FINAL PROJECT REPORT

Project Title: Non-toxic RNAi-based biopesticide to control SWD

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Other funding sources
Agency Name: OBC, WBC, ORBC, WRRC, ARF
Amount awarded: $120,000

Total Project Funding: $140,400

Budget History:

Item	Year 1: 2017	Year 2: 2018	Year 3: 2019
Salaries[1]	$25,000	$25,750	$25,750
Benefits[2]	$4,380	$4,510	$4,510
Wages[3]	$6,000	$12,000	$12,000
Equipment	$0	$0	$0
Materials & Supplies[4]	$8,000	$5,500	$5,500
Travel[5]	$500	$500	$500
Plot Fees	$0	$0	$0
Total	$43,880	$48,260	$48,260

Footnotes: USDA ARS in-house fund supports for equipment, facilities and supplies for RNAi project. [1]Salaries & [2]Benefit, 0.5 FTE Postdoc; [3]Wage, student assistant, $12/h x 20/w@4x6m & OPE $480; [4]Materialsand Supplies Molecular biology materials & supplies, RNAi materials & supplies (relatively expensive), and Insect rearing materials and supplies; [5]Travel, PI and/or postdoc to attend a cherry commission & grower meetings each year.

OBJECTIVES

Objective of this research is the development of a novel environmentally-friendly control that is non-toxic insecticide and non-transgenic strategy to control SWD as well as other potential cherry pests. Although RNAi technology is a new insight and promising tool for insect pest management, there are still technical huddles remaining to successfully develop a next generation pesticide. RNAi approach to pest management consider three major challenges: 1) selection and identification of suitable target genes and/or physiological system with high level of gene silencing, 2) cost effective RNAi material production, and 3) development of a suitable delivery method into target pest. A large scale production of RNAi in vitro using kits is too expensive, and not a practical approach for growers (#2). Therefore, there is required a mass production system to synthesis dsRNA through a microbial-based process such as a bacterial-based dsRNA production provides more practical application. To solve this problem, we have established a mass production system using a microbial-induced dsRNA production to increase the feasibility of RNAi application for SWD control (see previous studies and preliminary data 2). To control SWD the strategy of our RNAi approach is non-planted incorporated delivery method such as spray and/or bait-station application (#3).

In the present proposal, therefore we focus on the screening and identification of suitable RNAi target(s) from SWD (#1). A feasible approach for RNAi target gene screening is to search previous targets or systems observed already from same or similar insect groups. Another cost-effective approach is to search RNAi targets from known functional gene analysis and RNA mechanism studies from model insects to increase likelihood of success from the initial stage. Therefore, the screening strategy to identify appropriate RNAi target genes is particularly important. A feasible approach for RNAi target gene screening is to search previous targets or systems observed already from same or similar insect groups.

Our approach for RNAi target gene screening is based on our current RNAi research and previous RNAi results. We recently started the screening of RNAi candidates from SWD, and currently evaluate their impacts through various bioassay. In this proposal we continue to screen more target genes selected from SWD, and evaluate and identify suitable RNAi targets. To achieve this goal the following specific objectives need to be accomplished in this project:

1. Cloning and identify potential RNAi target genes from SWD
2. Construct, design and biosynthesis dsRNAs for target genes
3. Screen for efficacy using bioassay to measure RNAi impacts on SWD
   3-1. Inject dsRNA into adult flies and monitor RNAi impacts
   3-2. Feed dsRNA to larvae and adults, and evaluate RNAi impacts

SIGNIFICANT FINDINGS

- Selected, identified 32 genes, and constructed double-stranded RNAs (dsRNAs) for SWD RNAi
- Screened 3 housekeeping and 3 receptor for potential RNAi targets
- Established a SWD specific nanoinjection system
- Evaluated RNAi impacts by injection and in vitro (cell lines)
- Found the activity of dsRNases in the SWD mid-gut

RESULTS & DISCUSSION

For optimal impact of dsRNA delivered to target cells through feeding, RNAi target genes should focus on systemic RNAi if dsRNA can be internalized into the target cells through feeding. We have selected 32 RNAi targets based on the previous studies for insect RNAi targets and biological functions. These target genes include essential housekeeping genes that are required for the maintenance of basic cellular functions, neuropeptide (NP) hormones and GPCRs for SWD life stages.

