered postharvest treatments for managing brown rot, gray mold, and Rhizopus rot of sweet cherry. One commercial packingline study and several laboratory studies were done with emphasis on the recently registered BioSpectra (natamycin) and Chairman (a pre-mixture of fludioxonil and propiconazole). In the laboratory, fungicides were applied as aqueous solutions using an air-nozzle sprayer 12-14 h after inoculation with the decay pathogens. Two studies each were done with Chairman and with natamycin. In the commercial packingline studies, applications were done by two sequential T-Jets that were separated by a step on the belt so that fruit slightly tumbled and turned. Fruit wound-inoculated with 20 µl of a spore suspension of *M. fructicola, B. cinerea,* or *R. stolonifer* (30,000 spores/ml each) were used and additionally, treated fruit were spray-inoculated with *M. fructicola*. After treatment, fruit were 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.

***Initiate laboratory studies on new Phytophthora root rot fungicides*.** Baseline sensitivity data for oxathiapiprolin, mandipropamid, fluopicolide, and ethaboxam were developed for several *Phytophthora* spp. that occur on cherry and other tree crops in California. For this, isolates were recovered from cherry, walnut, and almond plant tissues (scion, crown, bark, trunk, root), from orchard soil, or were obtained from the G. Browne culture collection. Isolates were identified to species and EC_{50} values were determined using our standard spiral gradient assay.

RESULTS AND DISCUSSION

Evaluation of treatments for control of bacterial canker. Inoculations of treated, injured branches with a copper-resistant strain of *P. syringae* pv. *syringae* resulted only in a very low incidence of canker development in late winter and early spring in 2018, and data on treatment efficacy could not be obtained. This lack of disease development can be contributed to the unusual warm temperatures and low rainfall that occurred in the month (60.6ºF high, 34.1ºF low, and 46.5ºF avg with 0.03 in precip.) following inoculation when the study was conducted. Bacterial canker is generally associated with cool and wet environments.

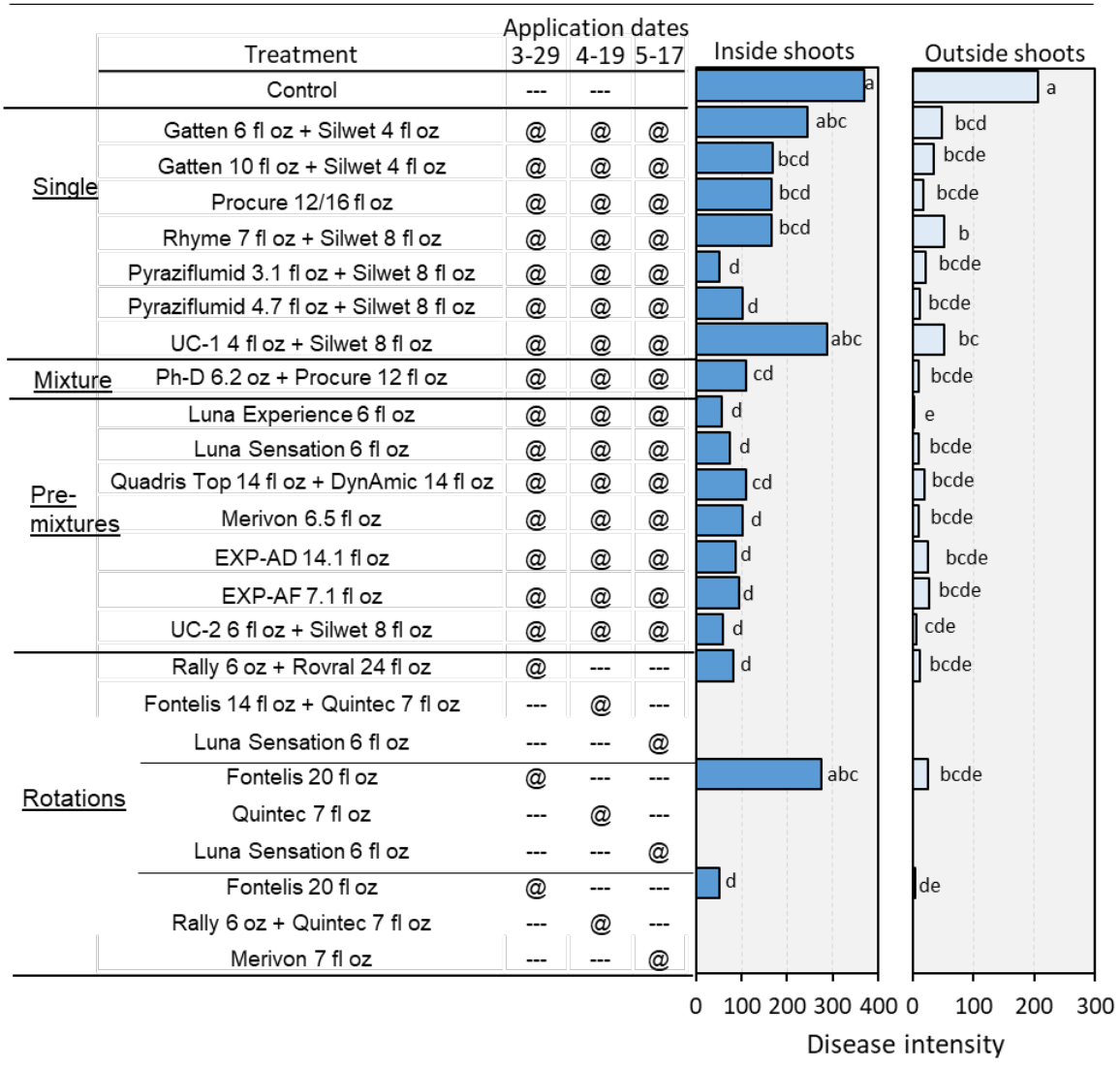
With widespread copper resistance in the bacterial pathogen *P. syringae* pv. *syringae*, new effective treatments are needed to manage bacterial canker and blast. These are important diseases of sweet cherry that can impact cherry production in seasons with favorable environmental conditions and can also have long-term effects on tree health. In our studies over the years, Kasumin was the most effective and consistent treatment against both phases of the disease, and based on these studies, this antimicrobial was recently registered. Oxytetracycline was also identified as a promising bactericide against *P. syringae*, and a registration on sweet cherry is currently pursued with support of the registrant through the IR-4 program. In our evaluations over several years, Actinovate also showed good efficacy in reducing blossom blast (but was less effective against canker), and Blossom Protect/Botector also reduced the disease.

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 epi- or mesocarp tissue of green fruit. We have observed that young leaves are more susceptible than old leaves. The efficacy of new fungicides and pre-mixtures was evaluated in a trial in San Joaquin Co. Three applications were done in ca. three-week intervals over a 7-week period starting at petal fall. Environmental conditions were highly favorable for powdery mildew development at our trial site in the spring of 2018. At evaluation time in late May, a high incidence of disease (>90%) was present on leaves of inside and outer shoots. Disease severity (leaf area affected), however, was lower on outside (rating 2.2) than on inside shoots (rating 3.7).

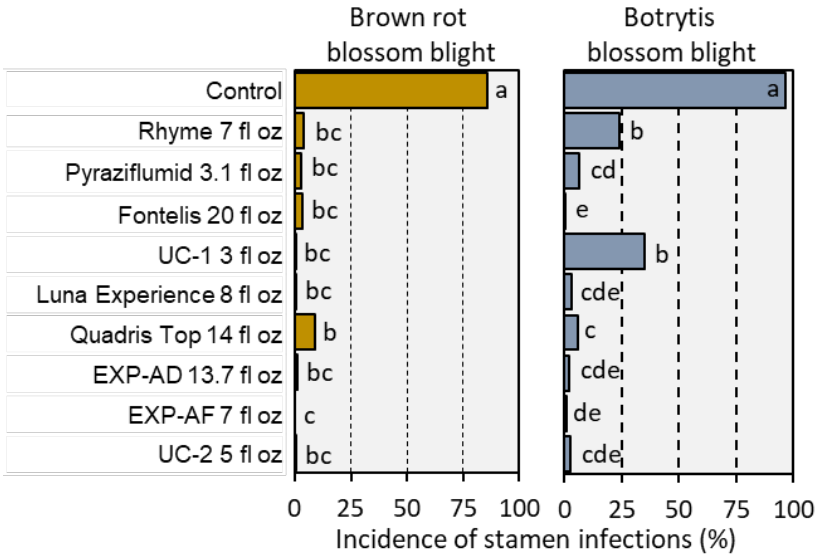
Based on disease intensity (the multiplication product of incidence and severity), treatments were mostly more effective on outside shoots where disease severity was lower than on the inside shoots (Fig. 1). Treatments that were highly effective on both types of shoots included the new FRAC 7 pyraziflumid

Fig. 1. Evaluation of preharvest fungicide treatments for management of powdery mildew of Bing cherries in San Joaquin Co. 2018



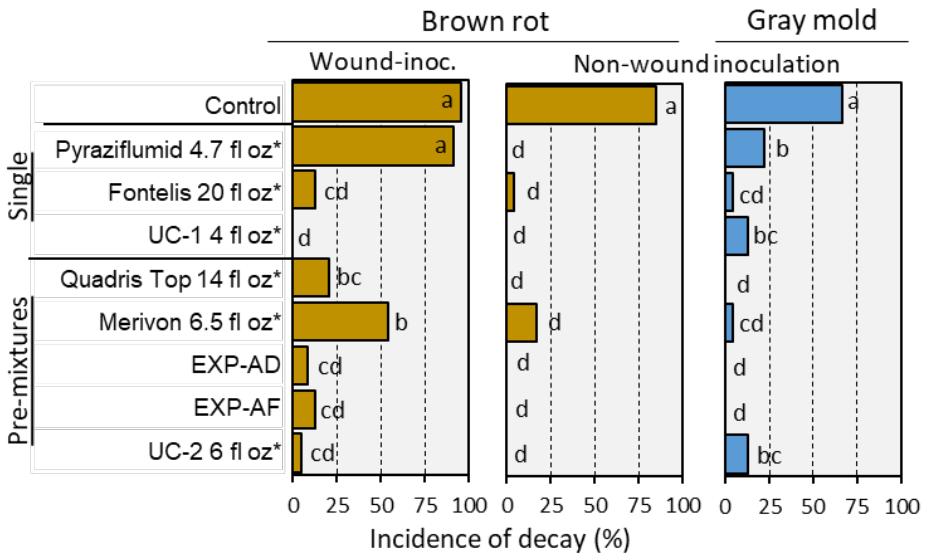
Applications were done using an airblast sprayer at 100 gal/A. Petal fall was on 3-29-18. For Procure,16 fl oz was used starting with the second application. For evaluation on 5-30-18, 20 leaves from 4 shoots each from inside or outside of the tree were sampled. The rating scale was: 0=healthy, 1=1-3 lesions/leaf, 2=<25%, 3=26-50%, 4 = >50% of leaf area diseased. The disease intensity was calculated by multiplying disease incidence with disease severity.

Fig. 2. Efficacy of post-infection treatments for control of brown rot and Botrytis blossom blight of Bing cherry – Laboratory studies 2018



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

Fig. 3. Efficacy of 1-day preharvest fungicide treatments for management of postharvest brown rot and gray mold of Bing cherries - UC Davis - 2018



Treatments were applied on 5-24-18 using an air-blast sprayer at a rate of 100 gal/A. Treatments marked with an asterisk were applied in combination with 6 fl oz of DynAmic. Fruit were wound-inoculated with *M. fructicola* (30,000 spores/ml) or non-wound-inoculated with *M. fructicola* (200,000 spores/ml) or *B. cinerea* (200,000 spores/ml cherry juice) and incubated at 20C for 6 days.

as well as registered (Luna Experience, Luna Sensation, Quadris Top, Merivon) and new (EXP-AD, EXP-AF, UC2) pre-mixtures. The registered pre-mixtures contain combinations of DMI, SDHI, and QoI compounds which are known to have high activity against powdery mildews. The new fungicide Gatten (flutianil) was moderately to highly effective in these studies. Quintec (FRAC 13) that was highly

effective in the first years after its registration on cherry showed reduced performance over the last several years. In 2018, it was used in three rotation/mixture programs. The program where Quintec was used by itself at one of the three application timings showed the lowest efficacy with no difference from the control for the inside shoots, but a significant reduction in disease on outside shoots. This again indicates that the pathogen population at the trial location has developed insensitivity against Quintec and was only effective in mixture with other FRAC codes. Reduced sensitivity to Quintec is still localized and use of the fungicide in mixtures with other fungicides should prolong its efficacy for the industry.

Thus, this research demonstrated excellent activity of several newly registered, as well as of experimental 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 FRAC 3, FRAC 7, FRAC 11, and FRAC 19 fungicides will be most sustainable. This limits the use of any single-site mode of action fungicide (i.e., single FRAC numbers) and reduces the selection pressure for selecting for fungicide resistance. Limiting the number of applications of any one mode of action (i.e., FRAC) 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.

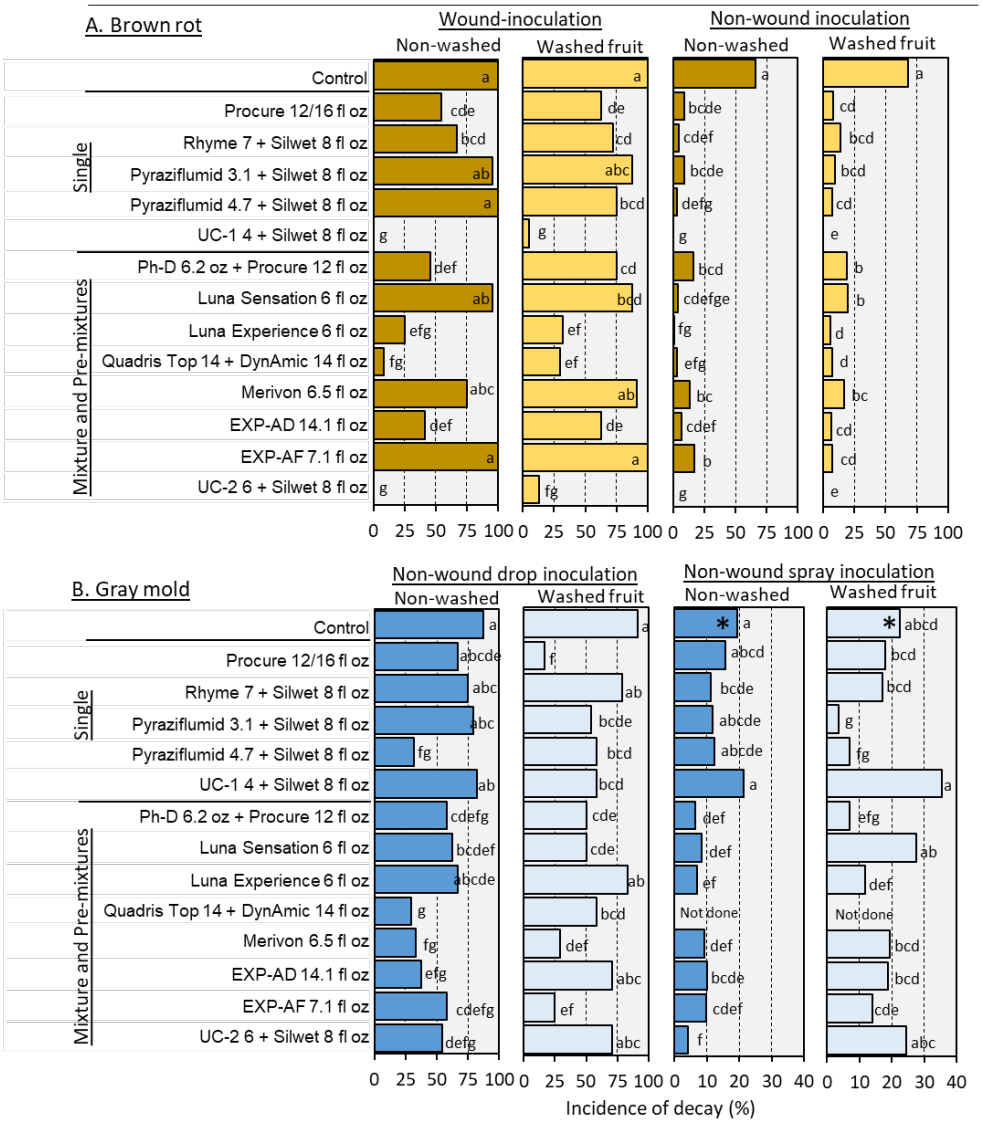
Efficacy of new fungicides for control of brown rot and Botrytis blossom blight. Selected fungicides were evaluated for their post-infection activity on detached opened blossoms in laboratory studies. For brown rot blossom blight, all of the registered and experimental compounds evaluated were highly effective, whereas for gray mold blossom blight, all treatments significantly reduced the disease from the control, but Rhyme and UC-1 were less effective than the other fungicides tested (Fig. 2). Thus, treatments with excellent activity for management of blossom blight caused by both pathogens are currently available and include Fontelis, Luna Experience, and Quadris Top.

Due to the good pre- and post-infection activity of most of the conventional fungicides that was demonstrated previously, 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 bloom treatments on sweet cherry. Although having reduced efficacy, selected biological treatments were identified previously (Botector, Serenade Opti) that are not as effective as conventional fungicides but can benefit disease management in organic production systems.

Evaluation of preharvest treatments for fruit decay control without postharvest washes and for postharvest decay control after postharvest washes. Two preharvest efficacy trials with 1- or 6-day PHI applications were done in 2018 (Figs. 3, 4). In wound inoculation studies using non-washed fruit, several fungicides provided excellent protection against brown rot and these included the registered pre-mixtures Quadris Top and Luna Experience, as well as the experimental compounds UC-1 and UC-2B (Figs. 3, 4). Fontelis was also very effective in the first study with 1-day PHI applications where this fungicide was evaluated. In non-wound inoculations, all fungicides evaluated significantly reduced the incidence of decay development in both studies. This emphasizes the importance of care in handling fruit to prevent injuries that by-pass the protective fungicides that are not locally systemic. With the gray mold pathogen, only non-wound inoculations were done but the pathogen was provided a nutrient source (i.e., cherry juice) to facilitate fruit infection. On fruit harvested and inoculated 1 day after application, all treatments significantly reduced gray mold development as compared to the control, and Quadris Top, Fontelis, Merivon, EXP-AD and EXP-AF were most effective (Fig. 3). In the 6-day PHI treatments that were not washed, efficacy was reduced compared to the 1-day PHI interval, but fungicides with good activity against gray mold were identified and these included Quadris Top, Merivon, EXP-AD, and pyraziflumid (Figs. 3,4B).

Harvested fruit were washed, simulating a hydrocooler treatment, in the second trial with 6-day PHI treatments (Fig. 4). In wound inoculations, a reduced efficacy was observed for some fungicides in reducing brown rot, but Luna Experience, Quadris Top, UC-1 and UC-2 (all containing a DMI fungicide) were still very effective (Fig. 4A). After non-wound spray inoculation with *M. fructicola*, fungicides mostly were similarly highly effective as with non-washed fruit. The incidence of gray mold on spray inoculated, washed fruit was significantly reduced by pyraziflumid (both rates) Ph-D+Procure, Luna Experience, and EXP-AF (Fig. 4B).

Fig. 4. Efficacy of 6-day preharvest fungicide treatments for management of postharvest brown rot and gray mold of Bing cherries - Orchard 2 San Joaquin Co. - 2018

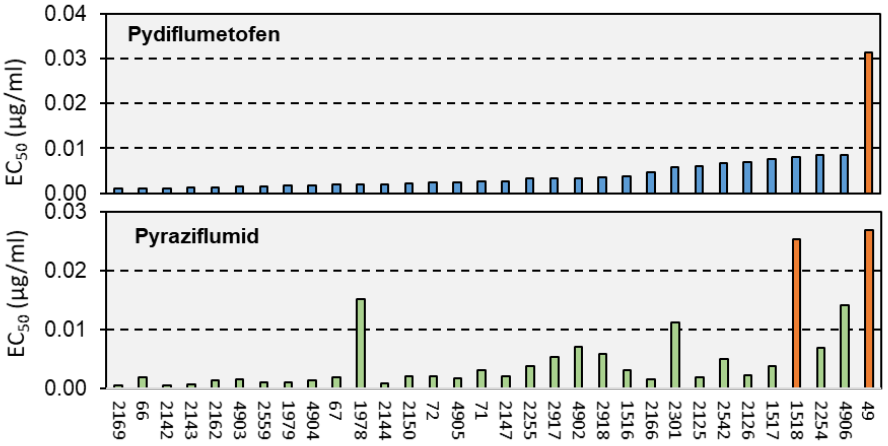


Treatments were applied on 5-17-18 using an air-blast sprayer at a rate of 100 gal/A. Washes of harvested fruit were done by spraying with high volumes of water for 3 minutes. Fruit wound-inoculated with *M. fructicola* (30,000 spores/ml), or non-wound drop-inoculated with *B. cinerea* (200,000 spores/ml in cherry juice), or spray-inoculated with a mixture of *M. fructicola* (15,000 spores/ml) and *B. cinerea* (30,000 spores/ml).
*The incidence of gray mold in these controls is likely under-estimated. Fruit were spray-inoculated with a mixture of both pathogens, and when a high incidence of brown rot was present, gray mold may have been over-grown.

