Spotted Wing Drosophila 2019 Progress Report

Project Title: Engineered transgenic *Drosophila suzukii* for wild population suppression and eradication.

Principal Investigator
Omar S. Akbari, University of California, San Diego

OBJECTIVES (2019)

Objective A- *Medea*-based drive system:

Previously, we have described a functional population replacement system in *D. suzukii* termed *Medea*, and have shown that it is capable of working in diverse genetic backgrounds and of maintaining itself at high frequencies in a population. We have now molecularly developed a second-generation optimized *Medea* system that can spread to fixation quickly and can be used to replace the first population should a recall ever be necessary, and are working on testing this system in flies (to be completed within a year). We are also working, in collaboration with the Montell Lab at UCSB, to test effector genes capable of bringing about conditional lethality in *D. melanogaster*, which should also be completed within a year. Once such genes are characterized, we can then link them with our optimized *Medea* system to generate a fully functional gene drive system capable of population suppression.

Objective B- CRISPR/Cas9-based drives:

Y-drive: We are also working on engineering a second type of suppression system termed Y-drive that relies on CRISPR/Cas9 to bias sex ratios by shredding the X chromosome, leading to an all-male population crash. We have already developed several components required to generate Y-drive: we have engineered multiple Cas9 strains, optimized transgenic gRNA designs, demonstrated efficient CRISPR/Cas9 function in *D. suzukii*, and developed a method to dock transgenes on the Y chromosome of flies[16]. We have also been testing X chromosome-targeting gRNAs in *D. melanogaster*. We now plan to combine these components to attempt to generate *D. suzukii* gRNA transgenes capable of shredding the X chromosome, and to express said transgenes from the Y chromosome.

Homing drive: We propose to engineer a Cas-9-mediated suppression homing drive. This system has a self-replicating (i.e., homing) Cas9-based transgene that targets a region within a gene necessary for female fertility, which over time would facilitate the sterilization of all females in a target population thereby resulting in a population collapse. 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 to generate a self-replicating transgene that could continuously mutate a target gene every generation and/or carry a transgene into the population. We have identified and characterized several promising candidate target genes, including *dsx, tra*, and *sxl*, which are all essential for female development in fruit flies.

Objective C- pgSIT:

We propose to create a precision guided sterile insect technique SIT (pgSIT) as a new, genetic-based methods functions by exploiting the precision and accuracy of CRISPR to simultaneously disrupt genes essential for either female viability or male fertility. It utilizes a simple breeding scheme requiring two homozygous strains - one expressing Cas9 and the other

expressing double guide RNAs (dgRNAs) (Figure 9). A single mating between these strains mechanistically results in synchronous RNA-guided dominant biallelic knockouts of both target genes throughout development, resulting in the complete penetrance of desired phenotypes in all progeny.

PROGRESS TO DATE

I. Objective A - *Medea*-based drive system

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 last 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. We are currently finishing the crosses to confirm reduced resistance in the new design. 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 have started testing them in *D. suzukii*.

Summary of Objective A:

A. We have developed a modified version of our original *Medea* system that is designed to reduce resistance to the drive. 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 are currently rigorously testing this second-generation *Medea* element and planning for longer term population cage studies.

B. We have 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. 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. We are currently testing this system and planning for longer term population cage studies.

C. We have identified several promising putative cargo genes that could be spread with the *Medea* gene drive to cause population suppression. 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*. However, multiple genes have been tested in *D. melanogaster* as proof of principle and are now being transitioned to *D. suzukii*

II. Objective B- CRISPR/Cas9-based drives

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*.

A. Last year, we developed and characterized multiple Cas9 transgenes in *D. suzukii* that are highly functional and enable efficient Cas9-mediated mutagenesis. So far this year, we have expanded and optimized multiple Cas9 expression lines with female germline specific promoters (*BicC* and *Dhd*), male and female germline specific promoters (*vasa* and *nanos*) or strong full body expression (*ubiq*).
B. Last year, we developed several ways to efficiently express gRNAs from the *D. suzukii* genome and we have used these systems to build and evaluate multiple gRNA expression systems. Now that we have a highly functional gRNA expression configuration, we have started to clone X chromosome-targeting gRNAs into our gRNA expression cassettes and test them.

C. Previously, we developed/optimized several components needed to build Y-gene drive including identifying X and Y chromosome regions, putative X chromosome specific target sites and we have efficiently engineered the Y chromosome of flies. We are now testing whether we can reliably 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.

D. We have developed/optimized several components needed to build Cas9-based suppression gene drive, including:

1. Building a proof of principle Cas9-based homing system in the *white* gene to test its ability to self-replicate. Specifically, we demonstrated that a genetically 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. These experiments allowed us to determine whether we can dock transgenes in a site-specific location using CRISPR/Cas9 and observe the efficiency of self-replication/homing of this Cas9-based transgene in *D. suzukii*.