1. SWD RNAi target genes: The 32 SWD genes include 17 housekeeping genes, 10 neurohormone receptor genes, 3 neurohormone genes, and 2 chemosensory genes, for potential SWD RNAi targets (Table 1).

Table 1. SWD RNAi candidate genes, GFP, and nucleotide lengths of dsRNAs.

RNAi candidates	DNA template for RNAi synthesis	Gene family
SWD ID1	296 nucleotides	Neurohormone
SWD ID2	195 nucleotides	Neurohormone
SWD ID3	399 nucleotides	Hormone receptor
SWD ID4	244 nucleotides	Housekeeping
SWD ID5	253 nucleotides	Housekeeping
SWD ID6	255 nucleotides	Housekeeping
SWD ID7	253 nucleotides	Housekeeping
SWD ID8	250 nucleotides	Housekeeping
SWD ID9	251 nucleotides	Housekeeping
SWD ID10	254 nucleotides	Housekeeping
SWD ID11	254 nucleotides	Housekeeping
SWD ID12	250 nucleotides	Neurohormone
SWD ID13	299 nucleotides	Hormone receptor
SWD ID14	377 nucleotides	Housekeeping
SWD ID15	374 nucleotides	Housekeeping
SWD ID16	325 nucleotides	Hormone receptor
SWD ID17	299 nucleotides	Hormone receptor
SWD ID18	315 nucleotides	Hormone receptor
SWD ID19	325 nucleotides	Hormone receptor
SWD ID20	261 nucleotides	Hormone receptor
SWD ID21	362 nucleotides	Hormone receptor
SWD ID22	240 nucleotides	Hormone receptor
SWD ID23	360 nucleotides	Hormone receptor
SWD ID24	363 nucleotides	Housekeeping
SWD ID25	308 nucleotides	Housekeeping
SWD ID26	200 nucleotides	Housekeeping
SWD ID27	378 nucleotides	Housekeeping
SWD ID28	200 nucleotides	Housekeeping
SWD ID29	200 nucleotides	Housekeeping
SWD ID30	240 nucleotides	Housekeeping
SWD ID31	250 nucleotides	Chemosensory
SWD ID32	240 nucleotides	Chemosensory
GFP	350 nucleotides	Control, unrelated gene

Some of genes identified in this study were very different nucleotide sequences from the gene sequences published on the SWD genome data. Housekeeping genes as constitutive genes are expressed in all cell types at a level that does not fluctuate with the cell cycle. Functional examples of housekeeping genes for RNAi targets are related in the muscle physiology, detoxification, ATP metabolism, protein sorting and transporting, and cell membrane structure in cells.

For neurohormone receptor genes we particularly focused on G-protein coupled receptors (GPCRs), 7-transmembrane receptors, because the receptors are belonged in the largest superfamily of integral cell membrane proteins and activated for crucial roles of wide physiological processes including behavior, olfactory, hormones, reproduction and development through the life stages. Therefore, GPCRs have great potential for RNAi targets. Five SWD specific neurohormone and chemosensory genes were identified for RNAi candidates.

2. Designed, and constructed double-stranded RNA (dsRNA)

The dsRNAs for each SWD genes were designed and synthesized approximately 250 nucleotides from the parent genes (Fig. 1).

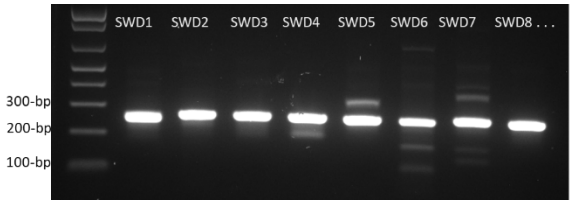

Figure 1. Examples of dsRNA synthesized for SWD RNAi test on injection and feeding.