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. Hydrocooling removes fungicide residues from the fruit as shown by generally higher disease levels in our studies. This

emphasizes that postharvest fungicides are still warranted for shipping and marketing fruit to distant markets.

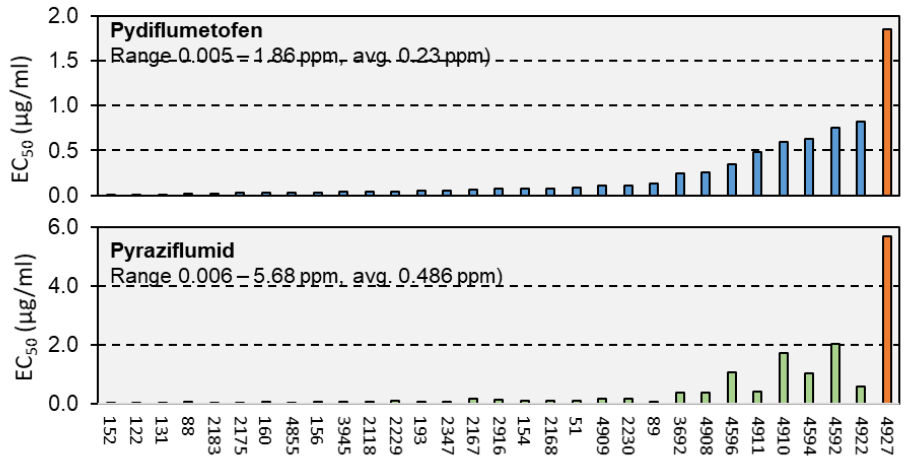
Fig. 5. Baseline sensitivities of 32 isolates of *Monilinia fructicola* to SDHI fungicides



SDHI Subgroup	Fungicide	Range (µg/ml)	Average (µg/ml)
Pyridinyl-ethylbenzamide	Fluopyram	0.005 – 0.132	0.022
Pyrazole-4-carboxamides	Fluxapyroxad	0.004 – 0.050	0.020
N-methoxy-phenylethyl)-pyrazolecarboxamides	Pydiflumetofen	0.001 – 0.031	0.004
Pyrazinecarboxamide	Pyraziflumid	0.001 – 0.027	0.005

In vitro sensitivities were determined using the spiral gradient dilution method. In the histograms, isolates are in the same order the two fungicides.

Fig. 6. Baseline sensitivities of 32 isolates of *Botrytis cinerea* to two SDHI fungicides



Sensitivities were determined using the spiral gradient dilution method. In the histograms, isolates are in the same order for the two fungicides. An additional isolate (from plum) was highly resistant to three SDHIs.

Develop baseline sensitivity data for new fungicides. Ranges and averages of EC_{50} values for inhibition of mycelial growth of 32 isolates of *M. fructicola* by fluopyram, fluxapyroxad, pydiflumetofen, and pyraziflumid, each belonging to a different SDHI sub-group, are presented in Fig. 4. One isolate (No. 49) was less sensitive against pydiflumetofen and pyraziflumid (EC_{50} values 0.031 and 0.027 mg/liter, respectively) and another isolate (i.e., 1518) was less sensitive to pyraziflumid as compared to the remaining isolates (Fig. 5). These latter isolates, however, were highly sensitive to fluopyram and

fluxapyroxad. Another isolate (isolate 2542) that was highly sensitive to pydiflumetofen and pyraziflumid showed reduced sensitivity to fluopyram with an EC_{50} value of 0.132 mg/liter. These three isolates were collected in 2003 or earlier, before field use of the newer SDHI compounds. Therefore, less sensitive isolates are present in natural field populations. Currently, all isolates evaluated are still considered sensitive to the SDHI compounds tested, but the results indicate possible cross-resistance patterns among the sub-groups and a risk of selection of isolates with lower sensitivity or resistance.

In contrast to *M. fructicola*, a much wider range of sensitivities was determined for 32 isolates of *B. cinerea* (Fig. 6). Although the majority of isolates was highly sensitive, several isolates had EC_{50} values >0.5 ppm. Sensitivity characteristics of isolates were similar for pydiflumetofen and pyraziflumid. One isolate was found to be highly resistant against these two SDHI compounds. This indicates that *B. cinerea* is at risk to develop resistance. Furthermore, cross resistance is present among SDHI sub-groups, and SDHI fungicides should always be rotated with different FRAC codes.

Efficacy of new postharvest treatments for managing brown rot, gray mold, and Rhizopus rot of sweet cherry. Laboratory studies and a commercial packingline trial focused on the evaluation of Chairman and BioSpectra (natamycin). In laboratory studies with wound-inoculated fruit, spray treatments with three rates (e.g., 8, 12, and 16 fl oz) of the recently registered Chairman each completely prevented the development of brown rot, gray mold, and Rhizopus rot (Fig. 7). Thus, this pre-mixture of fludioxonil and propiconazole not only has a wide spectrum of activity (brown rot, gray mold, Rhizopus rot, sour rot), but also is highly effective at low rates.

Two formulations of natamycin, the currently registered BioSpectra and the experimental Nata High Solution, each at three rates, were compared in other laboratory studies. All rates and both formulations completely inhibited brown rot development (Fig. 8). Overall, Rhizopus rot was also very effectively reduced by all treatments. As in previous studies, natamycin was less effective against gray mold, but decay was still significantly reduced from the control. There was not significant difference in efficacy among rates of BioSpectra or Nata High Solution, but the 250-ppm rate of Nata High performed significantly better than the same rate of BioSpectra. In our postharvest work on other fruit crops we also

Fig. 7. Postharvest treatments of inoculated Bing cherry fruit with different rates of Chairman in laboratory studies 2018

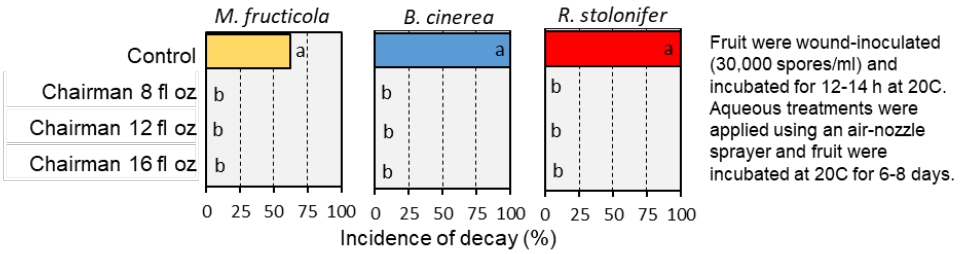
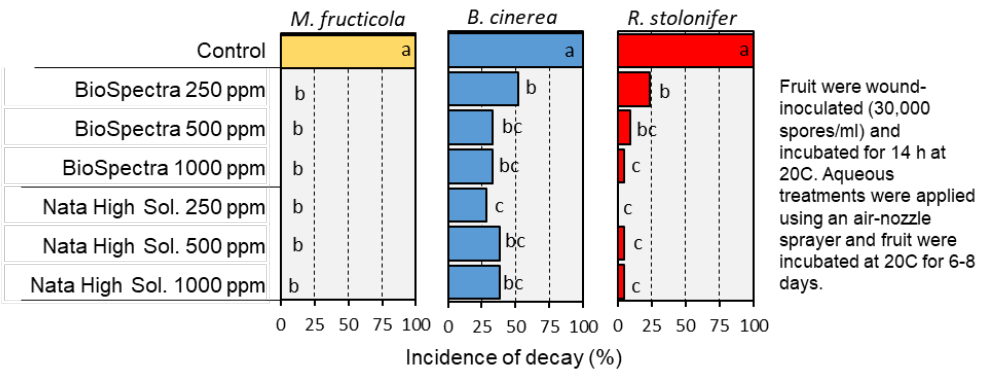


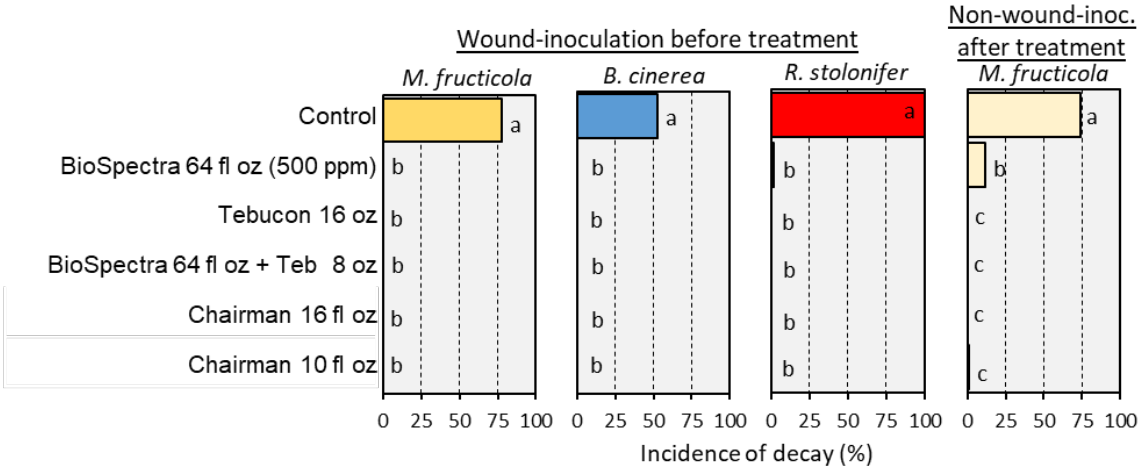
Fig. 8. Comparison of the efficacy of different rates and formulations of natamycin for postharvest decay control of inoculated Bing cherry fruit in laboratory studies 2018



noticed differences between formulations of natamycin. The registrant has indicated to us that therefore, different formulations may become available for different fruit crops.

In the commercial packingline study, all treatments evaluated in T-Jet applications including BioSpectra (used at 500 ppm), Tebucon, a BioSpectra-Tebucon mixture, and Chairman were highly effective in reducing brown rot, gray mold, and Rhizopus rot of inoculated fruit (Fig. 9). These treatments were also highly effective in reducing brown rot when treated fruit were non-wound inoculated. Thus, BioSpectra, in contrast to the laboratory study was also very effective against gray mold. Different incubation periods were used for laboratory and packingline studies before treatment, and the 2-h shorter incubation time used in the commercial study could have allowed for better gray mold control. Under commercial conditions, the 10 fl oz rate of Chairman was as effective as the 16 fl oz rate and should allow for flexible usage under low and high disease pressure.

Fig. 9. Postharvest treatments of inoculated Bing cherry fruit in a commercial packingline study 2018



Fruit were wound-inoculated with *M. fructicola*, *B. cinerea*, or *R. stolonifer* (30,000 spores/ml) and incubated for 12 h at 20C. T-Jet treatments were done by 2 sequential spray bars separated by a step on the belt to promote turning of the fruit. Additional treated fruit were non-wound spray-inoculated with *M. fructicola* (200,000 spores/m). Fruit were then incubated for 5-10 days at 22C.

In summary, in our postharvest studies, we identified, optimized, and helped registered two new postharvest treatments, BioSpectra and Chairman, for sweet cherry. Excitingly, resistance has never been reported to the active ingredient 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, however, MRLs have not been established for natamycin in many countries and use is suggested only for domestic markets (including Canada). A FAT in Japan is expected for natamycin in the next few years. With increasing emphasis on food safety and consumer concerns, natamycin with 'exempt from tolerance status' will likely become an important component of postharvest decay management in the future. MRLs have been established and FATs for propiconazole were approved in June 2018 and thus, Chairman can be used for cherries (and other stone fruits) exported to Japan. The registered use of propiconazole for both pre- and postharvest use will also provide numerous options for the packinghouse manager and marketing teams. We will continue our evaluations of these treatments in 2019 in cooperation with commercial packinghouses.

Evaluate the in vitro toxicity of new fungicides against selected *Phytophthora* species occurring on sweet cherry and other tree crops. Eighty isolates of *Phytophthora* were obtained from diseased tree fruit samples and from orchard soil. Species identification based on DNA sequencing revealed the presence of 11 species, with the majority of isolates belonging to *P. niederhauseri, P. syringae, P.*

citricola/P. citricola complex, and *P. cactorum*. Species less common included *P. chlamydospora, P. cinnamomi, P. gonapodyides, P. lacustris, P. megasperma, P. obscura*, and *P. rosacearum.*

All isolates were most sensitive to oxathiapiprolin with EC_{50} values for mycelial growth inhibition of $\leq$0.001 mg/liter (Table 2). A rather narrow range of EC_{50} values (0.001 to 0.01 mg/liter) among all isolates was also found for mandipropamid, whereas for ethaboxam and fluopicolide generally higher rates were needed. Higher concentrations of fluopicolide (0.104 - 0.229 mg/liter) were needed to inhibit the 7 isolates of *P. cactorum*, and a wider range of sensitivities (0.021 - 0.318 mg/liter) was determined for the 16 isolates of *P. syringae.* All isolates were also inhibited by mefenoxam, but rates of 0.122 to 0.155 mg/liter were needed for some isolates of *P. citricola*/*P. citricola* complex and *P. rosacearum*. For the 24 isolates of *P. niederhauseri*, a wide range from 0.012 to 0.209 mg/liter was determined. Thus, although a wide range of sensitivities was sometimes present for some of the fungicides and species, no resistance was detected. These isolates were never exposed to any of the new fungicides, and the wide range of sensitivities sometimes encountered for fluopicolide (similarly to our previous evaluations of *Phytophthora* isolates from citrus) may indicate that this fungicide has at higher risk for selection of resistance. Additional isolates will be collected and evaluated in 2018/19.

Table 2. Effective concentrations of four new fungicides and mefenoxam for inhibition of fifty percent mycelial growth (EC_{50} values) of 11 *Phytophthora* species collected from cherry and other tree crops in California[a]

		EC_{50} value ranges for mycelial growth (µg/ml)				
***Phytophthora* spp.**	**No. of isol.**	**Mefenoxam**	**Oxathiapiprolin**	**Mandipropamid**	**Ethaboxam**	**Fluopicolide**
P. cactorum	7	0.009 - 0.023	0.0005 - 0.001	0.007 - 0.009	0.026 - 0.088	0.104 - 0.229
P. chlamydospora	1	0.017	0.0003	0.002	0.053	0.035
P. cinnamomi	5	0.007 - 0.038	0.0002 - 0.0004	0.002 - 0.005	0.006 - 0.017	0.041 - 0.078
P. citricola complex	14	0.061 - 0.155	0.0003 - 0.0006	0.002 - 0.004	0.083 - 0.258	0.027 - 0.047
P. gonapodyides	4	0.004 - 0.015	0.0002 - 0.0005	0.001 - 0.005	0.007 - 0.047	0.026 - 0.072
P. lacustris	2	0.003 - 0.004	0.0002 - 0.0004	0.002 - 0.004	0.054 - 0.137	0.015 - 0.025
P. megasperma	4	0.01 - 0.013	0.0003 - 0.0005	0.002 - 0.005	0.04 - 0.079	0.082 - 0.24
P. niederhauseri	24	0.012 - 0.209	0.0001 - 0.0004	0.003 - 0.01	0.031 - 0.105	0.041 - 0.067
P. obscura	1	0.003	0.0003	0.002	0.033	0.018
P. rosacearum	2	0.105 - 0.122	0.0002 - 0.0004	0.003 - 0.005	0.06 - 0.089	0.06 - 0.08
P. syringae	16	0.002 - 0.041	0.0002 - 0.0004	0.001 - 0.004	0.017 - 0.13	0.021 - 0.318
Total	80					

[a] The majority of *Phytophthora* isolates were recovered from cherry, almond, and walnut plant tissues (scion, crown, bark, trunk, root), from orchard soil, or from the culture collection of G. Browne, USDA, ARS.

Annual report 2018

Project Title: IMPROVED MANAGEMENT OF FUNGAL CANKER DISEASES OF SWEET CHERRY

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Interpretive summary
Canker diseases of sweet cherry constitute an important problem for the production of sweet cherry in California, causing dieback of branches, scaffolds and trunks and reducing yields and longevity of orchards. In order to provide an integrated management strategy for cherry canker diseases, we first evaluated the sanitary status of sweet cherry plant materials to determine if commercial nursery stocks were contaminated with canker pathogens and thus the likelihood for growers of planting infected trees. Second, we assessed the possibility of spreading canker diseases within orchards by pruning tools, and, third, we tested the efficacy of 12 compounds to protect pruning wound infections by canker pathogens. Although the main canker pathogens (*Calosphaeria pulchella*, *Eutypa lata* and *Cytospora sorbicola*) were not found in nursery stocks, approximately 50% of the nursery planting material examined had symptoms of wood cankers, decay and lesions from which several known fungal pathogens were isolated. Planting with such infected material would doom an orchard to low productivity and reduced longevity. Our results also showed that the transmission of *Calosphaeria pulchella*, *Eutypa lata* and *Cytospora sorbicola* could be achieved by pruning tools when first cutting through a dead branch carrying fungal fruiting bodies and making a subsequent cut into a healthy branch. These findings highlight the necessity of developing sanitation strategies for pruning tools in order to minimize canker disease transmission during pruning periods. Deccosan 321, a quaternary ammonium compound used for equipment disinfestation was shown to be effective for pathogen disinfection of pruning tools. Finally, of the 12 compounds evaluated for protecting pruning wounds infections by *Eutypa lata* and *Cytospora sorbicola*, 4 compounds provided significant protection and can be considered promising candidates to be integrated in disease management programs for cherry canker diseases.

Objective 1: Determine latent infection of canker pathogens in nursery stocks (on-going)

Material and methods

We examined 161 plants originating from two commercial nurseries in order to determine if nursery stocks could be infected before planting for commercial production.

We obtained 90 trees corresponding to 5 rootstock/scion combinations from Nursery 1 and 71 trees corresponding to 4 rootstock/scion combinations from Nursery 2.
All 161 trees were sectioned transversally and longitudinally in order to examine the presence of vascular discolorations, wood rot and wood cankers at four sampling locations: within the rootstock, just below the graft union, just above the graft union, and within the scion (Figure 1).

From these symptomatic tissues, approximately 10 wood pieces were surface-sterilized by immersion for 2 mins in a 1.5 % sodium hypochlorite solution, and washed twice with sterile distilled water. Small pieces of healthy as well as necrotic tissues were selected and placed onto petri dishes filled with potato dextrose agar (PDA) amended with 100 ppm tetracycline (PDA-tet) for isolation of fungi. Fungal identification was conducted using DNA based techniques including the polymerase chain reaction (PCR), amplification and sequencing of the internal transcribed spacer region (ITS) of the rDNA using primers ITS1 and ITS4.

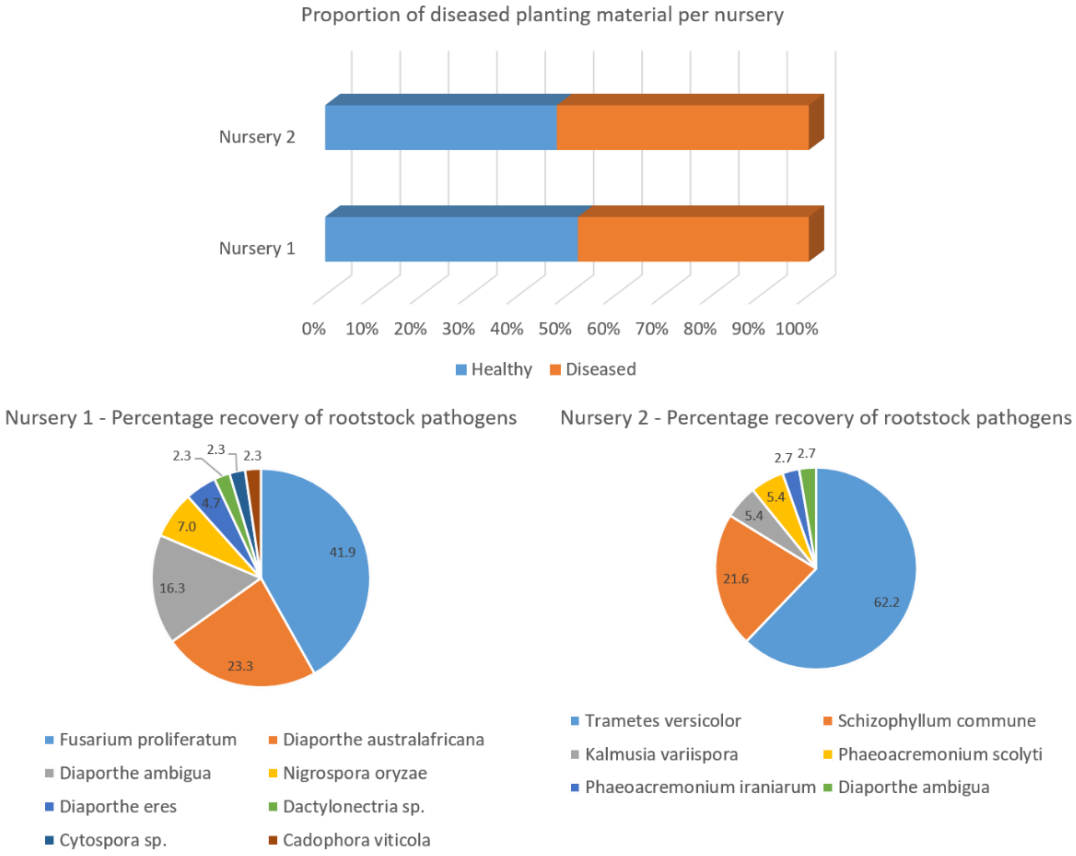
Figure 1. Sampling methodology for evaluating fungal infections in cherry nursery stocks. Left: 4 wood samples per tree were examined and sampled. Right: cross–sections of stems below the graft union frequently revealed wood rot from trees originating from Nursery 2.

