Final Project Report – SRSFC Project # 2014-01

Proposal Category:

Research

Project Title:

A proactive approach to understanding insecticide resistance as a strategy for sustainable management of spotted wing drosophila

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Objectives:
1) Determine the levels of resistance and cross-resistance to currently used insecticides in field-collected populations of spotted wing drosophila (SWD); 2) assess the genetic potential (risk) of SWD to develop resistance against new chemistries; and 3) determine biochemical and molecular basis of underlying mechanisms of resistance.

Justification:
Fruit production in the US has recently been challenged with a new invasive insect pest, spotted wing drosophila (SWD), *Drosophila suzukii* Matsumura (Diptera: Drosophilidae). The SWD, a native of Eastern and Southeastern Asia (1), is a devastating pest of small and stone fruits. Since it's first detection in California in 2008 (2, 3), the SWD has spread throughout the United States (4) causing significant losses in crop yield and quality, and risk of even more profound damage.

The SWD is highly polyphagous insect (5, 6) and presents a major threat to soft- and thin-skinned

fruit crops including blueberries, caneberries (blackberries and raspberries), cherries, strawberries, peaches, and grapes worldwide. SWD larvae have also been observed feeding on other wild and cultivated hosts including pears, persimmons, figs, loquat, currents, mulberry, buckthorn, and dogwood (5).

Based on descriptions of SWD biology, SWD adults mate within 1-2 d of emerging. Females can lay 300-600 eggs in their lifetime, usually 1-3 eggs per fruit. Eggs are inserted just under the skin of fruit, but paired respiratory filaments are left projecting from fruit, which allow the number of eggs to be assessed microscopically. Eggs develop in 1-3 d, larvae in 5-7 d, and pupae in 3-15 d. Therefore, under optimal conditions of about 20 °C, one generation can complete development in about 8-10 d leading to 15 generations per year (7). Because of this short generation time, SWD populations can increase to potentially devastating levels rather quickly. Estimates of 100-fold population increase every 2 wk are plausible. Actual loss statistics have been more difficult to generate, however, potential losses due to damage caused by SWD in fruit crops in the United States have been estimated at $850-900 million annually (8, 9).

Georgia has become number one blueberry producing states in the US (NASS 2012). Blueberries are number one fruit crop in Georgia with an annual farm gate value of $250 million (10) and economic impact of $1 billion on the state economy (Sial, Pers. Comm. with Georgia Blueberry Growers Association). Since its first introduction in Georgia in 2010, SWD infestations have led to 15-20% loss of blueberry crop annually (Sial, Pers. Comm. with Georgia Blueberry Growers Association). Blueberries produced in the Southeastern states are primarily marketed as fresh fruit in the US as well as export markets and the fresh fruit marketers have zero tolerance for SWD infestation. Detection of a single larva in fruit samples can result in rejection of the entire shipment.

It can be difficult to determine if fruit are infested by SWD at harvest because they often appear otherwise sound. Unfortunately, currently available traps and baits are useful for determining fly presence only but are not reliable predictors of fly density and fruit infestation risk. While this aspect of SWD monitoring is actively being investigated (11, 12), SWD management is currently achieved primarily through preventative insecticide applications (2, 13, 14, 15). The number of insecticides available is limited to those with SWD activity and sufficiently short preharvest intervals (≤3 days) to allow their use on frequently picked crops such as blueberries. The most effective insecticides available for use against SWD are primarily broad-spectrum chemicals including organophosphates, pyrethroids, and spinosyns (13, 14, 15, 16), the use of which is further complicated by annual application restrictions, pre harvest intervals, and trade related issues with residue tolerances.

The zero tolerance policy for SWD by marketers has led growers to make calendar day weekly insecticide applications, which are reapplied if feasible in the event of rain, resulting in as many as twice weekly applications. The repeated applications with the similar broad-spectrum materials could lead to resistance development in SWD rather quickly, compromising the useful life span of these products, and threaten the sustainability of SWD management programs. Insecticide resistance development in SWD is also one of the grave concerns because SWD is closely related to *D. melanogaster* (17) which has been shown to develop insecticide resistance at a much faster rate than predicted by earlier population genetic models (18). A significant level of resistance to permethrin in a field population of SWD has already been reported (19). Moreover, the fact that Georgia blueberries have the longest production season than any other state in the US (starting mid-April until end of July) indicates that the likelihood of resistance development in SWD populations in

Georgia is much higher.