3. Screen for efficacy using bioassay to measure RNAi impacts on SWD

3-1. Inject dsRNA into adult flies and monitor RNAi impacts: In this project, total 32 RNAi candidates plus two controls were injected over 4,000 flies for the project period (Fig. 2). This system is particularly important to inject a nano-liter (50nL = 0.05μL) of solution into small insects such as SWD with minimal or no physical damage to the fly. After injecting a sham or water control, we found that most SWD adults (> 90%) were not physically affected and survived at least for two weeks. Although individual RNAi injection into SWD is not a practical approach, it is the best and fastest method to screen RNAi targets from impacts on pheno-and genotypic effects. In this initial screening, dsRNA is injected into the hemocoel (= blood vessel). The injection system developed in this research is especially important for adult flies.

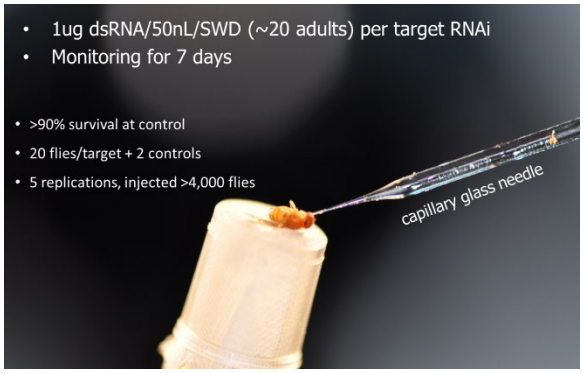

Figure 2. Photos of microinjection system equipped with micromanipulator and a vacuumed tubed plate, and SWD adult injected by a capillary glass needle.

Phenotypic impact of RNAi in SWD adults:

Phenotypic impacts after dsRNA injection into the flies, showed mainly mortality, and some of flies have recovered within few hours (Figs. 3 and 4). We found the maximum mortality up to about 60% on SWD flies injected with the specific dsRNA within 48h (Fig. 4).

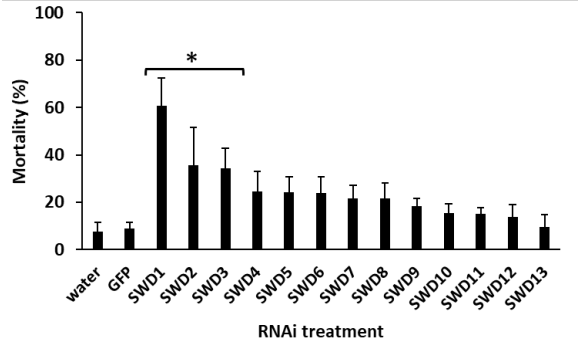

Figure 3. SWD mortality after injected dsRNA (1ug/fly) for 48 hrs. Replicated five times at least.

Figure 4. Photos of dead SWD flies after RNAi injected into adults within 48h.

Genotypic impact of RNAi in SWD adults: We narrowed down three housekeeping genes (SWD1, SWD2, SWD3), and further investigated the gene expression levels to find whether those genes are being suppressed or not after target RNAi (dsRNA) injected into SWD. Using a quantitative real-time PCR (qRT-PCR) three target genes were analyzed for the gene expressions. The result showed three RNAi target genes have been knock downed by dsRNA introduction to SWD (Fig. 5). The reduced

expressions of the genes were resulted from 33% to 67%, SWD3 was the highest genotypic impact, followed SWD1, and SWD2. The result is slightly different compare to the phenotypic impacts.