Results and Discussion

From the 90 trees examined from Nursery 1, 43 had symptoms of fungal infections, mainly cankers and vascular discolorations developing in the rootstock section of trees below pruning wounds: 48% of trees were found diseased based on symptom occurrence and isolation of known wood pathogens (Figure 2). From these diseased trees, we isolated fungal pathogens principally (62% of diseased wood samples) belonging to the genus *Fusarium* and *Diaporthe* (*Phomopsis*), two important genera of plant pathogenic fungi known to cause canker and crown rot diseases in various perennial crops. One *Cytospora* isolate was recovered from one symptomatic tree.

From the 71 trees examined from Nursery 2, 37 had symptoms of fungal infections, mainly wood rot developing within the inner wood of the rootstock section of trees, just below the main pruning wound occurring at the bud union. Wood discolorations were also found associated with topping of these young trees. Overall, 51% of trees were found diseased based on symptom occurrence and isolation of known wood pathogens (Figure 2). From these diseased trees, we isolated two main fungal pathogens, *Trametes versicolor* and *Schyzophyllum commune* (84% of diseased wood samples), two Basidiomycete fungi known to be responsible for white rot and wood degradation in many tree crops.

Figure 2. Sanitary status of cherry planting material (161 plants from two nurseries) regarding fungal infections. Ninety plants were examined from nursery 1 and 43 had symptoms of fungal infections from which we recovered potential fungal pathogens. Seventy-one plants were examined from nursery 2 and 37 had symptoms of fungal infections from which we recovered potential fungal pathogens.



Canker pathogens have been found previously to be present within nursery planting materials in other crops such as almond and grapevine, suggesting infections can take place during the plant propagation process (Gramaje and Armengol, 2011; Themis Michailides, personal communication). Introducing canker diseases into new orchards via planting material would make disease control inefficient. Further collaborations with nurserymen will be necessary to implement production practices that minimize the risks of nursery stocks contaminations by fungal pathogens.

Objective 2: Investigate the role of pruning tools on canker disease transmission (on-going)

Material and methods

A first experiment was conducted in April 2018 to evaluate the possibility of transmitting canker diseases from diseases branches to healthy branches while making pruning cuts with pruning shears. Also, we evaluated the efficacy of Deccosan 321 (Decco US, Monrovia, CA), a quaternary ammonium compound used for equipment disinfestation in other crops, as an effective method for pathogen disinfection of pruning tools.
Pruning experiments were conducted in April 2018 in one orchard. For each of the 3 pathogens (*Calosphaeria pulchella*, *Eutypa lata* and *Cytospora sorbicola*) 5 treatments were tested and applied to 6 branches (6 repetitions):

1. Canker transmission: non-disinfected pruning shears are first used to make a cut through a wood canker in a branch affected by Calosphaeria, Eutypa or Cytospora canker before making a new cut into a healthy branch.
2. Canker transmission + Deccosan 321: same as #1 except the pruning shears are sprayed with Deccosan 321 before the second cut into a healthy branch.
3. Fruiting body transmission: non-disinfected pruning shears are first used to make a cut through a dead branch carrying fruiting bodies of either Calosphaeria, Eutypa or Cytospora pathogens before making a new cut into a healthy branch.
4. Fruiting body transmission + Deccosan 321: same as #3 except the pruning shears are sprayed with Deccosan 321 before the second cut into a healthy branch.
5. Positive control: artificial inoculations of clean pruning cuts using spore suspensions of the three pathogens.

Following pruning, wounds were protected with Parafilm to prevent natural infection. Six months after pruning, pruned branches were brought to the laboratory for examination of wood lesions from pruning cuts and recovery of canker fungi from these lesions. For fungal isolations, 10 wood pieces per pruned branches were selected approximately 1 cm below the pruning cut. These wood pieces were placed onto petri dishes filled with potato dextrose agar (PDA) amended with 100 ppm tetracycline (PDA-tet) for isolation of fungi. Successful recovery of *Calosphaeria pulchella*, *Eutypa lata* and *Cytospora sorbicola* were evaluated based on colony morphology.

In August 2018, all six branches corresponding to each of the 15 treatments (3 pathogens, 5 treatments per pathogen applied to six pruning cuts) were collected to

determine if the disease was successfully transmitted by pruning tools. Pruned branches were brought to the laboratory for examination of wood lesions from pruning cuts and recovery of canker fungi from these lesions. This experiment was designed to test the hypothesis that cutting through a canker (Figure 3, top-left panel) or cutting through fruiting bodies of canker pathogens present at the surface of dead branches (Figure 3, bottom-left and top-right panels) can transmit the disease to a new, clean branch after a subsequent cut (Figure 3, bottom- right panel).

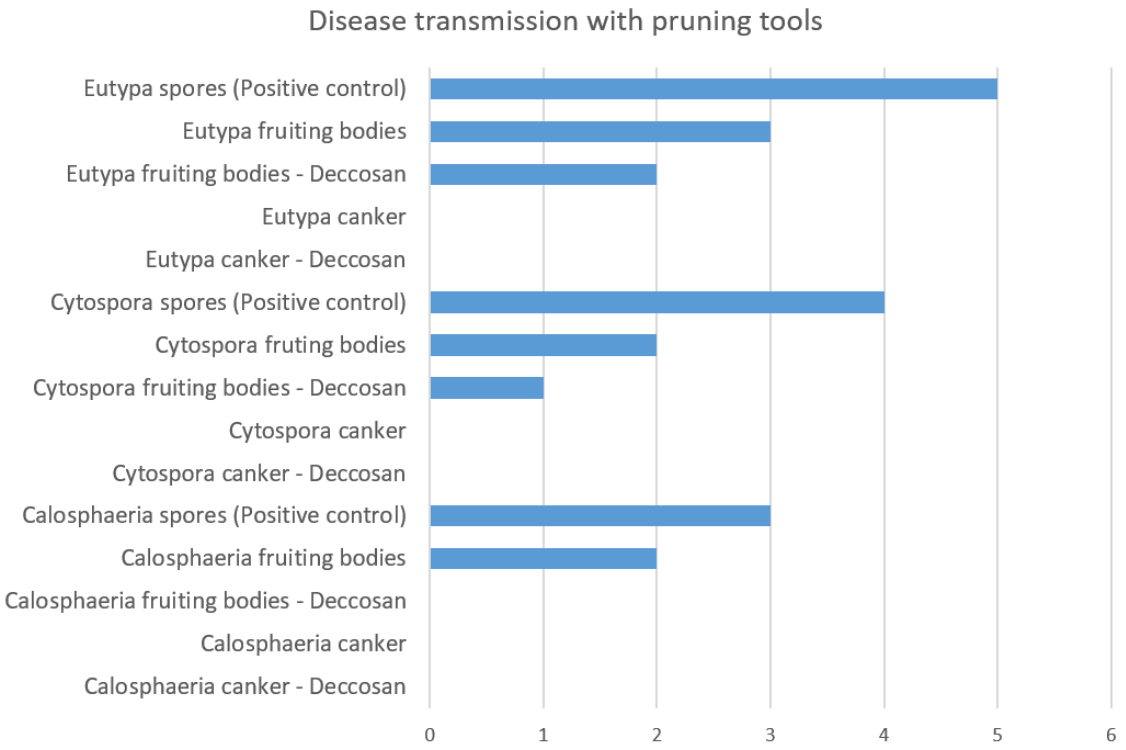
Figure 3. Illustrations of the infection sources (canker, top-left panel; fruiting bodies, bottom-left and top-right panels) used to evaluate the potential of disease transmission to a new branch after a subsequent cut (bottom- right panel) with pruning tools.

Results and Discussion

For each pathogen, spore inoculations (positive control) resulted in infections of fresh pruning cuts: 5/6 for Eutypa, 4/6 for Cytospora, and 3/6 for Calosphaeria (Figure 4). Cutting through cankers first and making a new cut to a healthy branch did not transmit the disease for any of the three pathogens. However, disease transmission with pruning tools was achieved when cutting through branches carrying pathogen fruiting bodies: 3/6 for Eutypa, 2/6 for Cytospora and Calosphaeria. The spray-disinfestation of pruning blades with Deccosan 321 reduced but did not prevent disease transmission to new, clean pruning wound for the 3 pathogens.

Our findings regarding the possibility of transmitting canker diseases with pruning tools confirm previous findings regarding Calosphaeria canker of sweet cherry and other canker diseases of grapevine in Spain (Agustí-Brisach et al., 2015, Berbegal and Armengol, 2018). These findings raised the question of the importance of this infection process in comparison with natural infection of fresh pruning wounds by airborne inoculum. This work also suggest that pruning tool disinfestation would require further research.

Figure 4. Number of branches out of 6 branches tested with successful transmission of canker pathogens following the various treatments.



Objective 3: Test the efficacy of various compounds for the protection of pruning wounds (on-going)

Material and methods

A field trial was conducted in Davis from February to May 2018. Lignified branches (2nd to 3rd year wood) of 12-year-old cherry trees were pruned in order to make a flat wound. Wounds were treated with either sterilized water (negative control) or one of the 12 compounds listed in Table 1. Applications were made with hand-held spray bottles at the label rate, and wounds were sprayed until runoff. Approximately 48 hours after wounds were treated with fungicidal products, wounds were misted with sterilized water to provide high relative humidity and these wounds were inoculated with 100 µL of a spore suspension of the fungal canker pathogens *Calosphaeria pulchella*, *Eutypa lata* and *Cytospora sorbicola* at a concentration of 1,000 spores per wound. Nine replicates per treatment (treatment = protection product + fungal isolate) were established in a randomized complete block design.

Treated branches were collected approximately 14 weeks after inoculation and brought to the laboratory for fungal isolations. Presence (1) or absence (0) of the inoculated fungal species was recorded and averaged for each treatment. Low rates of fungal recovery were correlated with high product efficacy. Infection rate was calculated as the percentage of pruning wounds from which the pathogen was recovered, out of the total number of inoculated pruning wounds. To assess the effect of each product on infection rate,

generalized linear mixed models were performed using the GLIMMIX procedure in SAS, which utilizes the *logit* link function to accommodate binomial data (0/1). Mean comparison with control treatment was performed using a Dunnett test in SAS. Mean percent disease control (MPDC) was calculated as the reduction in Mean Percent Recovery (MPR) as a proportion of the inoculated control (MPDC = 100 × [1 – (MPRtreatment/MPRI control)]).

Table 1. Compounds tested for preventing cherry pruning wound infections by spores of *Calosphaeria pulchella, Eutypa lata and Cytospora sorbicola*.

Compound	Active ingredient
Trichoderma sp.	Trichoderma atroviride
Rally	myclobutanil
Topsin M	thiophanate-methyl
Quash	metconazole
Quadris Top	azoxystrobin + difenoconazole
Inspire Super	difenoconazole + cyprodinil
Quilt Xcel	azoxystrobin + propiconazole
Luna Experience	fluopyram + tebuconazole
Merivon	fluxapyroxad + pyraclostrobin
Luna Sensation	fluopyram + trifloxystrobin
Polymer 1	polymer of cyclohexane
Trichoderma spp.	T. harzianum + T. virens
Control (water)	Water

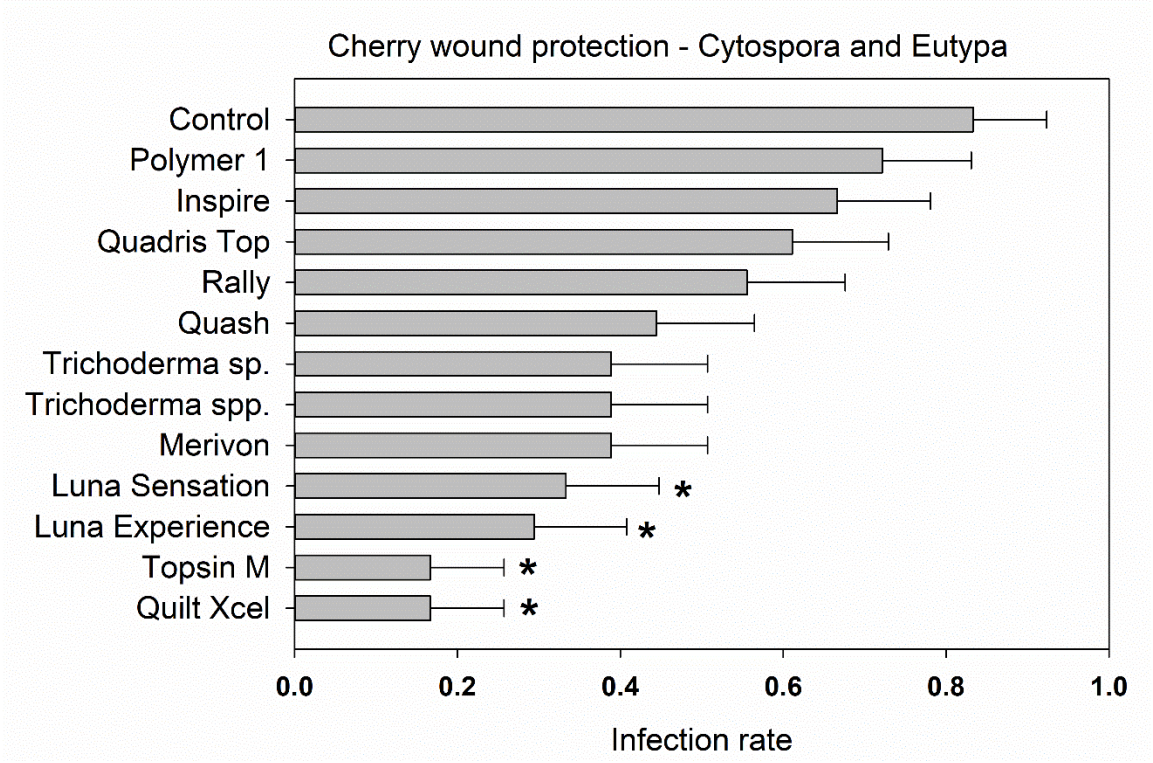
Results and Discussion

In this trial no pruning wounds treated with *Calosphaeria pulchella* had successful infections so no data for this pathogen are presented. Instead all the results concern *Eutypa lata* and *Cytospora sorbicola*. Approximately 83% of pruning wounds treated with water and inoculated with spores of *Cytospora sorbicola* and *Eutypa lata* had successful infections, providing high infection rates in our control treatments. Of the 12 compounds tested, Topsin M and Quilt Xcel performed best, only allowing 17% infections of pruning wounds. Overall, these two products provided 80% disease control. Luna Sensation and Luna Experience also provided significant control (60 to 65% disease control; Figure 5). Pathogen recovery rates from the pruning wounds treated with the 8 other compounds tested did not significantly differ from fungal recovery rates of the water control. Percent disease control ranged from 13% for Polymer 1 to 53% for Merivon.

The efficiency of Topsin M for pruning wound protection against *Eutypa lata* has been demonstrated in the past for grapevines (Rolshausen et al., 2010) so our findings in cherry confirmed the efficacy of this product against the same pathogen in a different crop. Our results are preliminary and based on a single field trial in sweet cherry so these

efficacies results will need to be further evaluated. However, these results corroborate with findings from work conducted in almond canker diseases (Trouillas lab). Here, the lack of positive infection of Calosphaeria in pruning wounds raised numerous questions about the disease epidemiology, which will require further investigation.

Figure 5. Infection rates of cherry pruning wounds by Eutypa and Cytospora depending on the fungicide applied.



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Rolshausen PE., Úrbez-Torres JR, Rooney-Latham S, Eskalen A, Smith RJ, Gubler WD. 2010. Evaluation of Pruning Wound Susceptibility and Protection Against Fungi Associated with Grapevine Trunk Diseases. Am J Enol Vitic. March 2010 61: 113-119;

Spotted Wing Drosophila 2018 Final 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:
Omar S. Akbari, University of California, San Diego
Bruce A. Hay, California Institute of Technology

ORIGINAL OBJECTIVES

Drosophila suzukii is a major invasive pest of ripening small fruit including raspberries, blueberries, strawberries, and cherries [1,2]. It has caused significant worldwide economic losses including significant damage in the berry- and cherry-growing industries of western North America [2–5]. Achieving effective control of *D. suzukii* has been difficult in a number of crop systems including cherries [6,7], and control measures have largely relied on prophylactic application of expensive broad spectrum insecticides [6–8]. This is problematic, as the repeated use of broad-spectrum insecticides has led to disruption of integrated pest management systems developed for crops such as cherries and berries, and has had a serious impact on beneficial arthropods, resulting, for example, in an increased use of miticides [4]. Additionally, broad use of insecticides makes it inevitable that resistance will become a major problem in the foreseeable future [8] (in fact, the first incidence of spinosad resistance in the US has just been reported in California [13]). Finally, broad insecticide use increases the risk of residues on fruits [8], and arouses public concern [6]. However, there are no effective alternatives to managing *D. suzukii* infestation, and it is likely that, unless more effective control measures are developed, this pest will continue to spread [8].

An alternative, highly promising approach that could complement existing control methods is genetic pest management [9], which includes strategies such as gene drive [10,11] and transgenic-based sterile insect technique (SIT) [12,13]. In particular, engineered *D. suzukii* gene drive strains can be utilized to spread desirable genes (e.g., susceptibility to a novel bio-friendly pesticide) throughout, or to entirely suppress/eradicate, wild *D. suzukii* populations. Such an approach is catalytic, with release of only modest numbers of engineered insects required to spread desirable genes or achieve population suppression. Additionally, since such a system relies on only a few releases of transgenic insects to do the all of the work on an ongoing basis, it is cheap as compared to the use of insecticides, which need to be applied regularly. Finally, a major appeal of this approach is that it is environmentally friendly and entirely insect-specific, and would have no effect on crops or on beneficial organisms.

Our objective over the last year, therefore, was to make progress towards engineering gene drive systems in *D. suzukii*. Specifically, out of the multiple types of gene drive systems that can be utilized in a genetic pest management program [11,14], we decided to focus our efforts on developing *Medea* and Cas9-mediated systems. Our goals were to evaluate the feasibility of engineering each strategy in *D. suzukii*, and to take concrete steps towards developing a product (a genetically modified *D. suzukii*) that can be mass-reared and deployed into the wild to catalytically suppress, and completely eliminate, the wild populations of this significant pest.