The total cost of insecticide resistance is difficult to assess, but the loss of insecticide effectiveness directly results in increased application frequencies and dosages, and the cost of switching to more expensive replacement compounds. The continued development of new compounds to replace the old ineffective ones as a result of resistance development has already placed agriculture on a pesticide treadmill. Efforts to manage pest populations on sustainable basis, and our ability to predict genetic changes caused by resistance have been limited by our understanding of the genetic basis of insecticide resistance (20), which is crucial for the development of tactics to slow the evolution of resistance. In this situation, characterizing resistance in various field populations could be extremely valuable for SWD management programs by detecting potential problem of resistance at an earlier stage, thereby allowing growers to change their SWD control strategies and slow the spread of resistance.

The successful management of insecticide resistance depends ultimately on a thorough understanding of the resistance mechanisms involved at the biochemical and molecular level which is extremely important for both fundamental and applied research related to pest management. Resistance management may not be possible without complete assessment of genetic, ecological, as well as operational factors associated with resistance. Strategies to manage resistance are usually developed after it has occurred in the field, which is too late. On the other hand, understanding of genetic, biochemical and molecular basis of resistance before its occurrence in the field would be a proactive approach, and could be extremely valuable in developing strategies to manage susceptibility leading to delay the development of resistance. Because frequent use insecticides to control SWD is a fairly recent phenomenon in the Southeast (2010-2011), this is the best time for us to be proactive and generate as much information as possible regarding current levels of resistance, cross-resistance, and underlying mechanisms; and utilize that information to design sustainable IPM programs for SWD not only in blueberries but also other small fruits in the Southeastern United States.

Therefore, in this project we propose to assess the current levels of resistance and cross-resistance in SWD populations, genetic potential of SWD to develop resistance, and biochemical and molecular basis of underlying mechanisms. This information will not only allow growers to detect the occurrence of resistance in SWD at early stages of its development, but also help them to use effective insecticides in a way that will minimize selection pressure on SWD populations leading to delay the resistance evolution as much as possible, thereby providing for successful control of SWD on a more sustainable basis.

Materials and Methods: We standardized bioassay protocols to test susceptibility of adult SWD to the selected insecticides. For insecticides with contact activity, a residual bioassay protocol was developed in which formulated insecticides were used. Appropriate amount of each insecticide was diluted in acetone or deionized water to prepare 100 mL of stock solution. A series of dilutions at desired concentrations were prepared for each insecticide. The bioassay chambers (225 mL glass jars) and their lids were labeled with appropriate dilution using paper tape. One milliliter of appropriate insecticide dilution was added to each of the pre-labeled glass jar. The lids were put back on the corresponding glass jars. The glass jars were swirled and inverted to insure that all surfaces inside the bottle are coated with insecticide residues. The glass jars were opened and placed inside fume hood to dry. While glass jars were drying in the fume hood, 5-7 d old SWD females were aspirated from the vials used to maintain SWD colonies in the laboratory into 22 mL glass vials. Once glass jars and lids

were completely dry, a set of 10 SWD females was transferred to each of the pre-treated glass jars. The glass jars treated with acetone or deionized water served as control. Fly mortality was evaluated after 2-6 hours of exposure depending on nature of the insecticides tested. All bioassays were replicated 3-5 times. Median lethal concentration (LC_{50}) values were estimated using probit option of the POLO software (21).

Bioassays, Sample Preparation, Total RNA Extraction, and Transcriptome Sequencing: Based on results of preliminary bioassays, 5-7 d old female SWD from a field population collected from Pierce County, GA and a laboratory colony were treated with spinosad, zeta-cypermethrin, and malathion at LC_{50}. A total of 10 survivors were collected from both treated and untreated groups and immediately stored at -80 °C. All bioassays were replicated three times. Total RNA was extracted using TRIzol reagent (Ambion) and quantified with NanoDrop Spectrophotometer (N-1000). KAPA Stranded mRNA-Seq Kit (KK8420) was used to create paired-end sequencing libraries with an average insert length of around ~300 bp. Transcriptome libraries were sequenced using 75bp paired-end Illumina NextSeq 500 platform at the Georgia Genomic Facility at the University of Georgia.

Transcriptome Assembly and Quality Control: Raw reads for each replicate were aligned independently to the SWD reference genome (SpottedWingFlybase v.1) using the Bowtie-based TopHat program. Pearson's correlation was calculated using the cor() function in R, and the distribution plots of FPKM (Fragments Per Kilobase per Million mapped reads) values between biological replicates were generated using ggplot2 and cummeRbund R libraries.

Differential gene expression analysis: We used Cufflinks to calculate the expression value FPKM with the Cuffdiff 2 default geometric normalization and identified differentially expressed genes ($P < 0.05$ and the $FDR < 0.05$ after Benjamini-Hochberg correction for multiple-testing) between two sets of samples: (1) lab-reared populations that are treated or untreated; and (2) field-collected populations that are treated or untreated.