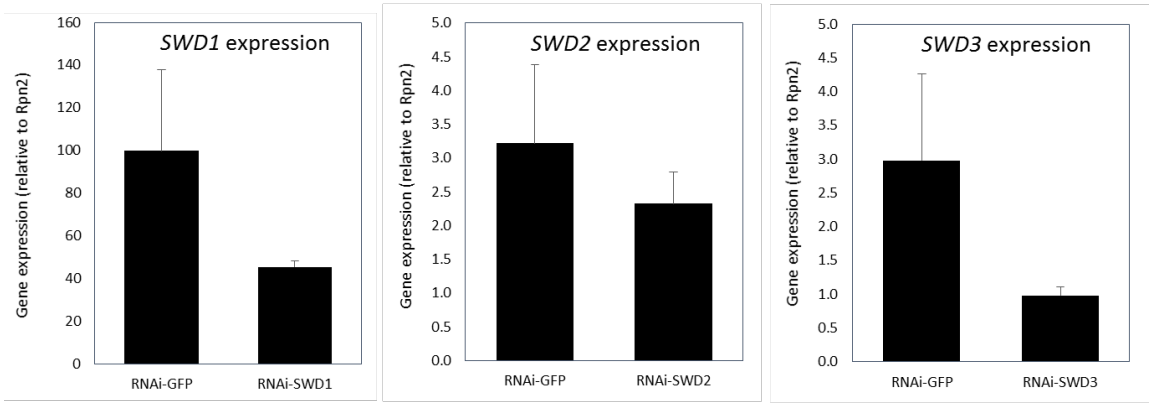

Figure 5. Knock-down of housekeeping genes expression by RNAi. The mRNA expression levels of SWD1, SWD2, and SWD3 were compared between RNAi-GFP and RNAi-target in SWD 12h after dsRNA injection of SWD1, SWD2, SWD3, and GFP. Gene expression estimates are given per a copy of mRNA for the reference gene Rpn2

3-2. RNAi impact of the feeding by SWD adults: SWD1 dsRNA was mixed in the food, and introduced in 4-5-day old adults, then monitored the fly mortality for 7 days (Fig. 6). The result showed no significant between the treatment and control that flies fed sugar solution only. The feeding result was very different from the injection that dsRNA was directly introduced into the fly hemocoel. From two controversial results, we examined RNAi impact *in vitro* system which is using *Drosophila* cell line (S2 cell) because the *in vitro* cells could take dsRNA directly without the midgut membrane barrier (see the section 5).

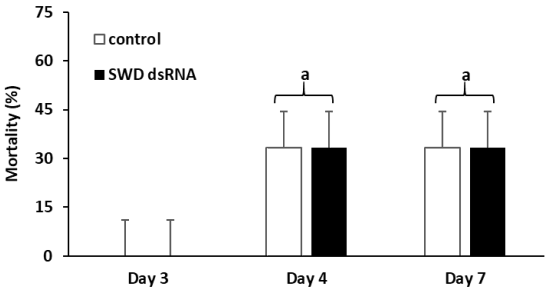

Figure 6. SWD mortality of SWD1 dsRNA-fed flies (1ug/fly) for 7 days. Replicated five times at least.

4. RNAi impact of a neurohormone receptor on SWD adult: Insect GPCR is a great potential target to develop biologically-based insecticide, because GPCRs are key elements to initiate almost all biological signals and physiological functions during insect life stages even the expression levels are very low.