SIGNIFICANT FINDINGS

I. Objective A - Development of CRISPR/Cas9-based drive systems in *D. suzukii*
   A. Achieved an efficient means of transgenesis (required to test any gene drive components)
   B. Developed and characterized multiple Cas9 transgenes in *D. suzukii* that are highly functional and enable efficient Cas9-mediated mutagenesis
   C. Developed several ways to efficiently express gRNAs from the *D. suzukii* genome
   D. Developed/optimized several components needed to build Y-gene drive
      1. Identified *D. suzukii* X and Y chromosome regions

2. Identified putative X chromosome specific target sites
3. Efficiently engineered the Y chromosome of flies

E. Developed/optimized several components needed to build Cas9-based suppression gene drive
1. Identified promising suppression drive candidate target genes
2. Identified *D. suzukii* homologues of target genes and selected suitable gRNA target sites within these genes
3. Designed gRNA-expressing transgenes to test ability to target these genes
4. Built a proof of principle Cas9-based homing system in the *white* gene to test its ability to self-replicate

II. Objective B - Development of a *D. suzukii Medea*-based drive system

A. Finished characterizing and testing previously developed *D. suzukii Medea* drive system
1. Characterized resistance to this drive system, which could hinder the spread of such a drive

B. Developed a modified version of this same system that should obviate the observed resistance
1. Currently testing this system; preliminary evidence suggests that it does, as expected, function better that the original *Medea*

C. Developed a second-generation "reversal" *Medea* system that should be more robust in the face of genetic diversity in general and could be used to replace the original *Medea* in case a recall is necessary
1. Currently testing this system

D. Identified several promising putative cargo genes that could be spread with the *Medea* gene drive to cause population suppression
1. Currently testing these in *D. melanogaster* as proof of principle

RESULTS & DISCUSSION

(A) Development of CRISPR/Cas9-based drive systems

Summary

CRISPR/Cas9 technology has great applicability to the development of genetic pest management approaches, and can be used to build various gene drives - including Y-chromosome drive and Cas9-mediated homing-based drive - that can be employed to suppress and eliminate pest populations. We have made significant progress in developing the tools needed to engineer both of these types of gene drives in *D. suzukii*. Specifically, we have developed and characterized multiple Cas9 transgenes in *D. suzukii* that are highly functional and enable efficient Cas9-mediated mutagenesis in this pest. We have also developed several ways to efficiently express gRNAs from the *D. suzukii* genome. Together, these tools enable efficient CRISPR/Cas9-based manipulations of the *D. suzukii* genome, and provide the basis for building Cas9-based gene drives. Furthermore, we have developed/optimized several components needed to build Y-gene drive, including identifying *D. suzukii* X and Y chromosome regions, identifying putative X chromosome specific target sites, and efficiently engineering the Y chromosome of flies. Additionally, we have also taken steps towards engineering Cas9-based suppression gene drive, including: identifying promising candidate genes to be targeted by this drive; finding *D. suzukii* homologues of, and selected suitable gRNA target sites within, these genes; designing gRNA-expressing transgenes to test our ability to target these genes; and building a proof of principle Cas9-based homing system in the white gene to test its ability to self-replicate. We can now begin putting these components together to generate functional suppression gene drives in *D.suzukii*.

Background

The arrival of CRISPR technologies heralded a new era for traditional genome manipulation and site-specific transgenesis [15,16], and for advanced engineering of target genomes including the construction of gene drives [14,17]. Out of all the types of gene drives that have been proposed, drives based on the

CRISPR/Cas9 gene-editing system may be the simplest to build (especially given CRISPR's functionality in many insects [18–26]) and the most effective [11]. Most CRISPR technologies used in insects utilize a simplified two-component system consisting of a *S. pyogenes* Cas9 endonuclease (SpCas9) and a single chimeric guide RNA (gRNA) [27] that can generate DNA double-strand breaks (DSB) in a location of one's choosing. These breaks can them be repaired either randomly (via non-homologous end-joining, NHEJ) or based off a template (via homology-directed repair, HDR) [27,28]. The functionalities of CRISPR/Cas9 systems can be exploited to bring about gene drive-based population suppression.

For example, distortion of the sex ratio in favor of males can lead to a gradual population reduction and eventual elimination of a target population [29–32], and natural so-called meiotic driving Y-chromosomes have been described [33–35]. A system for sex-ratio distortion can also be engineered by designing CRISPR-based transgenes that target the X-chromosome during spermatogenesis [36,37](Figure 1). This Y-gene drive approach would depend on the destruction of X-bearing sperm to produce males that only give rise to male progeny [14,38], and would require the ability to meiotically express an X-chromosome targeting element from the Y-chromosome [36,39]. Importantly, CRISPR/Cas9 technology could straightforwardly be utilized to engineer Y-gene drive elements by designing gRNAs that target only the X chromosome [36,37]. Such a system has already been developed in one species of mosquito [36,40,41], and should be portable to *D. suzukii*.

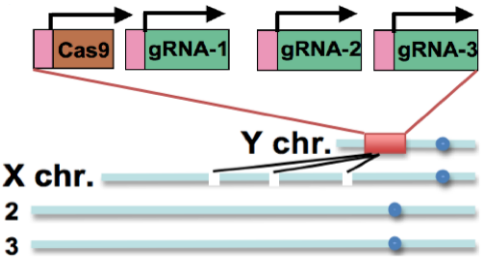

Fig. 1

Another way CRISPR/Cas9 can be utilized to bring about population suppression is via Cas9-mediated homing-based gene drive [14]. This concept is based on the idea of using homing endonuclease genes (HEGs) to manipulate populations [42]. These genes are extraordinarily selfish, and this property can be exploited for both population suppression and replacement. HEGs have the ability to "cheat" during meiosis by converting their corresponding allele on the opposite chromosome into an exact copy of themselves, by encoding a sequence-specific endonuclease that severs and disrupts their competing chromosomal allele, which can force the call to use the HEG as a template for homology-directed repair (HDR), resulting in the HEG copying itself (i.e., homing) into its competing allele. If the latter repair option occurs in the germline, or early embryo, then the proportion of offspring that receive the HEG will be above that expected with normal Mendelian transmission (i.e., 50%), allowing for rapid invasion of the HEG into a target population [43]. A HEG can be used to spread a payload gene (replacement drive) or for population suppression and possibly eradication by homing into a target gene, the disruption of which leads to recessive lethality or sterility (Figure 2). In such a suppression approach, homing must be confined to the germline during gamete formation, leading to sterility/non-viability only in homozygotes that receive the HEG allele from both parents. Consequently the HEG can rapidly spread, and once a large fraction of the population is heterozygous, it can cause a population crash as heterozygote pairings will produce sterile/non-viable offspring.

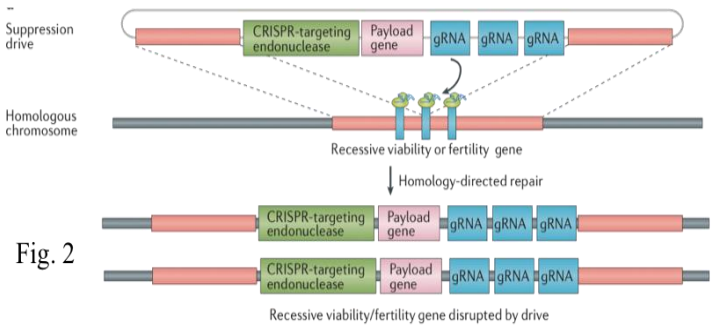

Fig. 2

Although several proof-of-principle studies have shown the utility of HEGs as gene drives prior to the advent of CRISPR/Cas9 (e.g., [44]), this powerful system is enabling the efficient design of homing-based drive systems in many contexts [17]. Several replacement Cas9-mediated homing-based gene drives have been developed [18,45,46]; additionally, several Cas9-based suppression drive systems have recently been engineered in fruit flies [47,48] and one species of mosquito [20,49], and should also be possible to transfer to *D. suzukii*. However, neither this approach nor Y-gene drive have been developed in this pest species.

Results and Future Directions

Efficient Transgenesis in *D. suzukii*

In order to engineer any type of gene drive system in *D. suzukii*, we first have to be able to efficiently generate transgenic flies. Although transgenesis in *D. suzukii* has been previously established [50], it is not very efficient [51], and we had previously struggled with obtaining *D. suzukii* transgenic fly lines. However, a recent work [52] described the generation of a "jumpstarter" *D. suzukii* strain that carries the *transposase* gene necessary for *piggyBac* transposition, and reported that performing germline transformation in this strain dramatically increased transgenesis rates (in some cases 40- to nearly 60-fold [52]). Since increased rates of transgenesis would help us accelerate our gene drive development efforts, this past year we obtained the USDA/APHIS permits necessary to acquire this transgenic strain from the researchers that developed it, have expanded the obtained stocks into a large colony, and are carrying out all microinjections for transgenesis into this strain. This has been greatly helpful, as we are now able to obtain transgenic lines with much greater efficiency.

Development of Cas9 Tools in *D. suzukii*

The development of both Y-gene drive and Cas9-mediated suppression drive in *D. suzukii* requires functional CRISPR/Cas9 tools in this fly. Although Cas9-mediated genome editing had been previously demonstrated in *D. suzukii* [53], it was carried out by microinjection of gRNAs and Cas9 protein into embryos. Conversely, the building of a gene drive requires a germline source of Cas9 and gRNAs driven by an effective promoter, typically a PolIII promoter such as U6.

Leveraging our experience in designing and optimizing CRISPR/Cas9 tools in *D. melanogaster*, we have generated both of these components. Specifically, we have generated four distinct functional transgenic Cas9 lines, where expression of Cas9 is driven by either strong female germline specific promoters (*BicC* and *Dhd*) or by male and female germline specific promoters (*vasa* and *nanos*) that have been previously validated in *D. melanogaster* [12,46]. We have tested these Cas9 lines, and have shown that all four work, with up to 100% mutagenesis efficiency (for *vasa*-Cas9). We have also generated several functional gRNA-expressing transgenes by targeting the *white* gene, which gives flies a red eye color, as a proof of principle. Specifically, after several failed attempts, we have demonstrated that a genomically encoded, PolIII U6:3 promoter-driven gRNA targeting *white* produces up to 100% mutated (white and mosaic-eyed) progeny when crossed to a Cas9 expressing line (Figure 3). We have also shown that a genomically encoded tRNA-gRNA expression cassette [54], driven by a PolII germline specific promoter, also functions to produce mutated progeny (albeit at a more modest frequency of ~15-30%).

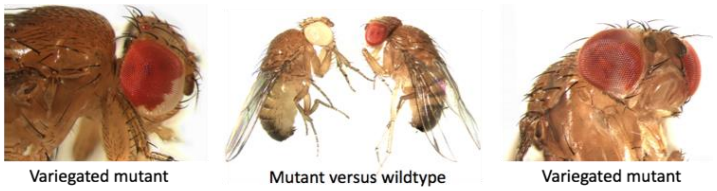

Fig. 3

The development of these tools lays the foundation for the ability to engineer Cas9-based gene drives in *D. suzukii*.

Engineering a Y-gene Drive System

Assuming that efficient CRISPR/Cas9 tools are available, the ability to build a Y-gene drive requires three further components: the ability to identify X and Y chromosomes in *D. suzukii*; the ability to insert large transgenes on the Y-chromosome; and the ability to target and cut sequences only present on the X-chromosome.

Identifying, and inserting genes on, the Y chromosome in D. suzukii

The current genome annotation of *D. suzukii* (http://spottedwingflybase.org) is divided into over 29, 000 contigs (independent fragments that have not been brought together to make a clear linear

sequence map of each chromosome), and it is not entirely clear which of these contigs comes from the *D. suzukii* Y and X chromosomes. Therefore, we have used a bioinformatic approach to try to identify fragments of these chromosomes. To do this, we took the entire *D. melanogaster* Y chromosome sequence and carried out a search for related sequences (a BLAST homology search) among the *D. suzukii* contigs; essentially, we looked for regions of *D. suzukii* that were nearly identical to those from the melanogaster Y chromosome, as these are likely to represent *D. suzukii* Y chromosome sequence. We identified a total of 134 contigs that had extremely high homology (E-value = 0) to the *D. melanogaster* Y chromosome. Given this high homology, we are confident that these contigs are pieces of the *D. suzukii* Y chromosome. From this data we have identified several regions of the putative *D. suzukii* Y chromosome that should be ideal locations for integrating an X chromosome targeting Cas9/gRNA cassette (outside of any known transcribed regions, in unique, non-repetitive DNA).

In order to assay whether we could use CRISPR/Cas9 to dock transgenes on the Y chromosome, we first set out to develop a CRISPR/Cas9-based technique for site-specific engineering of the *D. melanogaster* Y chromosome as a proof of principle [55], as it is much easier and faster to test and troubleshoot components in this species before porting them to *D. suzukii*. To do this, we engineered a vector comprising a fluorescent marker (tdTomato) driven by the eye-specific 3xP3 promoter and flanked by the gypsy and CTCF insulators, with unique restriction sites upstream and downstream for cloning specific homology arms (Figure 4). We then selected ten distinct intergenic regions spanning the Y chromosome for targeting, identified a suitable sgRNA target site in each region, and cloned in homology arms, corresponding to ~800-1,000 base pairs of sequence 5' and 3' of each selected target site, upstream and downstream of the insulator-flanked 3xP3-tdTomato element to generate ten unique Y chromosome targeting transgenes. Each transgene was then injected, along with the appropriate in vitro transcribed sgRNA and Cas9 protein, into a transgenic line expressing a germline source of Cas9 using standard procedures, and G1 progeny were screened for presence of the transgene marker. Two of the injected transgenes inserted in the correct positions on the Y chromosome, demonstrating that we can use the above approach to insert, and detect expression from, a fluorescently marked transgenic cassette at specific locations on the Y-chromosome in *D. melanogaster* using CRISPR/Cas9-mediated HDR.

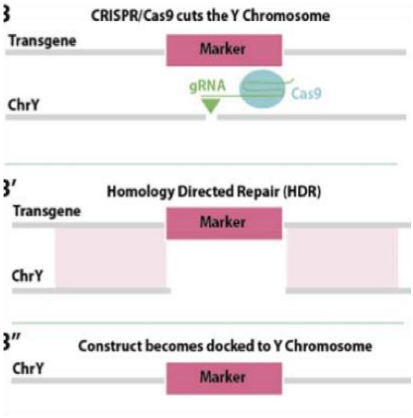

Fig. 4

We are now testing whether we can insert, and detect expression from, Cas9-containing transgenes at these same Y chromosome locations, as we will need to be able to express Cas9 cassettes from the Y in order for the Y gene drive approach to work. Once these experiments are complete, we plan to port this approach to *D. suzukii*.

Identifying and cutting the X chromosome in D. suzukii

We performed a similar bioinformatic analysis to the one described above identify the X chromosome of *D. suzukii*, and identified 388 contigs from *D. suzukii* as being X-linked. Then, to identify potential gRNA sequences specific to the *D. suzukii* X chromosome, and present in multiple copies, we first developed a program to predict all possible Cas9 cleavage sites on the X-chromosome by searching for the PAM motif (XGG in the target sequence N(21)XGG). Once potential X-chromosome cleavage sites were identified, they were aligned to the rest of the genome (all the other non-X contigs) and those that showed a sequence match to these contigs were eliminated. The final output of this program was a conservative list of X chromosome specific Cas9 cleavage sites.

From all of this, we conservatively predicted several potential target sequences repeated exclusively on the X chromosome in up to ten locations, making them ideal for the development of guide RNAs to cleave the *D. suzukii* X chromosome. However, our initial attempts at testing these gRNAs for their ability to cut the X did not succeed because, as discussed above, our initial gRNA-expression configuration were not functional. However, now that we have a highly functional gRNA expression

configuration, we can proceed to clone X chromosome-targeting gRNAs into our gRNA expression cassettes and test them.

Engineering a Cas9-mediated Suppression Drive System

To engineer a Cas9-mediated suppression homing drive, we need to introduce the coding sequence for Cas9 and gRNA into the genomic site targeted by the Cas9/gRNAs [11] to generate a self-replicating transgene that could continuously mutate a target gene every generation and/or carry a transgene into the population. This self-replicating (i.e., homing) Cas9-based transgene would need to be placed within a gene necessary for female fertility, so that eventually all of the females in a target population would become sterile and the population would collapse [49].

As described above, we now have working Cas9 and gRNA transgenes that we can utilize as the basis for such a gene drive. After analyzing recent efforts to develop such suppression drive systems in fruit flies [47,48] and mosquitoes [49], we have also identified several promising candidate target genes, including *dsx*, *tra*, *sxl*, and *zpg*, which are conserved in *D. suzukii*. After analyzing the sequences of the *D. suzukii* homologues of these genes to find regions that are highly conserved and thus unlikely to contain sequence variation, we have selected two gRNA target sites within each gene, and have engineered separate U6-driven gRNA transgenes targeting each gene to test whether the selected gRNA sequences will work to efficiently cut the selected targets. (We are currently working on obtaining transgenic lines for these transgenes.) After we verify that the gRNAs work, we will proceed to construct full Cas9-based suppression drive cassettes targeting the most promising candidates (based on gRNA function). In parallel, we are also testing a split Cas9-based gene drive cassette [56] targeting the *white* eye color gene as a proof of principle, to determine whether we can: a). dock transgenes in a site-specific location using CRISPR/Cas9 in *D. suzukii*; and b). observe the efficiency of self-replication/homing of this Cas9-based transgene in *D. suzukii*.

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

Summary

Previously, we had developed the first *D. suzukii* functional replacement gene drive system termed *Medea*, had rigorously tested it in laboratory cage populations, and had characterized it in different genetic backgrounds to determine effectiveness and fecundity (our results on this project were published in *PNAS* this year [57]). We found that this first-generation *Medea* system was capable of biasing Mendelian inheritance rates with up to 100% efficiency and could maintain itself at high frequencies in a wild population; however, drive resistance, resulting from naturally occurring genetic variation and associated fitness costs, was present and could hinder the spread of such a drive. Therefore, since mathematical modeling indicated that our *Medea* drive system could spread to fixation if either its fitness costs or toxin resistance were reduced [57], we have developed a modified version of this same system that should obviate the specific resistance that we observed, and have preliminary evidence to suggest that it does, in fact, function better that the original *Medea* we tested. We have also developed a second-generation *Medea* system in *D. suzukii* that should be more robust in the face of genetic diversity in general and could be used to replace the original *Medea* in case a recall is necessary. Finally, we have identified several promising putative cargo genes that could be spread with the *Medea* gene drive to cause population suppression, and are moving forward with testing them in *D. suzukii*.

Background

Medea was first discovered in the flour beetle [58], and multiple versions were later reverse engineered from scratch and shown to act as robust gene drives in the laboratory fruit fly, Drosophila melanogaster [59,60]. Such engineered *Medea* systems rely on a *Medea* element consisting of a toxin-antidote combination (Figure 5). 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

heterozygous 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 high temperature or diapause - a state that allows insects survive periods of adverse conditions such as cold [61]. 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 [62,63] – can be engineered to drive expression of an insect-specific toxin (e.g., [64]). 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 [59]. Furthermore, a *Medea* element can be utilized to spread a thermally activated TRPA1 cation channel [65] that, upon exposure to a specific threshold temperature, renders flies paralyzed or dead. However, although transgenesis of *D. suzukii* has been established [50], no effective suppression gene drive systems in this major pest have yet been engineered.

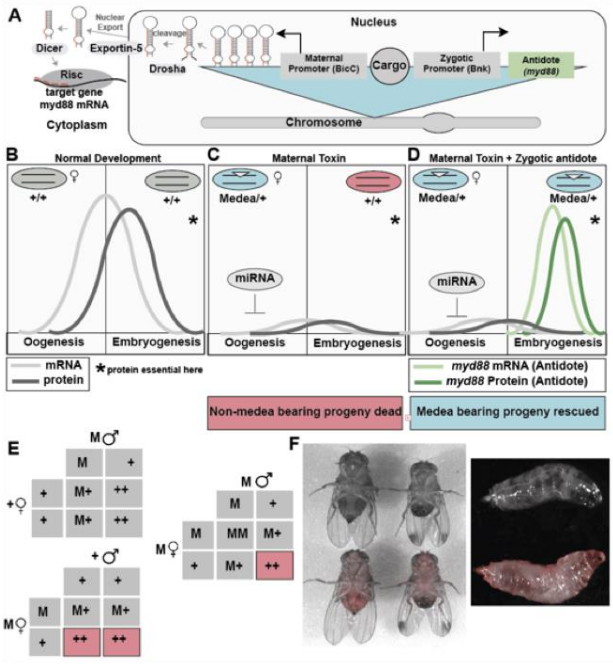

Fig. 5

Results and Future Directions

Generation of Synthetic *Medea* Gene Drive

To create a synthetic *Medea* gene drive in *D. suzukii*, we engineered a *piggyBac* vector comprising a miRNA toxin coupled with a toxin-resistant antidote, inspired by the architectures used to generate previous *Medea* systems in *D. melanogaster* [66,67]. We designed synthetic miRNAs to target *D. suzukii* myd88, a highly conserved gene shown to be maternally deposited and required for dorsal-ventral patterning in the early embryo in *D. melanogaster* [68]. We used the predicted *D. suzukii* female germline-specific bicoid (BicC) promoter to drive expression of a "toxin" consisting of a polycistronic array of four synthetic microRNAs (miRNAs) each designed to target the 5' untranslated region (UTR) of *D. suzukii* myd88 (Figure 5). Importantly, to ensure these miRNAs could target the desired sequence, we performed genomic DNA sequencing of the myd88 5'UTR target region in our reference *D. suzukii* strain (collected from Corvallis, Oregon) and designed the miRNAs against this sequence. This *Medea* drive also contained an "antidote" consisting of the *D. suzukii* myd88 coding region, insensitive to the miRNAs as it did not contain the miRNA-targeted 5'UTR, driven by the predicted *D. suzukii* early embryo-specific bottleneck (bnk) promoter, and two separate transformation markers – eGFP driven by the eye-specific 3xP3 promoter [69], and dsRed driven by the ubiquitous hr5-IE1 promoter [70].