Results and Discussion:

Transcriptome overview: A total of 12,490 genes were found to be expressed in at least one of the replicates. Estimates of gene abundance (FPKM) were highly correlated across biological replicates, however, biological replicates for field-collected populations showed higher variance as compared to the lab-reared populations indicating more genetic variation and thus higher potential for adaptation to environmental stressors such as insecticides.

Analysis of differentially expressed metabolic detoxification enzyme genes: A total of 2791 genes were up-regulated in malathion, spinosad, and zet-cypermethrin treated field population of which 1223 genes were common among malathion, spinosad, and zeta-cypermethrin treated SWD. In contrast, only 317 genes were up-regulated in the lab population of which 29 genes were common among all three insecticides (Fig. 1a).

For lab-reared SWD ((Fig. 1b-e)), only one and four CYP genes were significantly up-regulated after spinosad and zeta-cypermethrin treatment, respectively (False discovery rate – $FDR < 0.05$). There was only one UGT gene up-regulated after treatment with malathion and spinosad, and two UGT genes were up-regulated after treatment with zeta-cypermethrin, respectively, whereas none of the GST or CES genes were up-regulated. Unlike lab-reared SWD, the number of detoxification enzyme genes up-regulated in field-collected SWD after similar treatments was substantially higher. A total of

40 CYP genes were up-regulated in malathion, spinosad and zeta-cypermethrin treated SWD, of which 12 genes were common in all treatments (Fig. 1b). Likewise, a total of seven UGT genes were up-regulated in malathion, spinosad and zeta-cypermethrin treated SWD, respectively, of which two genes were common in all treatments (Fig. 1c). A total of eight GST genes were up-regulated after malathion, spinosad, and zeta-cypermethrin treatment of which three genes were common in all treatments (Fig. 1d). A total of six CES genes were up-regulated of which five genes were common in both spinosad and zeta-cypermethrin treatments and none of the CES genes were up-regulated in response to malathion treatment (Fig. 1e).

Overall, up-regulation of substantially higher number of detoxification enzyme genes in field-collected SWD than lab-reared SWD in response to malathion, spinosad and zeta-cypermethrin treatment indicates that SWD field population has much higher potential to detoxify these insecticides and develop resistance. Based on the number of genes up-regulated after treatment with malathion, spinosad and zeta-cypermethrin, cytochrome P-450 monooxygenases are likely the primary detoxification mechanism for malathion (organophosphate), spinosad (spinosyn) and zeta-cypermethrin (pyrethroid) in field-collected SWD. However, other families of metabolic enzymes such as UGTs and GSTs may have a secondary role in detoxification of these insecticides. Furthermore, the fact that majority of the up-regulated detoxification enzyme genes were common in all treatments indicates the potential risk of cross-resistance between these insecticides which threatens the sustainability of current SWD management programs in both conventional and organic production systems.

Conclusions:

The differential expression of metabolic detoxification enzyme genes in field-collected SWD as compared to the laboratory-reared SWD indicates significant risk of resistance evolution in field populations of SWD. These transcriptomes not only give us information about metabolic enzymes that are inducible with specific insecticide treatments and therefore might be involved as mechanisms of resistance, but also serve as the baseline that will be used to compare similar data from the field-evolved and lab-selected resistant SWD populations in the future. However, it is extremely important for us to be proactive and characterize specific resistance mechanisms in SWD by selecting for resistance in the lab and testing field populations, identify specific genetic changes/mutations, and develop molecular markers to screen SWD for resistance. It will help small and stone fruit producers detect resistance at an earlier stage of its development, and implement scientific knowledge based strategies in a timely manner to slow/delay the development of resistance in this devastating pest throughout the United States. Using these results as preliminary data, we submitted a proposal (currently being review by NIFA) to solicit funding through AFRI Foundational Program to pursue this work further. Now that we have standardized protocols and sequenced baseline transcriptome, we plan to continue this work and provide growers with more specific information on resistance mechanisms in SWD field populations and help them develop effective resistance management strategies.