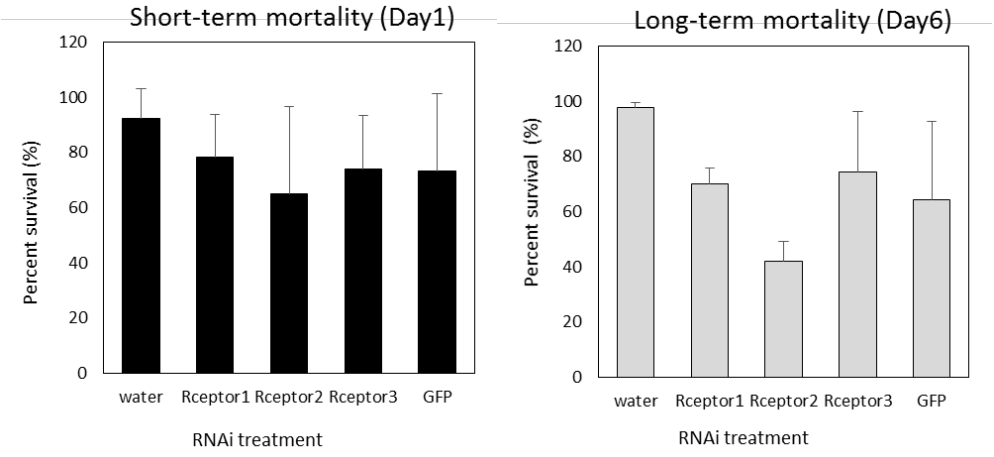

Figure 7. Knock-down of receptor genes expression by RNAi. The mRNA expression levels of Receptor 1, 2 &3 were compared between RNAi-GFP and RNAi-target for SWD 24h (left) and 6 days (right). Gene expression estimates are given per a copy of mRNA for the reference gene Rpn2

Therefore, we examined the gene expression levels of neurohormone receptors after target RNAi (dsRNA) applied to SWD. With qRT-PCR analysis, we found the receptor 2 was significantly suppressed at 48hr after the dsRNA introduced into SWD adults (Fig. 7). The receptor 2 RNAi is currently under more investigation with different bioassays.

5. Effect of RNAi in *Drosophila* cell line: S2 cells (*Drosophila* cells) were incubated, and transferred to a 96-well plate. Then, two amounts of the SWD dsRNAs (1ug – 5ug/well) were applied to the cells, and monitored the cell viability for 24h (Fig. 8). Surprisingly, many cells treated with the dsRNAs showed negative impacts such as cell death and/or decreased cell growth compare to the controls, water or GFP dsRNA treatment.

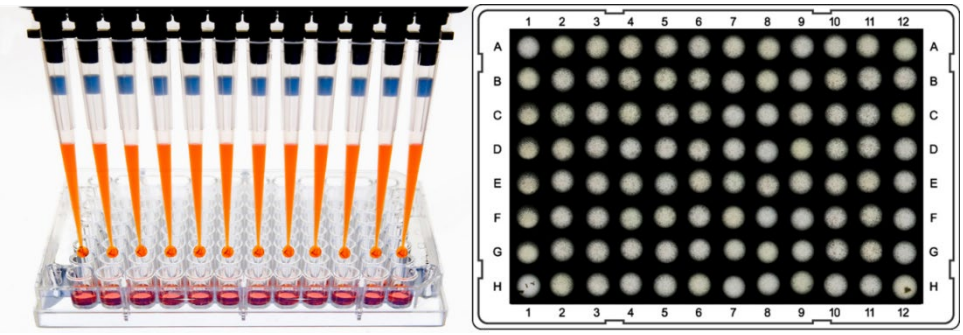

Figure 8. Photo of S2 cells treated with nine different dsRNAs (left) and cell viability after 24h incubation in a 96-well plate (right).

To identify the RNAi genotypic impacts the nine target genes were analyzed their gene expressions by the qRT-PCR. Eight of nine target genes were significantly suppressed with the gene expressions in the cells after the dsRNA treatments (Fig. 9). The phenotypic and genotypic results were consistent from the *in vitro* tests.