Characterization of *Medea* Genetic Behavior

Following microinjection of the *Medea* transgene into *D. suzukii* embryos, a single G_1 transformant male was recovered, as identified by ubiquitous hr5-IE1 driven expression of dsRed (Figure 5), and weak eye-

specific 3xP3-driven eGFP. When outcrossed to several wildtype (non-*Medea* bearing; +/+) females, this male produced roughly ~50% *Medea*-bearing and ~50% wildtype offspring, as would be expected from standard Mendelian segregation without biased inheritance. Resulting heterozygous G_2 *Medea*-bearing progeny were individually outcrossed to wildtype individuals of the opposite sex to determine inheritance patterns, and these individual outcrosses were continued for six generations (Table 1). Remarkably, until the G_5 generation, all heterozygous *Medea*/+ mothers (n = 91) produced 100% *Medea*-bearing progeny (n = 1028), while heterozygous *Medea*/+ fathers (n = 16) produced ~50% *Medea*-bearing progeny (n = 268). While the majority of heterozygous *Medea*/+ G_5 (23/31) and G_6 (16/25) generation females also produced 100% *Medea*-bearing progeny, some heterozygous G_5 (8/31), and G_6 (9/25) females unexpectedly produced a small yet notable number (52/1219) of wildtype offspring. Although the exact reason for the difference is unclear, later analysis suggested that resistance to the miRNA toxin might explain this unexpected observation. Notwithstanding, individually these G_5 and G_6 heterozygous *Medea*/+ females displayed significantly biased biased inheritance rates ranging from 76%-96%, with an average rate of 86.4%. Overall, in six generations of individual female outcrosses, the percentage of *Medea*-bearing progeny borne by single heterozygous *Medea*/+ mothers (n = 147) was 97.7% (2195/2247) as opposed to the 50% that would be expected with standard Mendelian segregation, indicating that the *Medea* drive is extremely functional at biasing inheritance.

To further characterize the genetics behind the highly biased inheritance patterns described above, additional crosses between individuals of various *Medea* genotypes were performed, and confirmed that *Medea* exhibits maternal-effect lethality and zygotic rescue. For example, matings between heterozygous *Medea*/+ mothers and wildtype fathers resulted in 55.63±0.76% total embryo survival with 94.20±1.33% of the progeny being *Medea*-bearing, while matings between heterozygous *Medea*/+ mothers and heterozygous *Medea*/+ fathers yielded 79.11±3.95% total embryo survival with 94.12±0.67% of the progeny being *Medea*-bearing. The higher-than-expected embryo survival is consistent with the observation that not all heterozygous *Medea*/+ mothers give rise to 100% *Medea*-bearing progeny, indicating that not all wildtype progeny from a heterozygous *Medea*/+ mother perish.

<u>*Medea* Functionality in Geographically Distinct Populations</u>

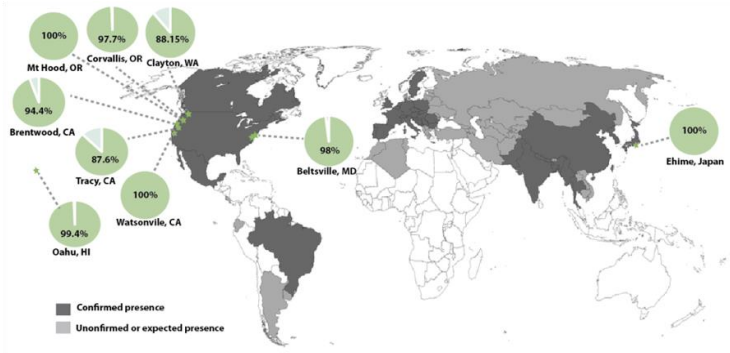

Fig. 6

To assess whether the *D. suzukii Medea* could function in geographically distinct populations that possibly harbor genetic variability in regions that canonically have less conservation such as the 5'UTR, heterozygous *Medea*/+ flies were tested in eight additional *D. suzukii* strain backgrounds. These strains were collected from various locations around the world, including: Mt. Hood, OR; Clayton, WA; Brentwood, CA; Tracy, CA; Watsonville, CA; Oahu, HI; Beltsville, MD; and Ehime, Japan. Interestingly, for 3/8 strains, the *Medea* inheritance rate from heterozygous *Medea*/+ mothers was 100%, while from 5/9 strains the inheritance rate ranged from 87.6% to 99.4%, with an overall transmission rate of 94.2% (Figure 6). These results strongly demonstrate that the *Medea* drive described here can dominantly bias transmission in diverse *D. suzukii* populations.

<u>Long Term Population Cage Experiments</u>

The above observations suggested that *D. suzukii Medea* should be able to drive robust population replacement. To test this prediction, we performed several long term multi-generational population cage experiments specifically challenging the *Medea* drive with a wildtype strain that harbored pre-existing resistance (Corvallis, OR). We set up these population cage studies after maintaining this

population for approximately ten generations, we mated *Medea*-bearing fathers to wildtype Corvallis, OR, strain mothers at three distinct introduction (G_0) frequencies: low frequency (25 heterozygous *Medea*/+ and 25 wildtype +/+ males mated to 50 wildtype +/+ virgins, *Medea* allele frequency of ~12.5% and genotype frequency of ~25%); medium frequency (50 heterozygous *Medea*/+ males mated to 50 wildtype +/+ virgins, *Medea* allele frequency of ~25% and genotype frequency of ~50%); and high frequency (50 homozygous *Medea*/*Medea* males mated to 50 wildtype +/+ virgins, *Medea* allele frequency of ~50% and genotype frequency of ~50%). These experiments were conducted in separate bottles in biological triplicate for the low and medium threshold and quadruplicate for the high threshold drives, producing ten distinct populations with G_1 *Medea* allele frequencies ranging from ~12.5-50% and genotype frequencies ranging from ~25-100%. Altogether, these population cage experiments were followed for 9 generations (for lower allele frequency populations, as the *Medea* allele disappeared from the population by that time) or 19 generations (for higher allele frequency populations), counting the number of *Medea*-bearing adults each generation to determine the genotype frequency, as described previously [60,67]. Interestingly, the observed changes in *Medea* frequency over time indicated that, for release proportions (defined as the genotype frequency in the G_1 population) of 50% or smaller, the *D. suzukii Medea* drive was unable to drive into the wildtype population, likely because of selected drive resistance combined with high fitness costs outweighing the effect of drive. However, at higher release proportions of >90%, similar to classical chromosomal rearrangement thresholds [71], the drive largely compensated for the fitness cost, allowing the gene drive to remain in the population at high frequencies for the duration of the experiment (19 generations). Although unintended, the self-limiting dynamics of the generated *Medea* system may be useful in achieving a transient population transformation of the type associated with other proposed gene drives (e.g.,[72]).

Mathematical Modeling

To characterize the population dynamics observed in the above cage experiments, we fitted a mathematical model to the observed data in which the *Medea* drive had an associated fitness cost in heterozygotes and homozygotes and there was a *Medea*-resistant allele present in the population that reduced toxin efficiency. For the fitted model, the *Medea* drive was estimated to have a toxin efficiency of 93% in individuals homozygous for the resistant allele (95% credible interval (CrI): 90-95%) and was assumed to have a toxin efficiency of 100% in individuals lacking the resistant allele. The *Medea* drive was estimated to confer a large fitness cost on its host - 28% in heterozygotes (95% CrI: 27-30%) and 65% in homozygotes (95% CrI: 62-67%) - and the resistant allele was estimated to have an initial allele frequency of 78% in the population (95% CrI: 57-97%).

Predictive mathematical modeling based on these parameter estimates suggests that the *Medea* drive would spread to fixation in the absence of toxin resistance if released above a threshold frequency of 79%. Spread to fixation would also be expected if the fitness costs of the generated *Medea* drive were halved, even if all individuals in the population were homozygous for the *Medea*-resistant allele, provided the drive was released above a threshold frequency of ~25-27%. Consistent with the experimental results, a *Medea* drive with a large fitness cost in a *Medea*-resistant population is expected to be maintained at high frequencies through its drive; however, its eventual elimination is inevitable unless supplemental releases are carried out. However, for high release frequencies (90-95%), the drive may be maintained at high frequencies (>75%) for ~20 generations, which likely exceeds the duration required for agricultural impact.

Improved *Medea* Construct and Reversal *Medea*

Given our observations regarding resistance and its effect on *Medea* function, we set out to engineer improved *Medea* systems that could reduce the chances of resistance acting as an impediment to spread. Specifically, we performed some sequencing-based characterization of naturally occurring genetic variation in various geographically distinct target populations to help guide selection of target sites that are well conserved across all populations in which the drive is intended to function. We then designed a modified version of the original *Medea* system that targeted different, conserved sequences (still in the 5'UTR of the *myd88* target gene), reasoning that such a *Medea* element should function very similarly to

the original element but not be impeded by the resistance we previously observed. We have obtained transgenic lines for this improved *Medea* element, and preliminary data indicates that it works better than the original *Medea*, producing 100% inheritance bias. We will continue rigorously testing this second-generation *Medea* element in the coming year.

Additionally, we hypothesized that to reduce resistance, miRNA target site selection could be limited to the coding DNA sequence regions of a genome, which tend to be strongly conserved, as opposed to regions such as the 5'UTR, which canonically have higher tolerance for sequence variation. We have therefore also developed a second-generation *Medea* system in *D. suzukii* that should be more robust in the face of genetic diversity in general (because it targets coding DNA regions as opposed to the 5'UTR) and could be used to replace the original *Medea* in case a recall is necessary. Specifically, to reduce risk and mitigate the spread of the *D. suzukii Medea* system into wild populations, it is important to develop a reversal Medea (RM) system and demonstrate that it can function as predicted. 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. We have finished designing and building a Reversal *Medea* system capable of spreading on its own and of replacing the first *Medea* described above, and are in the process of obtaining transgenic *D. suzukii* individuals containing this *Medea* and of rigorously characterizing this system.

Identification of Putative "Cargo" Genes

For *D. suzukii*, elimination of the pest populations is ultimately the goal. An engineered *Medea* system could achieve this by spreading a "cargo"gene proffering susceptibility to a particular pesticide, or a conditional lethal gene that would be activated by some substance or environmental cue such as high temperature or diapause. One promising type of candidate "cargo" gene is a thermally activated TRPA1 cation channel [65]. Specifically, TRPA1 is an ion channel located on the plasma membrane of many human and animal cells, and is finely tuned to detect specific temperatures ranging from extreme cold to noxious heat [65]. Upon exposure to a critical "threshold" temperature, this cation channel can "open" and modulate Ca2+ and Mg2+ entry into the cell [73]; when TRPA1 is overexpressed in an exogenous tissue (such as the fly brain, for example), this "opening" can lead to total fly paralysis and death. We therefore would like to engineer *D. suzukii* to express a specific TRPA1 channel in the brain, so that exposure of the engineered individuals to a threshold temperature (determined by the specific TRPA1 channel used) would paralyze/kill the flies. We should then be able to spread this temperature-activated "cargo" gene through wild populations by using our *Medea* system during cooler months, and achieve population suppression when the TRPA1 gene is activated in warmer months.

To achieve this, we are working to leverage data from the Montell lab (UCSD), which is developing this technology for mosquito control. The Montell lab is currently testing several TRPA1 channels with different activation temperatures (including rattlesnake TRPA1, python snake TRPA1, boa snake TRPA1 and fruit fly TRPA1) in *D. melanogaster* as a proof of principle, and has preliminary data indicating that at least some of the tested TRPA1 channels, when expressed in the fly brain, work as expected. Once we know which TRPA1 channel appears most promising, we will insert it into our best *Medea* element and begin testing this approach in *D. suzukii*.

Developing a field-ready strain

The above efforts should allow us to engineer both a robust gene drive system capable of spreading linked genes into varying geographic populations, and an effective "cargo" gene that can be activated to bring about population suppression. We will then engineer a strain containing both elements (i.e., a functional TRPA1 element linked to our best *Medea* element) that could be used for wild releases. Laboratory and caged field trials will then be conducted on this strain to determine mating competitiveness, longevity, and fitness compared to wild flies. This data will be used and fed into mathematical models to predict the introduction frequencies we will need to use to achieve suppression. Gene drive experiments will be initiated at various introduction frequencies to characterize the population

suppression dynamics. Modeling work will occur in collaboration with Dr. John Marshall, a mathematical biologist with whom we have worked on a number of modeling studies.

Since the ultimate goal here is to develop a product (a genetically modified *D. suzukii*) that can be mass-reared and deployed into the wild to catalytically suppress, and completely eliminate, the wild populations of *D. suzukii*, we will need regulatory bodies to permit such releases. In brief, this involves requesting a permit from USDA-APHIS BRS/PPQ. APHIS is responsible for issuing permits for the import, transit and release of regulated animals, animal products, veterinary biologics, plants, plant products, pests, organisms, soil, and genetically engineered organisms. These permits have been successfully issued for the release of transgenic insects in the USA. For example, in 2009 the USDA approved the integration of genetically engineered pest insects (including pink bollworm moth (*P. gossypiella*), Mediterranean fruit fly (*Ceratitis capitata*), Mexican fruit fly (*Anastrepha ludens*), and oriental fruit fly (*Bactrocera dorsalis*)) into ongoing SIT programs [51]. These insects have been engineered to carry either a heritable marker gene, or a heritable marker gene and a repressible female lethality gene resulting in the production of only males. Transgenic insects have also been developed, and released into the wild, to prevent human disease. For example, a biotech company based in the UK, known as Oxitec, is commercially generating genetically modified mosquitoes and releasing them, in populated cities, in many countries including the Cayman Islands, Malaysia, and Brazil [51.] These GM mosquitoes are likely going to be released in the USA. Therefore, the key point here is that obtaining regulatory approval for releasing transgenic insects in the USA, that are engineered to reduce wild populations and prevent crop damage, has been achieved in the past, and therefore we do not envision it to be a limitation with our approach.

We have been working with Nick Matteis from the CCB and others to begin the process of applying for an APHIS permit. Once a permit is granted, a possible experimental path to utilization would involve mark-release-recapture studies, and collections at different times of year to create a picture of the structure of *D. suzukii* population and the extent of migration. Simulation models parameterized with these data and field cage competition assays would then be used to propose release strategies that could be cost effective and yield population suppression or eradication as quickly as possible in specific high value environments.

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EXECUTIVE SUMMARY

Drosophila suzukii is a major invasive pest of many small fruits, and has caused significant damage in agricultural industries of western North America. Control measures have largely relied on prophylactic application of broad-spectrum insecticides, which is problematic, as repeated use of insecticides is expensive, has had a serious impact on beneficial arthropods, and makes it inevitable that resistance will arise in the foreseeable future. However, there are no effective alternatives to managing *D. suzukii* infestation, and it is likely that this pest will continue to spread.

An alternative, highly promising approach that could complement existing control methods is genetic pest management, which includes strategies such as gene drive. In particular, engineered *D. suzukii* gene drive strains can be utilized to spread desirable genes (e.g., susceptibility to a novel bio-friendly pesticide) throughout, or to entirely suppress/eradicate, wild *D. suzukii* populations. Such an approach is catalytic, with release of only modest numbers of engineered insects required to spread desirable genes or achieve population suppression, and can be cheap, since it relies on only a few releases of transgenic insects. A major appeal of this approach is that it is environmentally friendly and entirely insect-specific, and would have no effect on crops or on beneficial organisms. Our objective over the last year, therefore, was to make progress towards engineering *Medea* and Cas9-mediated gene drive systems in *D. suzukii*.

We had previously developed the first *D. suzukii* functional replacement gene drive system termed *Medea*, had rigorously tested it in laboratory cage populations, and had characterized it in different genetic backgrounds to determine effectiveness and fecundity (our results on this project were published in *PNAS* this year). We found that this first-generation *Medea* system was capable of biasing Mendelian inheritance rates with up to 100% efficiency and could maintain itself at high frequencies in a wild population; however, drive resistance, resulting from naturally occurring genetic variation and associated fitness costs, was present and could hinder the spread of such a drive. Therefore, since mathematical modeling indicated that our *Medea* drive system could spread to fixation if either its fitness costs or toxin resistance were reduced, we have developed a modified version of this same system that should obviate the specific resistance that we observed, and have preliminary evidence to suggest that it does, in fact, function better that the original *Medea* we tested. We have also developed a second-generation *Medea* system in *D. suzukii* that should be more robust in the face of genetic diversity in general and could be used to replace the original *Medea* in case a recall is necessary. Finally, we have identified several promising putative cargo genes that could be spread with the *Medea* gene drive to cause population suppression, and are moving forward with testing them in *D. suzukii*.

We have also made significant progress in developing the tools needed to engineer CRISPR/Cas9 based gene drives (including Y-chromosome drive and Cas9-mediated homing-based drive) in *D. suzukii*. Specifically, we have developed and characterized multiple Cas9 transgenes in *D. suzukii* that are highly functional and enable efficient Cas9-mediated mutagenesis in this pest. We have also developed several ways to efficiently express gRNAs from the *D. suzukii* genome. Together, these tools enable efficient CRISPR/Cas9-based manipulations of the *D. suzukii* genome, and provide the basis for building Cas9-based gene drives. Furthermore, we have developed/optimized several components needed to build Y-gene drive, including identifying *D. suzukii* X and Y chromosome regions, identifying putative X chromosome specific target sites, and efficiently engineering the Y chromosome of flies. Additionally, we have also taken steps towards engineering Cas9-based suppression gene drive, including identifying promising candidate genes to be targeted by this drive, finding *D. suzukii* homologues of and identifying suitable gRNA target sites within these genes, designing gRNA-expressing transgenes to test our ability to target these genes, and building a proof of principle Cas9-based homing system in the white gene to test its ability to self-replicate. We can now begin putting these components together to generate functional suppression gene drives in *D. suzukii*.

PROGRESS REPORT

Project Title: Electronic sensors to capture spatiotemporal population density of SWD

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Cooperators: Eamonn Keogh (UC Riverside, Dept. of computer science and engineering)

Budget: **Year 1**: $31,384

Percentage time per crop: Cherry: 100%

Budget
Organization Name: UC Davis **Contract Administrator: Yang Yeh**
Telephone: (530) 752-3794 **Email address:** ypyeh@ucdavis.edu

Item	2017-18 2018-19 (no cost extension)	(type additional year if relevant)	(type additional year if relevant)
Salaries	$16,016		
Benefits	$8,168		
Wages	-		
Benefits	-		
Equipment	-		
Supplies	$5,200		
Travel	$2,000		
Miscellaneous			
Plot Fees	-		
Total	$31,384		

Footnotes:
Salaries and Benefits are for one SRAI (technician) for sensor testing and insect collection (33.3% time)
Supplies include funding to construct 20 sensors for testing ($4000) and for insect capture and maintenance ($1,200)
Travel funds ($2,000) are requested for SRAI to travel to Washington or Oregon to conduct field sensor testing

JUSTIFICATION

Sensor technologies and automated insect identification models are currently being developed for the control of insects that spread human diseases. Our cooperator Dr. Keogh, a computer scientist at UC Riverside, has recently developed inexpensive pseudo-acoustic opto-electronic sensors and accompanying classification algorithm that can accurately classify multiple species of mosquitoes that vector pathogens such as Zika and West Nile virus (Chen et al. 2014) by using wing-beat frequencies, daily activity patterns, and geographical distribution. The ability to remotely capture real-time measurements and forecast insect density in a spatiotemporal manner allows for efficient and precise insect control response that could prevent public health crisis. ***The overall goal of this proposal is to adopt and translate this technology to optimize insect pest management programs and benefit agricultural stakeholders.*** We propose to develop and ultimately deploy opto-electronic sensors that can accurately identify Spotted Wing Drosophila (SWD) and differentiate it from other insect inhabitants of cherry orchards.

SWD are a highly invasive pest species that cause up to $500 million in annual losses in the western United States by ovipositing their larvae into marketable, ripening fruit (Goodhue et al. 2011, Wiman et al. 2016). An insect sensor utilizing wing beat frequency for classification can theoretically be applied to identify any flying insect, but the substantial economic loss caused by SWD warrants the prioritization of optimizing this new technology for its control. It is important to stress that the electronic sensor technology we propose to develop and optimize for SWD is not simply a modernized version of insect traps currently used for population monitoring. Besides supplanting conventional monitoring tools and greatly reducing the time necessary to process trap contents, we anticipate that the capability of the sensors to classify insects in real-time will revolutionize pest management research and lead to developments in precision agriculture. For example, current monitoring tools lack spatial and temporal resolution as conventional traps do not provide time-stamps for insect catches. Our sensors on the other hand can ultimately be connected to a central network and will be capable of reporting real-time movement between crop and non-crop host plants, providing opportunities to target SWD for sprays at times when they are at maximum density in non-crop plants. This can reduce insecticide residues on crops, a major concern for export markets.