2. We have designed and generated multiple gRNA-expressing transgenes targeting one or more essential genes (threshold dependent split drive) and dual gRNA and Cas9 expression lines (full drive). We are expanding these lines and have started testing their drive capabilities in small population studies. Our preliminary full and split suppression drives targeting the *doublesex* (*dsx*) gene have a reduced female to male ratio, but not all females are eliminated from the population; however, the viable females were all intersex and sterile, so these results may still be useful for a suppression. We are testing these lines further to determine the reproducibility of these results and we plan to optimize these lines and our others to achieve the female killing phenotype.

III. Objective C- pgSIT

In order to construct a pgSIT system, we previously created functional Cas9 tools (including gRNA lines that target genes essential for female viability and male sterility and Cas9 expressing lines) in *D. suzukii*. Also essential to building a pgSIT system are guide RNA (gRNA) lines that target genes essential for female viability and male fertility. We have previously identified genes essential for female viability or male fertility in *D. melanogaster*, and have shown that disrupting these genes via CRISPR/Cas9 produces the desired results (e.g., female death or conversion of females into sterile intersex individuals for the former group, male sterility for the latter. Since *D. melanogaster* is closely related to *D. suzukii*, we chose to evaluate whether targeting these same genes in *D. suzukii* would have a similar result. Specifically, we are building constructs to disrupt female viability by targeting several sex-specifically alternatively spliced sex-determination genes including *sex lethal* (sxl), *transformer* (tra), and *doublesex* (dsxF), as well as *zero population growth* (zpg), a germline-specific gap junction gene. We have identified *D. suzukii* homologues of all of these genes, and have carefully selected two gRNA target sites in each gene that are highly conserved and thus unlikely to harbor sequence variation. We have constructed double-gRNA transgenes targeting each candidate gene, and have begun generating and testing *D. suzukii* lines harboring these transgenes. Each of these lines are being crossed separately to our Cas9 strains to see whether the combinations of Cas9+gRNA will produce female lethality and male sterility. As soon as we identify the gRNA sets that produce the desired phenotypes, we can proceed to

combine best sets of gRNAs to produce a single transgene that crossed with a Cas9 strain kills female and sterilize male progeny.

A. Designed (>20) and injected (>10) constructs that express gRNAs targeting the female viability genes and *beta tubulin* (***β-tub***), a male fertility gene. We are expanding these lines and will test them in crosses to multiple Cas9 expression lines to determine the most efficient gRNA and Cas9 line combinations to generate sterile male progeny.
B. Established six transgenic gRNA lines targeting both *sxl* and ***β-tub*** simultaneously; Exciting preliminary results indicate that there are **no female transheterozygote progeny** from gRNA and vasa-Cas9 lines crosses (Table 1), which indicates females that inherit these transgenes are killed as we expected!!!! Additionally, when the transheterzygote progeny were crossed with wildtype (WT) females, as expected they **produced no viable progeny** (Table 2). Taken together, these data strongly indicate that we may **have a functioning pgSIT system in *D. suzukii***, however we need to continue to assess these lines in more replicates and also expand and homozygose these lines and measure fitness and mating competitiveness.

Table 1	**1056H L.3 ♂ x vas-Cas9 ♀**	**1056J L.2 ♂ x vas-Cas9 ♀**	**1056J L.3 ♂ x vas-Cas9 ♀**
WT M	36	16	34
WT F	-	-	-
Inherited 1056 M	3	12	12
Inherited 1056 F	-	-	-
Inherited Cas9 M	77	63	62
Inherited Cas9 F	-	-	-
Transhet Females	0	0	0
Intersex	2	0	3
Transhet Males	**23**	**42**	**9**
n=	141	133	120

Table 1. Preliminary results from vasa-Cas9 and *sxl* and *β-tub* gRNA line crosses demonstrate female killing. The table depicts the number of resulting progeny of crosses between three different gRNA lines that simultaneously target *sxl* and *β-tub* (genes required for female viability and male fertility, respectively) and a vasa-Cas9 line. The highlighted row shows that no transheterozygous (transhet) females were generated from these crosses demonstrating that females inheriting both transgenes are efficiently killed.

Table 2: Transhet Males x WT Females Embryo Counts					
Parental Genotype		**Replicate 1**			
		Genetic Cross			
Female	***Male***	***# of WT Females***	***# of transhet Males***	***Embryo Count***	***Emerged***
WT	**1056H L.3 ; vasa-Cas9**	15	5	385	0
WT	**1056J L.2 ; vasa-Cas9**	15	5	364	0
WT	**1056J L.3 ; vasa-Cas9**	15	5	in progress	in progress
WT	**1056J L.5 ; vasa-Cas9**	15	5	in progress	in progress
WT	**1056K L.1 ; vasa-Cas9**	15	5	194	0
WT	**1056K L.3 ; vasa-Cas9**	15	5	279	0

Table 2. Preliminary results from crosses between male transheterozygote progeny and wildtype (WT) females demonstrate male sterility. The transheterozygote male progeny generated from crosses between the gRNA and Cas9 lines (e.g. Table 1) were crossed to WT females to assess male fertility. No males resulted from these crosses (highlighted column) indicating that the male progeny are sterile.