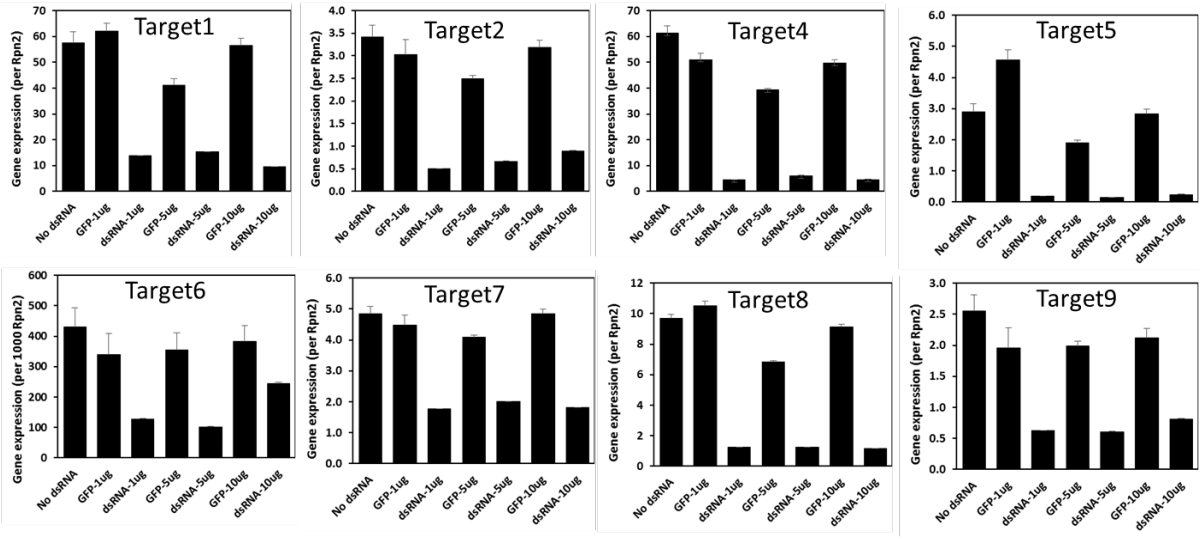

Figure 9. Knock-down of the genes expression by RNAi. The mRNA expression levels of eight genes were compared between RNAi-GFP after dsRNA introduced in the cells. Gene expression estimates are given per a copy of mRNA for the reference gene Rpn2.

From different outcomes from *in vivo* (= feeding) and *in vitro* (= injection and cell bioassays) experiments we particularly targeted the fly midgut because it is the most important component of the

digestive tract for absorbing nutrients from foods. RNAi activation material (= dsRNA) fed to insects should pass through the midgut barrier and then the dsRNA should be taken up by target cells.

6. Found RNase III type enzymes in the SWD mid-gut: We dissected the adult gut, separated the midgut and the other tissue. The tissues were homogenated, and incubated with the GFP dsRNA for 30 min. The amount of the dsRNA incubated with the midgut homogenate was significantly degraded as the RNase III incubation (Fig. 10), but the other gut tissues had no degradation activity (data not shown). The result indicates the midgut juice containing a dsRNA degradation enzyme. Currently, we characterize the enzyme genes, location, and activity in the midgut.

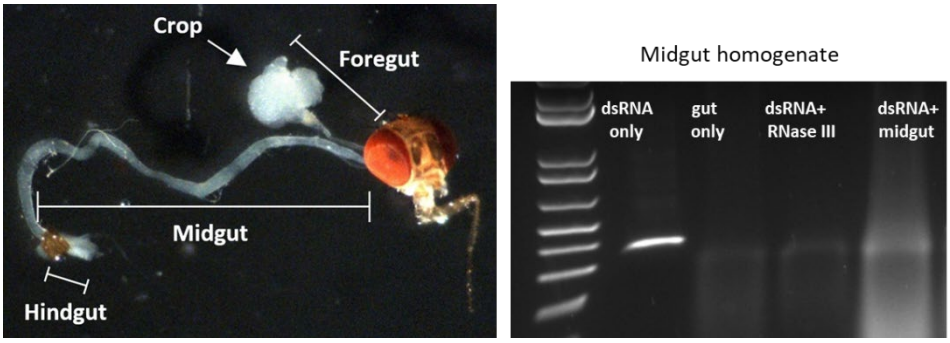

Figure 10. Photo of the adult fly digestive track (left), and gel photo of the GFP dsRNA incubated with RNase III or the midgut homogenate (right) for 30 min at the room temperature.