OBJECTIVES

Objective 1:

Measure wing beat frequency and circadian activity pattern of SWD to improve insect identification algorithm. Opto-electronic sensors will be installed in insect cages that house SWD to measure wing beat frequency and daily activity patterns simultaneously. Since biological parameters, e.g. sex, age, and seasonal morphology, may alter wing beat frequency and activity patterns, we plan to evaluate male and female SWD, different ages of SWD, and summer and winter forms of SWD. Various abiotic factors can also affect wing beat frequencies so we will evaluate recordings in a range of environmental conditions.

Objective 2:

Field recording to assess opto-electronic sensor and insect identification algorithm. We will deploy opto-electronic sensors housed in McPhail traps to assess the capability of the sensors to accurately identify SWD from other inhabitants of Cherry orchards.

METHODS

Objective 1: Refining insect identification algorithm using opto-electronic sensors

Overview: In order to automate the process of insect identification based on wing beat frequency, an algorithm must be created and refined to take into account biotic and abiotic factors that may result in changes to insect wing beat frequency and activity pattern. Our cooperators have already created an algorithm to accurately identify insects down to species and sex using wing beat frequency in controlled environments, which they have tested on mosquito species (Chen et al. 2014). To refine

this algorithm for SWD and use in the field, wing beat frequency of SWD and other insects commonly found in cherry orchards will be recorded in different environmental conditions (temperature, light cycle, humidity, etc.). The data acquired from these species in controlled environments will be sent to our cooperators to be incorporated in their insect identification models and refinement of the algorithm.

Collection of data for insect identification algorithm refinement

Flies of a known species and sex (N=60) will be placed into a modified McPhail trap outfitted with an opto-electronic sensor ring and connected to a recording device. This setup will then be placed into a Digitherm incubator (Tritech Research) that allows us to control the environmental conditions. Using this setup, we will record wing beat frequency data in different temperatures, humidity, light-dark cycles with different photoperiods, etc. as well as wing beat frequency of different species and sexes. The data collected in these controlled environments can be visualized using analysis programs written by our cooperators using MATLAB (Mathworks). General trends can be visualized using these analysis tools. Comparison between SWD and the closely related *Drosophila melanogaster* in controlled conditions show distinct wing beat frequency patterns. Based on live capture in field in CA, we identified several closely related *Drosophila* species such as *D. simulans*, *D. biarmipes* and *D. tristis*. Recording using these different species and other relevant species present in cherry orchards will be generated in order to refine the algorithm and improve identification accuracy.

Refinement of insect identification algorithm

Our cooperators have created an insect classification algorithm which they have used to accurately identify disease carrying mosquito species based on wing beat frequency alone (Chen et al. 2014). When more species were added or environmental conditions were changed the classification model was less accurate. Due to the large diversity of species present in the field and the heterogeneity of environmental conditions, it is important to have accurate classification established on a wide range of fluctuating parameters and species to mimic field scenarios. By creating a training dataset using the data we collect from flies in various conditions, our cooperators will be able to "train" the classification model to accurately identify insect pests in vastly different environments. Our cooperators have already "trained" the insect classification model based on geographical and circadian rhythm data to increase the accuracy of the model in identifying mosquitos down to the species level (Chen et al. 2014). By "training" the insect classification model to correctly identify insects using a larger number of variables we will be able to increase the accuracy of our identification process in the field. This will be an iterative process of testing and refinement.

Objective 2: Assessment of insect identification algorithm and field deployment of sensors

Overview: With current monitoring methods, it is extremely time consuming to monitor insect pest species in the field because it requires the presence of a specialist to manually identify individuals. In addition, the time lapse between trapping and identification constitutes an important limitation to initiate a quick and appropriate response to slow down crop infestation. Our goal in refining the insect identification algorithm is to develop an automated identification process that is easier and faster to identify insect pests then is possible with current pest capture and identification processes. We will be assessing the ability of the sensors to correctly identify and monitor pest species both spatially and temporally in and around cherry fields.

Deployment of insect sensors in the field

Once the classification algorithm is found to be highly accurate (>99%), we will deploy our system in cherry fields. We will use baited McPhail traps outfitted with sensors in the opening at the bottom to record the wing beat frequency and relevant environmental variables (temperature, humidity, time, etc.) of any insect that enters the trap and identifying them in real time. By deploying

multiple trap/sensor setups in and around cherry fields, we will be able to track the movements of SWD throughout the day, e.g. from crop to non-crop hosts. This will allow for the development of more precise strategies of pest management than are possible through conventional monitoring techniques using traps and manual identification. The automated process of insect identification also means that there will be far less processing time required to identify flies allowing growers and researchers to respond to the presence of pests as soon as they arrive and are detected in their fields.

RESULTS:

Objective 1:

Hardware optimization for insect sensors

We successfully went through several interactions of design and testing of the sensors. We have converged on a solution that we feel is robust, maintainable and cheap to produce in large numbers. Briefly, we use IR emitters and phototransistors working at a wavelength of 940nm, which is outside the visible light spectrum. Our emitters (OSRAM SFH 4043) and phototransistors (Everlight PT19-21C) use around ~20mA. There may be some other low power emitters and phototransistors out there that we can used to further improve our design in the future. We are currently using a cortex M4 MCU, which runs at 80Mhz. This chip has 32K of flash and 2K of RAM. It consumes around 5.5mA when running at 8Mhz but we can put it to sleep when there is no activity, in sleep mode it only consumes few micro amps. For transmission we are using Long Range Wide Area Network (LoRaWAN) technology. LoRaWAN is a wireless standard designed for long range communications at a low bit rate on a very low power budget. We use Semtech SX1272 LoRa module which has a range of 2 miles in non line-of-sight environment and up to 15 miles in line-of-sight environment. It can achieve data rates up to 50 kbps. SX1272 consumes ~15mA while transmitting/receiving and a negligible power (1.5 uA) in idle state. We have started to install solar panel to the sensor unit, so that the sensors can be left unattended in the field for weeks at a time (Figures 1 to 3).

Development of species ID algorithm

We have now completed most of the activity and wing beat frequency recordings for 5 different *Drosophila* species (*D. simulans, D. tristis, D. suzukii, D. biarmipies, D. melanogaster*) at various temperature and photoperiod (Figure 4). The accuracy of the resulting species ID algorithm is easily over 90% accuracy, and will continue to improve as we finished our planned recordings.

Objective 2:

Field deployment and testing

With the outfitting of the sensors with solar panel and cellular data transmission for remote sensing, we are ready for field testing of the sensors in this upcoming year of the project. Field testing and validation will be performed as described in the Methods section.

LITERATURE REVIEW:

There have been some efforts in identifying insects based on recordings of their wing beat frequencies and these attempts date back to the advent of commercially available computers and audio recording devices (Reed et al. 1942, Foster and Robinson 1991, Moore and Miller 2002, Raman et al. 2007). These attempts have not been successful in creating an automated and accurate identification process based on recordings of wing beat frequencies. In most studies, wing beat frequency has been recorded using acoustic microphones, which are susceptible to noise from the wind as well as any ambient noise in the environment (Reed et al. 1942, Mankin et al. 2006, Raman et al. 2007, Villarreal et al. 2017). This made it very difficult to get quality recordings of insect wing beat frequency with acoustic recording devices. Because of this difficulty, wing beat frequency data is sparse, low quality, and typically recorded in unnatural conditions (Moore et al. 1986). Despite the sparseness and low quality of available insect wing beat frequency data, some researchers have

attempted to create insect identification models with 300 or less recordings (Moore 1991). It is difficult to create models with such sparse data and this will cause the models to have very low accuracy in identifying insects (Banko and Brill 2001, Halevy et al. 2009). This is compounded by the fact that most attempts at classification of insects by recording wing beat frequency have used just one variable (wing beat frequency). Other environmental factors that cause wing beat frequency to change have also been ignored (Chen et al. 2014). By using pseudo-acoustic opto-electronic sensors, we will be able to record higher quality data. We will also be able to record larger volumes of data in more natural conditions than has been possible in the past, which will allow us to create a highly accurate insect classification model that can be used to identify SWD and differentiate it from other species in the field.

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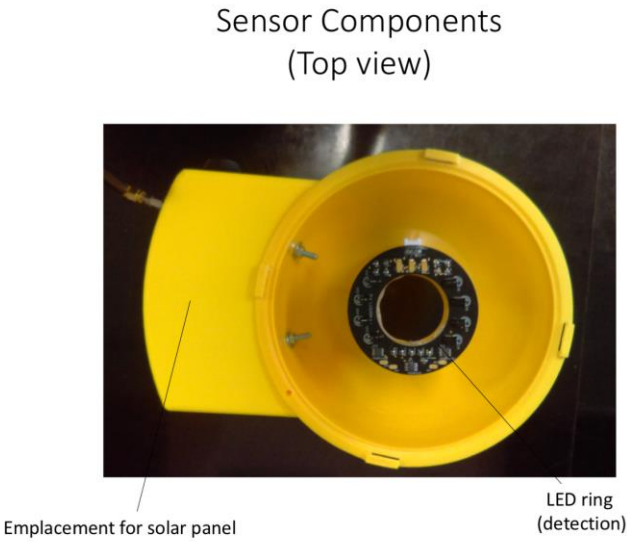


Figure 1: Top view of the modified Mcphail trap outfitted with the LED sensor ring, showing emplacement for solar panel.

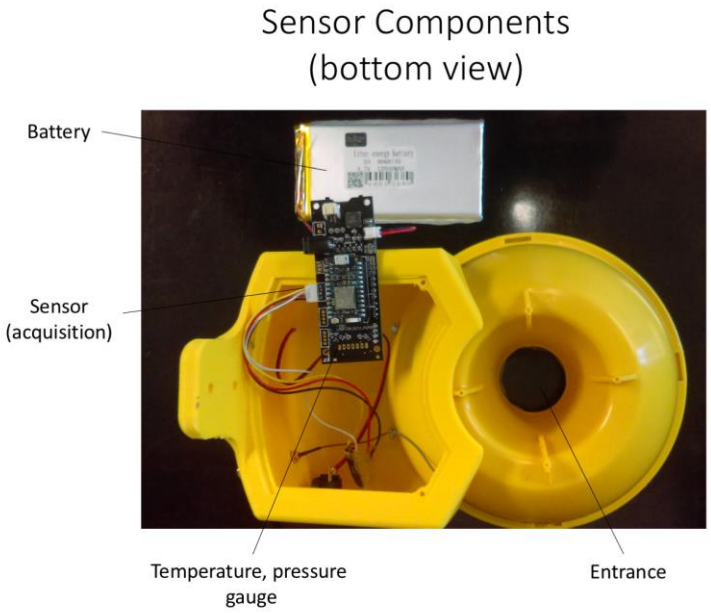


Figure 2: Bottom view of the modified Mcphail trap showing the battery unit, the data acquisition unit, the environmental measurement unit, and the entrance of the trap.

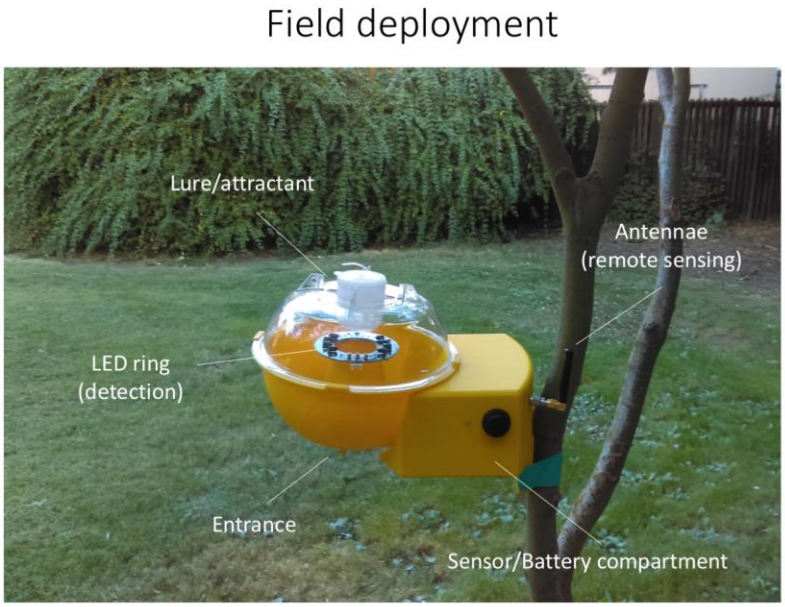


Figure 3: The modified Mcphail trap holding the sensor unit in a field setting. The antennae for transmitting remote sensing data is shown.

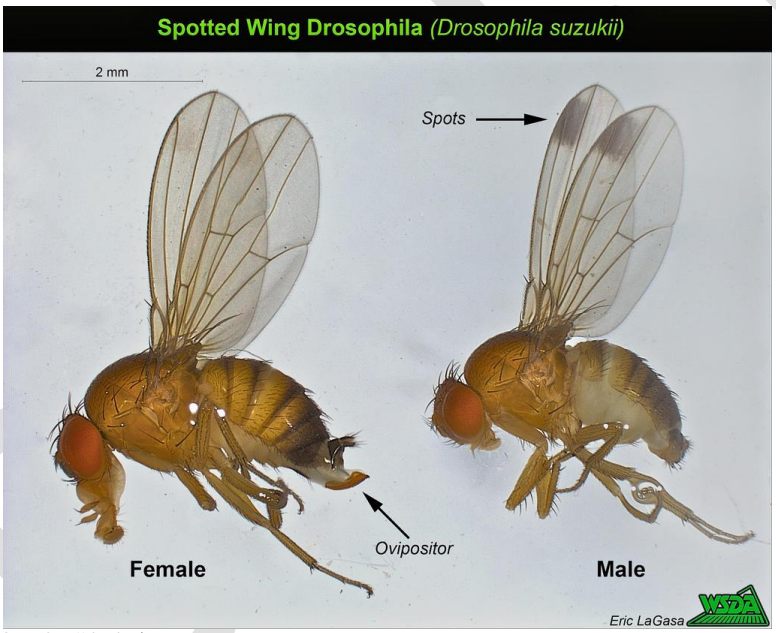
Temperature:		20C	25C	30C
D. sim	Male	x	x	x
	Female	x	x	x
D. tris	Male	x	x	x
	Female	x	x	x
D. suz	Male	x	x	x
	Female	x	x	x
D. biar	Male	x	x	x
	Female	x	x	x
D. mel	Male	x	x	x
	Female	x	x	x

Photoperiod		12:12
D. sim	Male	x
	Female	x
D. tris	Male	x
	Female	x
D. suz	Male	x
	Female	x
D. biar	Male	x
	Female	x
D. mel	Male	x
	Female	x

Figure 5: Temperature and photoperiod conditions for wing beat frequency recordings. Conditions marked with pink have been completed. *D. simulans (D. sim); D. tristis (D. tris); D. suzukii (D. suz); D. biarmipes (D. biar); D. melanogaster (D. mel).*

BETTER WAY BIOTECH

BUSINESS CASE



Oregon State University photo

December 16, 2018

SUMMARY

Science is the most powerful sociological construct that humans have ever developed. It's the means by which we unravel and understand nature. It's how we advance solutions to our ills and differentiate ourselves from all other forms of life. We are knowledge-seeking creatures, and we use that knowledge to advance our insight, perspective and well-being.[1]

Public trust in business, government, the media, and even civil society has fallen to the point where more than half of the world feels the current system is failing them. The widening gap in trust between those in their country's top income quartile and the rest of the population indicates that social cohesion is fragile at best, and very close to breaking down at worst. It is in this precarious political and social context that we face both the opportunities and the challenges of a range of powerful, emerging technologies -- from artificial intelligence, to biotechnologies, advanced materials to quantum computing -- that will drive radical shifts in the way we live, and which I have described as comprising the Fourth Industrial Revolution.[2]

These two statements reflect the foundation of a new company, Better Way Biotech, which is organized as a Benefit Corporation to introduce emerging technologies into the environment through thoughtful review and public discussion in a way that respects civil discourse. The mission of the company is to mitigate the impact of damage caused by a particular agricultural pest. The product/service will be the development and release of a modified Spotted Wing Drosophila (SWD) that has the purpose of eliminating the populations of the wild type of this invasive insect.

The many disruptive advances in science and technology offer great promise to address our most fundamental human challenges, but these emerging technologies have substantial uncertainties and risks that accompany the potential benefits. These risks and uncertainties are incompletely understood by the scientists, and the public is typically not even aware of the existence of unknowns to scientists. Because of this uncertainty, with the introduction of leading edge technology it is important to ensure that proper mechanisms are in place to get scientists out in front of the issues to properly frame them and to explain to the public, media, and government regulators what is known (and not known) about the risks and benefits before political or economic interests usurp the narrative. The case of the development of Genetically Modified Organisms (GMO) in food is a good example of the failure to educate and engage the public.

Commercialization of technology is predominantly done in the private sector by established and start-up companies. The primary driver of these entities is investor/shareholder return on investment expectations. Such structural primacy does not permit a corporate entity to adequately consider its transparency or social return on investment. To expand the obligation of its governance board, requiring it to consider environmental and social factors as well as the financial interests of shareholders, Better Way Biotech is committed to adopting a Benefit Corporation corporate structure. A Benefit Corporation gives directors and officers the legal protection to pursue a mission and consider the impact that the business has on society and the

[1] Michael Crow, President of Arizona State University. Posted on LinkedIn, June 22, 2017.
[2] Klaus Schwab, *Shaping the Fourth Industrial Revolution* (World Economic Forum, 2018), vii.

environment. This can affect a wide range of decisions, attitudes toward intellectual property, and levels and timing of financial return.

Management of a Benefit Corporation may be empowered to utilize broader metrics and tools to measure social return and to employ different approaches, such as design thinking and social strategic planning, for a longer term perspective than might otherwise be possible with a traditional corporate structure. To be successful, the Benefit Corporation will facilitate creation of a stakeholder ecosystem that links and builds trust among impacted parties, investors, government officials and others affected by and involved in the decision-making process.

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THE PROBLEM

Crop and public health damage caused by invasive insects and insects vectoring diseases.

Certain orders of insects have presented human society with some of its greatest development challenges by spreading diseases, consuming crops, and damaging infrastructure. Despite the massive human and financial toll of invasive insects, cost estimates of their impacts remain sporadic, spatially incomplete, and of questionable quality. However, one recent study estimated that invasive insects cost a minimum of US $70.0 billion per year globally, while associated health costs exceed US $6.9 billion per year.[3] Global climate change, rising human population densities, and intensifying international trade have allowed these costly insects to spread into new areas. Yet substantial savings could be achieved through effective surveillance, containment, and public awareness.

A grand challenge this century will be meeting the world's food requirements while maintaining economic productivity and conserving biodiversity. Globally, insect pests have been reported to reduce agricultural yields by 10–16% before harvest and to consume a similar amount following harvest.[4] In fact, the largest food-producing countries, China and the United States, exhibit the highest potential losses from invasive insects.[5] Other insect pests defoliate trees and degrade plant biodiversity, threaten commercial forestry, and hamper climate change mitigation via increased tree mortality and associated increased greenhouse-gas emissions. Many other insects are nuisance species or disease vectors that directly erode public health, and from the 17th to 20th centuries insect-borne diseases caused more human disease and death than all other causes combined.[6]

The introduction of invasive/non-native species can have a dramatic effect on natural resources, human health, and the economy. In a natural or native community, species evolve together in an ecosystem with many checks and balances that limit the population growth of any one species. These checks and balances form the complex web of life that makes up an ecosystem in which a native species competes for survival. When non-native species are introduced into an ecosystem in which they did not evolve they are without environmental or native predator control and their populations can flourish unchecked.

The immediate motivation of Better Way Biotech is to formally address methods to control damage caused by the invasive insect, *Drosophila suzukii*, commonly called the Spotted Wing Drosophila (SWD). In June 2009, spotted wing drosophila, formerly known as the cherry vinegar fly, *Drosophila suzukii* (Diptera: Drosophilidae), was trapped over a wide area in northern California including Santa Clara, San Benito, Santa Cruz and Monterey Counties. SWD was

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[3] Bradshaw, et al., *Massive yet Grossly Underestimated Global Costs of Invasive Insects,* Nature Communications. 2016; 7: 12986. Published online 2016 Oct 4.

[4] Bebber D. P., Ramotowski M. A. T. & Gurr S. J., *Crop Pests and Pathogens Move Polewards in a Warming World.* Nature Climate Change Journal, 3, 985–988 (2013).

[5] Paini , et al., *Global Threat to Agriculture from Invasive Species*. Proc National Academy of Sciences U S A. 2016 Jul 5; 113(27):7575-9.