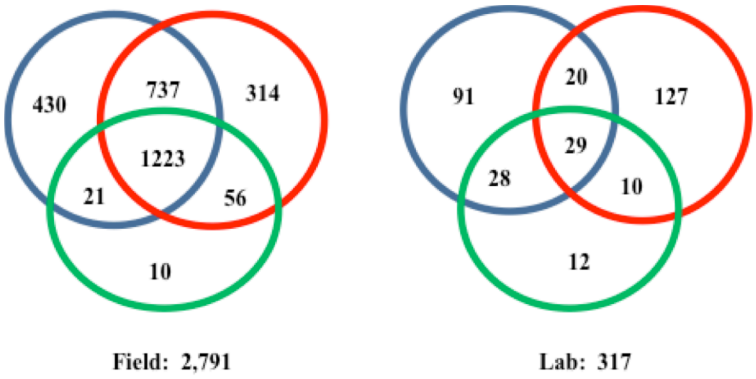


Fig. 1a: Venn diagrams showing total number of genes up-regulated in field-collected and laboratory-reared SWD after treatment with malathion, spinosad and zeta-cypermethrin. [O = Malathion, O = Spinosad =, and O = Zeta-cypermethrin]

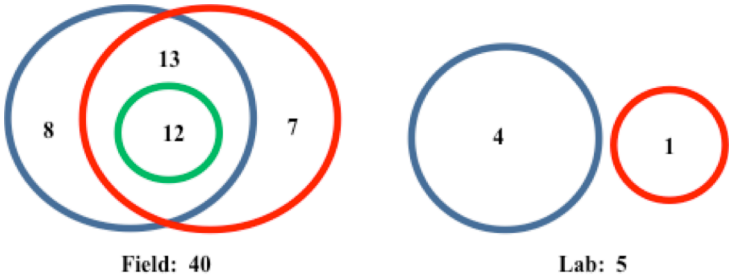


Fig. 1b: Venn diagrams showing number of cytochrome P-450 monooxygenase (*CYP*) genes up-regulated in field-collected and laboratory-reared SWD after treatment with malathion, spinosad and zeta-cypermethrin. [O = Malathion, O = Spinosad =, and O = Zeta-cypermethrin]

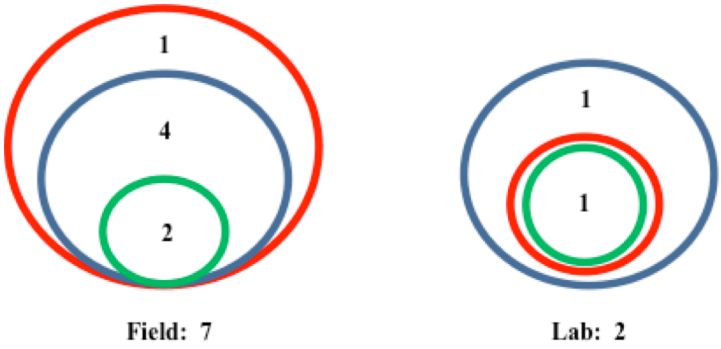
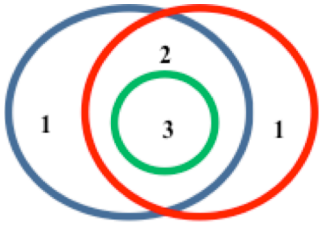
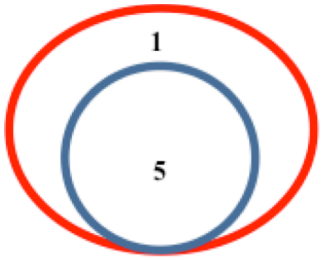


Fig. 1c: Venn diagrams showing number of UDP Glucuronocyltransferease (*UGT*) genes up-regulated in field-collected and laboratory-reared SWD after treatment with malathion, spinosad and zeta-cypermethrin. [O = Malathion, O = Spinosad =, and O = Zeta-cypermethrin]



Field: 7 Lab: 0

Fig. 1d: Venn diagrams showing number of Glutathion-S-Transferase (*GST*) genes up-regulated in field-collected and laboratory-reared SWD after treatment with malathion, spinosad and zeta-cypermethrin. [O = Malathion, O = Spinosad =, and O = Zeta-cypermethrin]



Field: 6 Lab: 0

Fig. 1e: Venn diagrams showing number of Carboxylesterase (*CES*) genes up-regulated in field-collected and laboratory-reared SWD after treatment with malathion, spinosad and zeta-cypermethrin. [O = Malathion, O = Spinosad =, and O = Zeta-cypermethrin]

Impact Statement:

For the first time, these findings provide scientific evidence to believe that the risk of insecticide resistance and cross-resistance in SWD is significant. These results will be presented at grower meetings to emphasize the need for implementation of resistance management strategies, such as rotation of insecticides from different mode of action classes which will help slow the process of resistance development in the field populations and delay the onset of control failures associated with insecticide resistance.

Publications arising from this project:

We would like to acknowledge significant support (in terms of technical expertise – bioinformatics) provided by Ruchir Mishra and Dr. Mike Adang (Department of Entomology, UGA), and Joanna Chiu (Department of Entomology, UC Davis). We are currently working on a research paper which will be submitted for publication in PLOS Genetics.

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