EXCUTUVE SUMMARY

Project Title: Non-toxic RNAi-based biopesticide to control SWD

Key words: Spotted wing drosophila, *Drosophila suzukii*, RNAi, Biopesticide

Spotted wing drosophila (SWD) is a destructive invasive pest introduced from Asia, and attacks a wide range of ripening fruits. The annual losses have been estimated at $800 million for the soft fruits and cherry industry in US. Recently, SWD management has been ranked a top research priority by small fruit growers. Currently the primary control methods rely on chemical pesticides despite environmental risks, and human health. Therefore, the heavy reliance on chemical insecticides should be replaced or at least complemented with environmentally friendly alternatives. To replace or reduce the use of chemical insecticides, currently alternative options are being developed, but there are still many critical gaps to be implemented against SWD in field.

RNAi for insect control represents a new direction for insect pest control. RNAi is the specific down-regulation or knockdown of gene expression that is a post-transcriptional gene-silencing mechanism insecticide. The mechanism of RNAi is introduced by the delivery of double-stranded RNA (dsRNA) into cells, resulting in interfere target messenger RNA (mRNA) molecules, and subsequently no specific protein produced. During the past decade the application of RNAi techniques has progressed rapidly, and it's becoming a promising pest control tool for chemical insecticide alternative because it poses little or no negative impact on the environment. To develop an RNAi-based biopesticide the major key step is to identify suitable RNAi target(s) from specific pest. Therefore, the selection of effective RNAi target(s) from multiple candidates is critical because RNAi impacts vary depending on insects and genes.

We focused on the identification of suitable RNAi target(s) for SWD. To screen RNAi targets our approach is based on current RNAi research and previous RNAi results. In this project, we proposed specific objectives: 1) cloning and identify potential RNAi target genes from SWD; 2) construct, design and biosynthesis dsRNAs for target genes; and 3) screen for efficacy using bioassay to measure RNAi impacts on SWD. F

In the research, the most significant outcomes are as follows. The first, we screened 32 RNAi candidates including 17 housekeeping genes, 10 G-protein coupled receptor (GPCR) genes, 3 neurohormone genes, and 2 chemosensory genes, from SWD. Second, we constructed all RNAi templates and dsRNAs. Third, we successfully established a specific nanoinjection system for SWD and other flies. Fourth, we screened 3 housekeeping & 3 receptor genes for potential RNAi targets through various bioassays. Unfortunately, the RNAi impacts from the feeding tests that the fly fed the diet or sugar solution mixed with dsRNA, or blueberry fruits sprayed with dsRNA, were not significant compare to the controls without dsRNA. Not like the *in vivo* tests (= feeding), however the *in vitro* test using *Drosophila* cells applied by various dsRNAs showed significant negative impacts such as cell death or decreased cell growth. In this test, eight of nine targets were significantly suppressed the mRNA expressions after dsRNA treatment.

Different RNAi outcomes between *in vivo* (= feeding) and *in vitro* (= injection and cells) results allowed us to focus the fly midgut, particularly, because it is the most important component of the digestive tract for absorbing nutrients from foods. Our RNAi delivery strategy is based on feeding/ingestion options including incorporation into baits for SWD. RNAi activation material (= dsRNA) fed to insects should pass through the midgut barrier and then the dsRNA should be taken up by target cells. We found a digestive activity of RNase III type enzyme (= dsRNA degradation enzyme) in the midgut. There are expected single or multiple enzymes to degrade the dsRNA delivered before pass through the midgut epithelium, so the RNAi impact would be significantly decreased or inactive.

Taken our results from this project suggest, SWD RNAi could be possible to develop a novel RNAi-based biopestide, since no or little Dipteran RNAi has been reported. Future studies should identify and characterize midgut enzymes for effective RNAi delivery through block the enzyme activity or avoid from the enzyme attacking.