[6] Ibid.

considered established in California in 2010. 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 multi-state Specialty Crop Research Initiative grant funded SWD research group to monitor the effects and phenology, study existing control solutions and develop new control methods of this insect. Their initial report for 2014 estimated that the potential national crop loss due to SWD was valued at $1.3 billion for cherry, berry, and other soft fruit in the U.S.[7] In an effort to seek alternative solutions to the current costly insecticide management, the California Cherry Board began funding research at Cal Tech and UC Riverside on gene drive technology. With promising results in the laboratory, the Cherry Board funded a roadmap for advancing this research for possible release of modified SWD in the field with the goal of exterminating this invasive pest.

This business case outlines a pathway for addressing complex new technologies for mitigating insect damage that require regulatory approvals. A benefit corporation is considered the appropriate legal entity to organize products and services to gain regulatory approvals and provide farmers with effective tools to control the insects. The product the benefit corporation will develop addresses SWD mitigation.

PARALLEL APPROACHES TO CREATE SOLUTIONS

New technologies for mitigating insect damage to crops may involve regulatory approvals. History shows that opaque attempts to gain regulatory approvals with poorly understood technologies can incite significant public opposition that can slow adoption and delay application. Thus, the challenge is twofold: 1) develop effective products that employ advanced technologies, and 2) engage the public and regulatory agencies to gain support for solutions that are publically acceptable to the times.

EMERGING TECHNICAL SOLUTIONS

Various methods are used to fight invasive plant and animal outbreaks, including manual, biological, and chemical control. Manual control, the most basic, includes hunting, digging out, flooding, or burning invasive plant or animal species. When biological control is thoroughly calculated and tested, it can be an effective tool. For example, the aquatic plant species Hydrilla, an invasive species in Florida, has had its numbers cut through a variety of biological control measures, including using sterilized Chinese grass carp to control the population.

However, biological control methods can be disastrous if implemented without enough prior research. In 1930, a few thousand South American cane toads were introduced to Australia in order to eradicate the beetles eating local Queensland sugarcane crops. The toads never accomplished this goal, but they did manage to successfully colonize most of Northeast Australia. Their numbers are now in the millions, and they cause irreversible damage in every ecosystem they invade. What's more, they're also highly venomous, causing injury or even death to any other species that decides to prey on them. Clearly, this method is a high-risk, high-reward gamble. Chemical control is also a viable option when battling invasive species. Many invasive plant species have had their populations controlled and even eradicated through the use of herbicide in the past. However, herbicide can also have adverse effects on human and wildlife health, and can pollute soil, water,

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

and non-target vegetation. As a result, many conservationists have called for herbicides to be used sparingly and not as a first response measure.

The current standard of control for managing SWD combines diligent surveillance with the use of insecticides. Unlike native fruit flies, female SWD have a serrated ovipositor, or egg-laying appendage, to puncture the skin of intact fruit to lay their eggs. This makes SWD a more significant pest than the native flies that use damaged fruit to lay eggs. Soft skinned fruit such as cherries, blueberries, raspberries, strawberries 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.

The current practice for mitigating SWD damage consists of several steps:

1) Monitor fields with traps and check them regularly.
2) Apply strong insecticides registered to protect the fruit at key color stage that attracts the first flies or on spec.
3) Continue monitoring to evaluate the orchard's management program, and respond quickly if needed.
4) 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 significant added costs to production. In addition, the effectiveness of most pesticides decreases over time as the insects develop resistance to the chemicals, thus requiring more of its use and/or rotation of active chemical ingredients to reduce populations. Also, when pesticides are sprayed, they could travel outside their intended area of use and kill native beneficial insects .

Employing new techniques that promote the inheritance of a particular gene to increase its prevalence in a population of SWD with the goal of reducing its numbers could offer better alternatives for controlling SWD populations. These approaches offer a targeted method for reducing populations of SWD, 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 have the added benefit of supporting conservation efforts in agricultural regions.

A. Gene Drive Technology

A gene drive is a mechanism that biases inheritance of a trait in a sexually reproducing species of organisms. In this context, 'bias' means to increase the odds of a parent passing on a portion of their DNA to above 50% of their progeny, or what is historically understood as Mendelian inheritance. Gene drive has proven effective in many test results. In fact, under laboratory conditions some gene drives have been shown to function at 95% efficiency or higher, meaning that a parent carrying a genetic sequence associated with a gene drive will pass on that sequence nearly every time that they reproduce. The key scientific breakthrough is that designed gene drives could theoretically allow humans to 'drive' a desired trait into an uncontrolled population of organisms. Importantly, this population could be wild – neither its habitat nor its breeding behavior need be under human control – and the desired trait might reduce the fitness of the organism, even while increasing in frequency throughout the population. At a crude level, gene

drives thus disrupt Mendelian and Darwinian assumptions about how to predict inheritance and measure evolutionary fitness.[8]

Enhanced versions of gene drives may include the ability to customize its applications to fit the desired goal for managing release of modified animals in the wild. These features under study include:

- The potential to limit the spread of modified organisms (global population replacement versus local)
- The mechanisms for limiting spread (self exhausting versus threshold dependent)
- The ability to reverse the drive at the trait or DNA sequence level

With further development and testing, these features could provide mechanisms for reversing the introduction of the modified insects. As a result, it is possible that these features could be employed as a safeguard against unintended consequences from a release.

B. Medea Gene Drive Systems

Synthetic gene drive systems possess enormous potential to replace, alter, or suppress wild populations of significant disease vectors and crop pests; however, their utility in diverse populations remains to be demonstrated. Building on the studies performed for the California Cherry Board, researchers recently reported on the creation of a synthetic "Maternal Effect Dominant Embryonic Arrest" (Medea) gene drive system for SWD.[9] The researchers demonstrated that this drive system, based on an engineered maternal "toxin" coupled with a linked embryonic "antidote," is capable of biasing Mendelian inheritance rates with up to 100% efficiency. However, the researchers found that drive resistance, resulting from naturally occurring genetic variation and associated fitness costs, can be selected for and hinder the spread of such a drive. Despite this hindrance, their results suggest that the Medea gene drive system could maintain itself at high frequencies in a wild population and spread to fixation if either its fitness costs or toxin resistance were reduced, providing a clear path forward for developing future such systems in SWD.

Specifically, Medea systems rely on expression of a toxin/antidote combination, such as a microRNA (miRNA) toxin, that is expressed during oogenesis in Medea-bearing mothers, and a tightly linked antidote expressed early during embryogenesis in Medea-bearing progeny. The toxin is inherited by all progeny from a Medea-bearing mother, resulting in miRNA-mediated suppression of an essential embryonic gene that causes disruption of normal development during embryogenesis. Offspring that inherit Medea receive a tightly linked antidote, consisting of a zygotically active miRNA-resistant copy of the targeted essential gene, which allows for restoration of normal development; non-Medea-bearing progeny from Medea bearing mothers lack this antidote and perish. Due to this biased inheritance, Medea is predicted to rapidly spread itself, and any linked cargo genes, through a target population.[10]

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[8] Jason Delborne, et al., (2018), *Mapping Research and Governance Needs for Gene Drives*, Journal of Responsible Innovation, 5:sup1, S4-S12, DOI: 10.1080/23299460.2017.1419413

[9] Buchman, et al., *Synthetically Engineered Medea Gene Drive System in the Worldwide Crop Pest Drosophila suzukii.* PNAS April 17, 2018. 201713139; published ahead of print on April 17, 2018.

[10] Ibid.

To assess whether the *D. suzukii Medea* could function in geographically distinct populations that possibly harbor genetic variability in regions which canonically have less conservation such as the 5'UTR, heterozygous *Medea*/+ flies were tested in eight additional *D. suzukii* strain backgrounds. These strains were collected from various locations around the world, including Mt. Hood, OR; Clayton, WA; Brentwood, CA; Tracy, CA; Watsonville, CA; Oahu, HI; Beltsville, MD; and Ehime, Japan. Interestingly, for three of eight strains the *Medea* inheritance rate from heterozygous *Medea*/+ mothers was 100%, while from five of nine strains the inheritance rate ranged from 87.6 to 99.4%. These results strongly demonstrate that the *Medea* drive can dominantly bias transmission in diverse *D. suzukii* populations. Moreover, modeling results suggest that a *Medea* drive having a high fitness cost and high (though imperfect) toxin efficiency may be capable of maintaining itself in a population for a period of several years following a series of large-scale releases of homozygous males.[11]

The authors of the study conclude that *"*Medea also has the added benefit that, if fitness costs decline over time, its drive is frequency-dependent and hence large, and intentional releases are more likely to lead to spread than small, unintentional ones. That said, the potential and implications of fitness costs' evolving will be further investigated".[12]

In summary, while the Medea system offers promise in its ability to significantly reduce populations of SWD over large areas. Additional research is needed to enhance its effectiveness and increase the clarity as to the sequence and specific release areas necessary for reduction of populations. SWD modified by a Medea system is still under development and not yet ready for release into the wild. Medea needs a cargo gene to reliably get the system into the population. In addition, further research needs to be performed to identify the best trait, such as temperature sensitivity, to act as a trigger to allow Medea system spread during off-season and induce population decline at the most advantageous time.

Intellectual Property Requirements

UC Riverside has applied for patents to these core technologies, and initial discussions with licensing officers in the Office of Technology Commercialization have begun. Additional licenses to the use of related technologies may also be required, so further detailed review is needed.

SOCIO-ECONOMIC SOLUTIONS

As quoted above, *"Science is the most powerful sociological construct that humans have ever developed."*[13] Yet given ever more complex emerging technologies, scientists and scientific institutions see an increasing need for outreach and communication to counter misconceptions about science or misinformation by special interest groups. Distinguishing science from pseudoscience, providing explanatory narratives based on science, and exposing the tactics used to mislead people can all help members of the public to better understand science.

[11] Ibid.
[12] Ibid.
[13] Michael Crow, President of Arizona State University. Posted on LinkedIn, June 22, 2017.

Yet there remains a growing distrust in science among the public. What accounts for this mistrust? Part of the answer involves a misunderstanding of science itself. As Atul Gawande[14] has defined it, science requires a commitment to a way of building knowledge and explaining nature through factual observation and testing. This "is not a normal way of thinking," he said. "Much of what we do in science may be counterintuitive. A scientific explanation will not necessarily be the same as the explanation that may come from the wisdom of divinity or the explanations that come from experience or common sense. We watch the sun move across the sky. It's common sense. It's moving across the sky. Or people get colds in cold weather, and cold must produce colds." In science, intuitions are hypotheses that need to be tested. Gawande particularly likes Edwin Hubble's description of a scientist as someone with "a healthy skepticism, suspended judgment, and disciplined imagination." Gawande's interpretation of this description is that scientists have an experimental mind, not a litigious mind. Scientists are not free of opinion, but evidence contradicting their explanations can always arise. Hubble also said that "the scientist explains the world by successive approximations." This approach to understanding has proved remarkably powerful over time.[15]

There can be a tendency among some academic researchers to use strong assumptions to derive persuasive findings to inform public policy. Credible research, though, often yields interval rather than point prediction of policy outcomes. Why do researchers express certainty when they should be expressing uncertainty? Charles Manski[16] suggests two reasons. The first is that the scientific community tends to reward strong and novel findings. The second is that the public wants unequivocal policy recommendations. In his book, Manski argues that society should face up to the uncertainties that attend policy formation. He observes that the current practice of policy analysis hides uncertainty. Analysts at the Congressional Budget Office (CBO), for instance, know that their point estimates should be accompanied by ranges of uncertainty. But they may believe, Manski has speculated, that the members of the U.S. Congress are psychologically or cognitively unable to deal with uncertainty. Or they may believe, since the CBO has established a long-term reputation for impartiality, that it is best to leave well enough alone and have the CBO express certitude when it scores legislation, even if the certitude is conventional rather than credible.

Public outreach has therefore become an issue of growing importance for science. And there remains the public's obligation to review and, where appropriate, regulate the use of scientific outcomes and their deployment in society. Given the uncertainties with diffusing new technologies, scientific experts and government officials may view the regulation of emerging technologies could require deeper engagement with public values and perspectives. Forums for interrogating what can be learned from available data combined with credible assumptions may lead to more resilient policy outcomes. These outcomes can be further strengthened by a system that is flexible enough to accommodate change in the face of new learnings from intended and unintended consequences of those policies. This iterative engagement process could lead to more reasonable policy choices that can be made with partial foresight of outcomes.

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[14] Atul Gawande, *The Mistrust of Science,* The Science of Science Communication III: Inspiring Novel Collaborations and Building Capacity: Proceedings of a Colloquium. (The National Academies Press. 2017)
[15] Ibid.
[16] Charles F.Manski, *Public Policy in an Uncertain World* (Cambridge, MA: Harvard University Press. 2013)

Gene drives, for example, are being developed in a landscape of pest management shaped by past and current approaches, experiences, regulations, public opinion, and pest invasions. Because gene drive insects may spread well beyond their release area, stakeholder groups at different spatial scales need to be engaged in decisions about their deployment. In contrast to control options like insecticides that are adopted on a farm-by-farm basis, area wide approaches have the potential to impact everyone within the release zone. Further, the molecular constructs in gene drive systems are designed to spread, and therefore may move throughout the target species' geographic range. The unique nature of gene drive insects necessitates both "big-picture" and long-term thinking in the identification and engagement of stakeholders. This new paradigm both complicates and offers great promise for future pest management efforts.

New methods of public engagement in the process of developing regulatory policies for emerging technologies may address the tension between democratic and expert-led decision making. These methods offer the opportunity for mutual learning by integrating formal expertise, local knowledge, and public engagement. They can provide transparency and offer a forum for better informed decision-making. Finally, active engagement can build trust among experts, stakeholders, and public audiences. Trust among industry leaders, scientists, government officials, and the general public is not a given, but there are modalities available for creating and strengthening the trust among these stakeholders. These modalities include:

A. Anticipatory Governance

Anticipatory governance is defined as a broad-based capacity extended through society that can act on a variety of variables to manage emerging knowledge-based technologies while such management is still possible. Anticipatory governance motivates activities designed to build capacity in foresight, engagement, and integration, as well as through their collective activities. These capacities encourage and support scientists, engineers, policy makers, and other publics to reflect on their roles in emerging technologies. Reflection in this instance means awareness of one's own position as a participant, with a specific set of roles and responsibilities in a field of other actors.[17]

Another definition of anticipatory governance is that it is seen as a "strategy to facilitate the acceptance of new techno-sciences by inviting people to voice their hopes and concerns in focus groups, science cafes, and computer-based interactive spaces before the innovations are actually implemented."[18] Both of the above definitions capture the essence to include collective action among stakeholders to better manage the employment of emerging technologies in society. The question then becomes specifically what can be done to accomplish these goals?

B. The Appropriate Legal Entity

In determining the most appropriate organizational entity to advance the technology to adoption in the environment, 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 a framework to help triage the first fundamental choice of currently available entities; for-profit or non-profit organization?

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[17] David H. Guston, *Understanding Anticipatory Governance,* Social Studies of Science, 2014, Vol. 44(2) 218–242

[18] Fuller, S. (2009) Review of the *Handbook of Science and Technology Studies*. Cambridge, MA: The MIT Press. Hackett EJ, Amsterdamska O, Lynch M and Wajcman J (eds) (2008)

1. How Does The Organization Plan To Fund Itself: How Much Earned Income?
If the primary way the organization plans to fund itself is through an earned-income strategy, the organization would normally be a for-profit entity. The first reason is that income derived from commercial operations which 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.

Second, if such income comprises a substantial portion of the organization's overall income (typically more than 20 percent), it is 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 typically funneled 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 the activities pursuant to the mission when it is difficult to monetize these activities.

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 first question above. If the venture's products or services primarily benefit those in need or the environment, with no clear stakeholders who are willing to pay for the product or service, then the venture should probably be classified as 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 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 certain stone fruits, and many of these benefits 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 public in general. There is also 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 novel 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 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 (FKMCD) has been working with the British company Oxitec to obtain 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 are expected to die.

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 the typical linear process of primarily working with government officials with limited engagement of the public. Oxitec's efforts, though, did culminate with FDA approval in August, 2016 and by

two successful referendums in the Florida Keys on in November 2016 to approve the release of Oxitec mosquitoes.

The question now occupying the Commissioners on the District's Board is when to conduct the first release. The U.S. Environmental Protection Agency (EPA) is continuing its review of Oxitec's' Experimental Use Permit (EUP) application and the comments received during the two recent 30-day public comment periods surrounding the release of Oxitec's *Aedes aegypti* mosquitoes in Monroe County. The Florida Keys Mosquito Control District had expected the review to be completed in July of 2018, but as of August 9th a press release from the FKMCD stated that the District anticipates a regulatory decision on the application by the end of 2018.

This delay of over two years from regulatory approvals and public referenda is largely attributable to the failure by Oxitec to proactively engage the public. It is 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, the timeline could have been significantly shorter to gain the approval of one mosquito control district to release the modified mosquitoes. Focused public engagement could 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 could have brought this complex issue to more clarity for decisions in favor of earlier release.

Returning to the question of the appropriate commercial entity, it is clear that there are significant, measurable damages caused by SWD. Cherry and berry growers are keenly aware of these costs, incurred 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 34 states have passed legislation allowing this legal charter and more states are expected to do the same. As of January, 2018, it was estimated that there are over 5,000 Benefit Corporations active in the U.S.[19]

The purpose of a traditional corporation 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 inhibits them from operating the company by also taking into account social and environmental factors. However, as a result of the legal protection offered to Directors and Officers of a Benefit Corporation, this new legal entity was 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 short, a Benefit Corporation is similar to a traditional corporation, except language in the corporate charter details the special purpose of the corporation and the various stakeholder groups that must be considered equally by the governance and operations of the Benefit

[19] https://frederickalexander.net/2018/01/09/benefit-corporations-are-ready-for-2018/

Corporation. A Benefit Corporation, by its nature, is more credible in seeking scientists and other knowledgeable communicators to put technical and social risk-versus-benefit arguments to the public.

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, as of August 2018, there are over 2,600 certified B Corporations across 140 industries in 60 countries.[20] 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 weighted equally.

Operationally, a Benefit Corporation can strengthen trust among stakeholders by promoting transparency in its structure and activities. For decades, companies could control a brand's narrative through marketing. Spin often took precedence over transparency, as customers' access to information was limited. But as access to online information proliferates, customers are getting smarter. Today, honesty is a marketing strategy. In fact, transparency can actually be a more effective marketing strategy than spin. Authentic transparency is a proxy for a company's values, which modern consumers increasingly care about. According to Nielsen, nearly two-thirds of global consumers are willing to pay more for products from companies committed to positive social and environmental impact. According to a survey by Label Insight, nearly 40% of customers say they would switch from their current preferred brand to one that offers more transparency. And what is the number one driver of purchasing decisions for sustainable products? Trust.

Transparency of Operations

Transparency can greatly benefit companies. Big brands tend to be the least trusted institutions in America (by some measures, only Congress ranks lower), and in the absence of information, consumers will typically assume the worst. Even a company's flaws are generally better handled out in the open.

Not surprisingly, brand loyalty is also on the decline. But transparency is a key tool that can combat this trend of mistrust and cynicism. Transparent companies garner more devotion from customers and can even turn them into advocates which, in a social network-driven world, can lead to outsized growth.

The concept of stakeholder paradigm has been gaining currency over the past few decades and technological breakthroughs have been influential in building its momentum. Transparency is emerging as a building block and as an indispensable concomitant of stakeholder paradigm. The crux of a transparent organization is trust. This new paradigm requires substituting translucent and opaque business practices with fully transparent ones under which lasting trust can be built between the organization and its stakeholders. Transparency is an integral part of corporate

[20] https://bcorporation.net/

social responsibility debate and an eristic issue for the stakeholders. Moreover, transparency empowers the stakeholders to considerably influence the decision making sphere.

A few examples below serve to demonstrate what operational transparency can look like:

- *Everlane*, an online retailer with a focus on sustainable apparel, breaks down the pricing for each of their products from manufacturing costs to import duties. Most brands would hesitate to prominently advertise their 50-60% markup, but Everlane banked that customers would appreciate and understand those numbers (particularly when compared to the much higher margins of their competitors). And that bet paid off for an estimated $100 million in sales in 2016.

- *Fishpeople*, a company headquartered in Portland, Oregon puts a bar code on the front of their meal kits so customers can look up exactly where their fish was caught.

- *RXBAR*, a Chicago-based company that makes whole-food protein bars, lists their ingredients in bold print right on the packaging. There are hundreds of snack bar products on the market, each purporting to be healthier, tastier, and cheaper than the others. RXBAR doesn't just stand out because of its simple ingredients, but because they are listed in plain language to catch the customer's eye.

These are just a few examples of the many companies that have excelled by treating transparency not as a handicap, but as a competitive advantage. These companies are ahead of the curve. And this trend is just getting started.[21]

Organizational Culture

The culture of any organization is a driving influence on its success. To support the Benefit Corporation's goal of transparency and trust, the company's cultural values must be clear and reinforced in daily operations. In response to the National Academies of Science, Engineering, and Medicine Report entitled *Gene Drives on the Horizon, Advancing Science, Navigating Uncertainty, and Aligning Research with Public Values*,[22] several scientists and organizations self-assembled to provide a response to the report and outlined five foundational principles for organizations involved in the research and use of gene drive technology.[23] These principles can be directly referenced in the charter and operations of the Benefit Corporation:

Advance quality science to promote the public good

The pursuit of gene drive research must be motivated by, and aim to promote, the public good and social value. Funded research shall embody the highest quality science and ethical integrity, consistent with the current best practice guidance set by the research community and relevant decision-making bodies.

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[21] Sophie Bakalar, *The New Era of Hyper-Transparency,* www.collabotrativefund.com, Jul 3, 2017.

[22] *Gene Drives on the Horizon, Advancing Science, Navigating Uncertainty, and Aligning Research with Public Values*, The National Academies Press (2016).

[23] Emerson, C., James, S., Littler, K. & Randazzo, F. (2017) Principles for gene drive research. Science, 358, 1135–1137.

Promote stewardship, safety, and good governance
Researchers and sponsors are stewards of science and the public trust. It is imperative that good governance is demonstrably shown in all phases of the research, and especially in relation to risk assessment and management. This requires compliance with applicable national and international biosafety and regulatory policies and standards. Research conducted with respect and humility for the broader ecosystem in which humans live, taking into account the potential immediate and longer-term effects through appropriate ecological risk assessment, is a hallmark of both good stewardship and good governance.

Demonstrate transparency and accountability
Knowledge sharing is not only essential for the advancement of science, but for transparency to foster public trust in and understanding of emergent technologies. The timely reporting of results and broad sharing of data shall be the norm in gene drive research, consistent with the tradition of openness established in its parent communities of genetic and genomic science. Measures of transparency and accountability that contribute to building public trust and a cohesive community of practice will be supported.

Engage thoughtfully with affected communities, stakeholders, and publics
Meaningful engagement with communities, stakeholders, and publics is critical for ensuring the best quality science and building and sustaining public confidence in the research. Funded research shall include the resources needed to permit robust, inclusive, and culturally appropriate engagement to ensure that the perspectives of those most affected are taken into account.

Foster opportunities to strengthen capacity and education
Strengthening capacities in science, ethics, bio-safety, and regulation is essential for enabling agile and steady progress in gene drive research globally. Opportunities to partner, educate, and train shall be supported throughout all phases of the research, from the early stages to deployment. Strengthening capabilities within countries for testing and deploying the technology is essential for informed decision-making.

For the Benefit Corporation, then, operating with a high degree of transparency will help engender trust among stakeholders. Transparency can be fostered through making available research reports, field trials, survey results, public discussion forums and other activities that demonstrate the culture of the organization.

C. Social planning for the diffusion of modified insects
For the adoption of emerging technologies to address agricultural challenges, it is critically important that farmers receive information from a monitoring system that provides the information in a form that is needed to manage a particular infestation, especially for an intervention that is available at an acceptable financial risk. The continuing challenge for researchers, then, is to construct tools relevant to farmers and their advisors that improve upon the farmers' current management practices. This goal requires an appreciation of growers' decision calculus in managing crop threats and, more broadly, their overall farm enterprise management.

Many factors influence how people form risk perceptions. Farmers' perceptions of risk and levels of risk aversion impact on decision-making about such things as technology adoption and disease management practices. Regardless of the underlying factors that affect risk perceptions, these

perceptions can be summarized by variables capturing impact and uncertainty components of risk.

A new framework has been developed that has the subjective probability of plant disease and the cost of decision errors as its central features, which could allow a better integration of social science and epidemiology, to the benefit of plant disease management. By focusing on the probability and cost (or impact) dimensions of risk, the framework integrates research from the social sciences, economics, decision theory, and epidemiology.[24] This model and approach can be adapted and directly applied for use in decision making for the management of invasive insects.

This sophisticated approach has been developed by Neil McRoberts at UC Davis and his colleagues. Professor McRoberts has developed a model for analyzing the socio-economic risks to agricultural crops in reaction to an invasive agent.[25]

The key variables to his model are:

- The rate of innovation from sources external to the agricultural system
- The rate of spread by word of mouth/demonstration in the population
- The degree of isolation of adopters and non-adopters, and
- The rate of rejection after trial interventions

In addition, he has developed surveys for eliciting opinions from various stakeholder groups on technology adoption and then analyzing preferred pathways by stakeholders for the use of complex solutions to agricultural challenges, such as crop damage. This survey and modeling process can be quite valuable for engaging public opinion and contributing to the dialogue with regulatory agencies. The Benefit Corporation, as the focal point for actions to mitigate the damage caused by SWD, is well positioned to monitor and influence all of these variables in the model.

D. Design Thinking

Design Thinking has emerged over the past few decades as an effective tool for identifying organizational or system components, how they interact with one another, and creating effective actions to take to move the organization or system to an idealized state. Design thinking is a solution-focused method that begins by identifying a goal instead of a problem. In this way, the process encourages an action-oriented approach and uses "logic, imagination, intuition, and systemic reasoning . . . to create desired outcomes."[26] Design thinking is also user-centric and thus requires an ongoing examination of the needs, experiences, and viewpoints of the user.

The design-thinking process involves successive steps, each of which utilizes input from all stakeholders to forge workable solutions. The best solutions are identified and then tested through experimentation with actual users in real-world situations. The feedback gained from those users is then utilized to redesign the possible solutions, with the resulting versions again

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[24] McRoberts, N., Hall, C., Madden, L. V., and Hughes, G., *Perceptions of disease risk: From Social Construction of Subjective Judgments to Rational Decision Making.* Phytopathology 101:654-665. 2011.

[25] Ibid.

[26] *What Is Design Thinking?*, DESIGN MGMT INST., http://www.dmi.org/WhatisDesignThink; *see also* Michael T. McHugh, *Driving Government Transformation Through Design Thinking*, FED. TIMES (Aug. 24, 2016)

sent out for testing. These successive feedback loops are reminiscent of the iterative cycles of agile product development, and help in the same way to quickly generate the information necessary to create the best possible results.

One set of specific steps of design thinking are as follows:[27]

- Identify the problem to solve
- Identify the stakeholders, who then set their individual goals (divergent thinking)
- Convene to brainstorm solutions with all stakeholders participating (convergent thinking)
- Choose and implement best solutions
- Solicit stakeholder feedback
- Revise and retest solutions
- Continue with feedback loops until the most appropriate solution has been reached
- Initiate the most appropriate solution to a small segment of the public in a beta test
- Iterate process again based on data from beta tests
- Test again and collect feedback
- Continue process when necessary to adapt to new innovations

Design Thinking has been used for creating new systemic interactions among stakeholders across many industries and government agencies. In many cases, Design Thinking has redefined the role of individual stakeholders within the system. In certain industries, however, technology is developing so rapidly that companies can have programs or business models up and running long before regulators learn of their existence. For regulated industries, then, Design Thinking offers the promise for reducing tensions and creating new frameworks for rules that govern emerging technologies or novel business models. While Design Thinking may not be a panacea for every issue that plagues regulatory agencies, its emphasis on innovation and collaboration helps to facilitate open communication with entrepreneurs and the companies they build.

In practice, the Benefit Corporation could convene Design Thinking workshops that are attended by stakeholder groups impacted by the Corporation's operations: farmers, regulatory officials, growers associations, the communities affected, and management and employees of the Corporation. The goal would be to solicit input for co-creating new regulatory frameworks for the deployment of modified insects. During this initial workshop, findings from the survey conducted during the strategic planning sessions can be presented and discussed. After the initial policies are developed and subsequent releases of modified SWD, additional workshops could be held to review the results and determine if modifications to the policies area needed. If new regulatory policies are needed for different types of modified insects, new Design Thinking workshops could be conducted that address new regulatory practices.

E. Risk Innovation

Another socio-economic tool that can be employed by the Benefit Corporation is the use of a process termed "Risk Innovation" analysis. In today's evolving social marketplace of needs, wants and opinions, technologies should be designed to navigate a complex landscape of potential risks

[27] Alice Armitage, Andrew K. Cordova, and Rebecca Siegel, *Design Thinking: The Answer to the Impasse Between Innovation and Regulation,* Georgetown Law Technology Review, vol. 3 (2017)

if they are to succeed and be beneficial. And it is not just health and environmental risks that are important – potential threats to beliefs, community, culture, even sense of identity, are becoming increasingly relevant.

Unfortunately, even if innovators want to steer safely through this evolving landscape, there is remarkably little help available. The methods that professionals in companies and regulatory agencies are taught to handle risk – even how they think about risk – are often as antiquated as the technologies being replaced.

In the case of regulations, for example, they are inevitably built around previous technologies: "dumb" digital systems, for instance gadgets that don't communicate through the Internet, or medical devices that don't talk back. When novel technologies arise – gene drive, for example, cloud-based Artificial Intelligence, or the Internet of Things – the overwhelming impulse is to maintain the status quo by forcing them into existing frameworks. However, they are usually not remotely the right shape to do so, never mind being an adequate fit.

This lack of creativity and flexibility in how potential risks are understood and addressed can easily increase the chances of things going wrong. Not only does it create uncertainty around the safe development of new products, but it obscures potential pitfalls.

Public engagement is therefore aimed at raising awareness around potentially transformative technologies so that investors, businesses, regulators and the public know what is coming in the near future. It is also an opportunity for stakeholders to think through possible unintended consequences or what might go wrong as the technologies mature. Putting fantasies aside, though, it is hard to predict the plausible downsides of emerging technologies. Yet this is exactly what is needed if society is to ensure that these are developed responsibly in the long run.

One way to tease out the subtler possible impacts of emerging technologies is to think of risk as a threat to something of value – an idea that's embedded in the concept of Risk Innovation. This "value" depends on what's important to different individuals, communities, and organizations. Health, wealth, and a sustainable environment are clearly important "things of value" in this context, as are livelihood, food, water, and shelter. Threats to any of these align with more conventional approaches to risk – a health risk, for instance, can be understood as something that threatens to make you sick, and an environmental risk as something that threatens the integrity of the environment.

But this concept can also extend from the idea of a threat to something that society values to less conventional types of risk: threats to self-worth, for instance, or culture, sense of security, equity, even deeply held beliefs. These values touch on things that define us as individuals and communities, and get to the heart of what gives us a sense of purpose and belonging. In this way, relevant threats might include inequity or an eroded sense of self-worth from new technologies that threaten to take away jobs. Or fear of becoming socially marginalized by the use of new technologies. Or even dread over sacrosanct beliefs – such as the sanctity of life, or the right to free choice – being challenged by emerging technological capabilities.

Threats like these are not easy to capture. Yet they have a profound impact on people – and as a consequence, on how new technologies are developed and used. Thinking more broadly about

risk as a threat to value is especially helpful to understanding the possible undesired consequences of tech innovation, and how they might be avoided.

This approach to risk also opens the door to considering the potential risks of not developing a technology. Beyond existing value, future value is also important to most people and organizations. By considering how emerging technologies potentially interact with what stakeholders consider to be important, it becomes easier to weigh the possible downsides of developing them – or at least developing them without deliberate consideration – against those of either impeding their development, or not developing them at all.

What emerges when risk is approached as a threat to value is a much richer way of thinking about how emerging technologies might affect people, communities, and organizations, and how they can be developed responsibly. It's an approach that forces us to realize that the consequences of developing new technologies are complex, and touch people in different ways, not all of them for the better. It is not necessarily a comfortable reconceptualization, but looking at risk from this new angle paves the way for technologies that benefit many people and disadvantage fewer, rather than the other way round.[28]

TEAM

Directors, Officers, Management, and staff.

Nicholas Matteis: CEO
Skills: Program and research management, project fund raising, marketing and promotions strategy and execution, policy/regulatory advocacy, board of directors and operations management.

Experience – Mr. Matteis has a background and expertise as an Executive Director of crop representative organizations with Ag Association Management Services Inc. for 11 years.

Matteis is the primary contact for Better Way Biotech
Office Phone: 916-492-7069
Mobile: 916-517-9169
Email: nick@agamsi.com

Tom Turpen: Director/Founder
Skills: New product and service commercialization through comprehensive technology, regulatory, intellectual property, cost and market risk assessments. Experienced at business planning, operations, governance, fund raising, team building, stakeholder and customer relations.

Experience - Dr. Turpen is currently a Principal with Technology Innovation Group and CEO of SensIT Ventures, Inc. He has over 30 years of both healthcare and agricultural biotechnology experience in start-up and Fortune 500 companies as a senior executive, researcher and inventor. Tom is a serial entrepreneur and founder of multiple for-profit and non-profit organizations that

[28] Andrew Maynard, *How risky are the World Economic Forum's top 10 emerging technologies for 2016?* The Conversation, June 23, 2016.

have successfully raised and invested over $100MM in funding from public, government and venture investors. He is a registered patent agent and received his Ph.D. in Plant Pathology from the University of California at Riverside. He was elected a Fellow of the AAAS in 2017.

Dan Hanson: Director/Founder
Skills: Governance, financial management, public private partnerships as well as stakeholder alignment, design thinking, risk innovation, and public engagement.

Experience - Mr. Hanson is Treasurer for both Technology Innovation Group and SensIT Ventures, Inc. He has over 30 years of experience in financial management, public policy development, stakeholder engagement, government agency governance, and innovation management.

Greg Costa: Director/Founder
Skills: Farm and business operations ownership and management, mushroom spawn production facility management, cherry packing facility management, orchard management, cherry marketing. Director of food safety and regulatory compliance for partnership cherry activities.

Donald Drake: Director/Founder
Skills: Industry leadership/representation, farm and business operations management, packing facility food safety program management, regulatory compliance, field technology and orchard management.

Experience – Mr. Drake currently serves on the San Joaquin County Farm Bureau Board of Directors as well as on the California Cherry Board Research Committee and is the Chair of the California Cherry Board Food Safety Committee. He is a cherry grower and is part of a large cherry grower packer company with growing and packing operations in California and Washington. His expertise is in orchard management and cherry packing and shipping.

FINANCIALS DISCUSSION

Initial Capitalization
The initial capitalization for the Benefit Corporation would consist of common stock issued to directors, officers, management and staff. A large reserve of shares (20-25 of total authorized) would be earmarked for researchers that contribute expertise and for future managers and employees. Also, the majority of authorized but unissued shares are reserved for investors. The initial board could also consider a small amount set aside for stakeholder groups affected by the products/services but who are not yet shareholders (such as environmental or organic fruit associations).

Targeted Return on Equity (ROE)
A key to pricing the Benefit Corporation's products/services is a unique method that is based on cost accounting. Cost accounting is an accounting method that aims to capture a company's costs of production by assessing the input costs of each step of production as well as fixed costs, such as depreciation of capital equipment. Cost accounting will first measure and record these costs individually, then compare input results to output or actual results to aid company management in measuring financial performance.

For pricing decisions, the Benefit Corporation will use the input gained from cost accounting and apply a profit return that is derived from a rolling three year average of the Return on Equity (ROE) in a comparable field, such as drugs-biotech industry. Also known as return on net worth, a company's ROE is a common metric used by investors to analyze profitability. Expressed as a percentage, ROE is calculated by dividing a company's' net income for the previous year by its shareholders' equity. The NYU Stern School of Business annually publishes an aggregate ROE by industry that could be used to calculate a three year moving average to set the metric for determining the target ROE for the Benefit Corporation and, therefore, the pricing of services to capture the Corporation's costs and target ROE (see Appendix 1).

Financial Forecast

BETTER WAY BIOTECH
FIVE YEAR FINANCIAL FORECAST*

		Year 1	Year 2	Year 3	Year 4	Year 5
REVENUE						
SWD Releases		0	0	0	1,711,271	1,403,500
Total Revenue		0	0	0	1,711,271	1,403,500
EXPENSES						
General & Admin		250,000	350,000	350,000	350,000	350,000
SWD Development		150,000	250,000	400,000	500,000	500,000
Strategic Planning		75,000	75,000			
Design Thinking			150,000			150,000
Risk Innovation			150,000			
Total Expenses		475,000	975,000	750,000	850,000	1,000,000
Net Income/(Loss)		(475,000)	(975,000)	(750,000)	861,271	403,500
Year End Cash Balance		2,525,000	1,550,000	800,000	1,661,271	2,064,771
Equity						
Capitalization		3,000,000	3,000,000	3,000,000	3,000,000	3,000,000
ROE Target	13.5%		403,500	861,271	403,500	403,500

* Approximate estimates only to illustrate pricing model.
Note: 1st paid release in the wild occurs at the start of Year 4.

APPENDIX 1

The table below was published by the Stern School in January 2018.[29]

Industry Name	*Number of firms*	*ROE*
Advertising	40	-0.88%
Aerospace/Defense	87	29.03%
Air Transport	17	24.78%
Apparel	51	7.62%
Auto & Truck	18	8.64%
Auto Parts	62	24.14%
Bank (Money Center)	11	9.93%
Banks (Regional)	612	9.03%
Beverage (Alcoholic)	28	25.16%
Beverage (Soft)	35	28.39%
Broadcasting	27	18.11%
Brokerage & Investment Banking	42	11.41%
Building Materials	39	23.81%
Business & Consumer Services	169	18.32%
Cable TV	14	11.05%
Chemical (Basic)	38	18.60%
Chemical (Diversified)	7	14.20%
Chemical (Specialty)	99	17.00%
Coal & Related Energy	30	NA
Computer Services	111	28.66%
Computers/Peripherals	58	28.55%
Construction Supplies	49	13.23%
Diversified	24	9.59%
Drugs (Biotechnology)	459	13.45%
Drugs (Pharmaceutical)	185	14.39%
Education	34	2.60%
Electrical Equipment	118	13.54%
Electronics (Consumer & Office)	24	-29.60%
Electronics (General)	167	11.30%
Engineering/Construction	49	7.71%
Entertainment	90	18.99%

[29] http://pages.stern.nyu.edu/~adamodar/New_Home_Page/datafile/roe.html

Environmental & Waste Services	87	9.47%
Farming/Agriculture	34	12.10%
Financial Svcs. (Non-bank & Insurance)	264	-0.29%
Food Processing	87	11.54%
Food Wholesalers	15	16.64%
Furn/Home Furnishings	31	17.62%
Green & Renewable Energy	22	0.66%
Healthcare Products	251	9.51%
Healthcare Support Services	115	17.40%
Heathcare Information and Technology	112	10.80%
Homebuilding	32	12.49%
Hospitals/Healthcare Facilities	35	8.21%
Hotel/Gaming	70	12.94%
Household Products	131	29.25%
Information Services	61	20.91%
Insurance (General)	21	1.97%
Insurance (Life)	25	4.17%
Insurance (Prop/Cas.)	50	7.29%
Investments & Asset Management	165	13.75%
Machinery	126	19.08%
Metals & Mining	102	10.78%
Office Equipment & Services	24	8.70%
Oil/Gas (Integrated)	5	5.90%
Oil/Gas (Production and Exploration)	311	-4.87%
Oil/Gas Distribution	16	1.97%
Oilfield Svcs/Equip.	130	1.97%
Packaging & Container	25	20.54%
Paper/Forest Products	21	6.15%
Power	61	15.55%
Precious Metals	111	1.89%
Publishing & Newspapers	41	-2.51%
R.E.I.T.	244	9.54%
Real Estate (Development)	20	4.62%
Real Estate (General/Diversified)	10	4.17%
Real Estate (Operations & Services)	60	15.06%
Recreation	70	5.26%
Reinsurance	3	6.05%
Restaurant/Dining	81	95.67%

Retail (Automotive)	25	34.26%
Retail (Building Supply)	8	91.26%
Retail (Distributors)	92	16.05%
Retail (General)	18	16.10%
Retail (Grocery and Food)	14	24.43%
Retail (Online)	61	18.72%
Retail (Special Lines)	106	16.59%
Rubber& Tires	4	21.44%
Semiconductor	72	19.87%
Semiconductor Equip	45	33.63%
Shipbuilding & Marine	9	-1.88%
Shoe	11	25.32%
Software (Entertainment)	13	16.33%
Software (Internet)	305	18.52%
Software (System & Application)	255	17.08%
Steel	37	12.23%
Telecom (Wireless)	18	1.87%
Telecom. Equipment	104	15.31%
Telecom. Services	66	16.34%
Tobacco	24	NA
Transportation	18	28.97%
Transportation (Railroads)	8	17.41%
Trucking	30	7.87%
Utility (General)	18	9.95%
Utility (Water)	23	10.00%
Total Market	**7247**	**13.63%**