Project Title: Novel control of Codling Moth with RNA interference

Report Type: Continuing Project Report

Primary PI: William Walker
Organization: USDA-ARS
Telephone: 509-454-6566
Email: william.walker@usda.gov
Address: Temperate Tree Fruit and Vegetable Research Unit
Address 2: 5230 Konnowac Pass Road
City/State/Zip: Wapato, WA, 98951

Co-PI 2: Rodney Cooper
Organization: USDA-ARS
Telephone: 509-454-4463
Email: rodney.cooper@usda.gov
Address: Temperate Tree Fruit and Vegetable Research Unit
Address 2: 5230 Konnowac Pass Road
City/State/Zip: Wapato, WA, 98951

Cooperators: Dr. Alex Flynt, University of Southern Mississippi, Hattiesburg, Mississippi, USA (Scientific consultant and collaborator on RNA interference in insects)

Project Duration: 3 Year

Total Project Request for Year 1 Funding: $69,317
Total Project Request for Year 2 Funding: $70,703
Total Project Request for Year 3 Funding: $69,680

Other related/associated funding sources: None

WTFRC Collaborative Costs: None

Budget 1
Primary PI: William Walker
Organization Name: USDA-ARS
Contract Administrator: Mara Guttman
Telephone: 510-559-5619
Contract administrator email address: mara.guttman@usda.gov
Station Manager/Supervisor: Rodney Cooper
Station manager/supervisor email address: rodney.cooper@usda.gov

Item	2022	2023	2024
Salaries	$43,683.00	$44,775.00	$45,894.00
Benefits	$13,979.00	$14,328.00	$14,686.00
Wages			
Benefits			
RCA Room Rental			
Shipping			
Supplies	$11,655.00	$11,600.00	$9,100.00
Travel			
Plot Fees			
Miscellaneous			
Total	$69,317.00	$70,703.00	$69,680.00

Footnotes: Salaries and benefits are requested for a full-time GS-7 Lab Technician. Costs for supplies are for molecular reagents for RNAi, materials for transcriptomic sequencing costs, and also for materials for insect colony rearing and experimental bioassays.

OBJECTIVES

Objective 1. Identify candidate target genes for RNAi through transcriptomic analyses.
Comprehensive knowledge of gene expression in the target organism at the appropriate life stages is a pre-requisite for identification of candidate target genes for RNAi-mediated disruption. In the past decade, whole transcriptomic sequencing has emerged as a robust methodology for examining the sum-total of gene expression in a specific biological sample, representative of different life stages or tissue types. Currently, limited transcriptomic information is available for the codling moth. Therefore, using in-house sequencing equipment and codling moth from our colony, transcriptomes will be generated for different larval stages, pupae, adults and embryos. Analysis of these transcriptomes would lead to identification of candidate genes expressed at each stage that would be targeted for disruption with the predicted outcome of codling moth mortality.

Objective 2. Conduct larval feeding bioassays with RNAi effectors combined with various feeding stimulators to optimize potential deliverables.
Results from objective one will directly be channeled into larval feeding assays. Initially, dsRNA molecules targeting identified candidate genes will be mixed with codling moth artificial diet and provided to codling moth larvae with unrestricted access. Since the RNAi effect is mediated primarily through disruption of expression of specific genes, quantitative real-time PCR (qRT-PCR) assays will be conducted in experimentally treated insects relative to controls to assess efficacy of disruption of gene expression for the targeted genes. At the same time, longevity bioassays will be conducted in experimental codling moth specimen across all stages of development relative to non-treated controls to determine which genes, when targeted for disruption by RNAi, yield the most effective impacts on codling moth mortality and development.

Objective 3. Perform controlled laboratory and field trials on efficacy of RNAi in neonate larvae towards preventing codling moth damage in apples.
Once suitable target genes have been identified through RNAi feeding experiments, controlled experiments will be conducted on apple trees at our experimental orchards in Moxee. Larval behavioral modulators have been developed and used to elicit increased codling moth larval feeding before they entire the apple, thereby increasing exposure to materials that are toxic to them. Experiments will thus be conducted with dsRNA provided in combination with the behavioral modulator and experimental feeding stimulants to assess enhancement of external feeding, and thus uptake of dsRNA. Formulations of dsRNA and the modulators, mixed with water, will be applied through spraying the formulations over apple tree rows during periods where codling moth is active in flight. Codling moth damage to apples will be assessed in treated versus untreated/control areas.

SIGNIFICANT FINDINGS

- No RNAi phenotype observed with multiple genes when long double-stranded RNA was overlayed on or mixed into larval diet for feeding uptake by neonate larvae.
- Small RNA transcriptome sequencing of larval tissues reveals candidate “trigger” sequences that may be exploited to induce RNAi effect through an alternate RNAi pathway, called piRNA that may be more viable for codling moth.
- No RNAi phenotype observed with multiple genes when using multiple trigger sequences in attempt to knockdown genes with RNAi through feeding uptake via the piRNA pathway.

METHODS

Objective 1. Identify candidate target genes for RNAi through transcriptomic analyses and injection trials

Procedures: Whole transcriptome datasets will be generated and analyzed for several life stages of codling moth, including early and late embryo, early and late larval instars, pupae and adults. Lead-PI Walker has extensive experience with this approach in entomology research (Walker et al., 2016; Walker et al., 2019; Walker et al., 2023). For each life stage an appropriate amount of individual specimen will be collected to ensure that a sufficient quantity of RNA may be extracted to generate high quality transcriptomes. Codling moth specimen will be taken from our in-house codling moth colony. Standard protocols will be used to extract RNA from all sample types and subsequently prepare sequencing libraries that will serve as substrate for next generation RNA sequencing (RNA-Seq). Sequencing will be conducted in-house with our recently acquired Oxford Nanopore Mk1C sequencer, and the output sequence data will be assessed for quality and arranged into whole transcriptome data sets containing consensus transcripts for each gene that is expressed at each life stage. Bioinformatic analyses will be conducted on output sequence data to assess which genes are expressed and relative expression abundances compared to all other genes in each sample. Further analyses will be conducted to compare codling moth expressed genes to transcriptomic data sets of other related insects to characterize unique and conserved genes in the codling moth.

Expected Results: Comprehensive gene expression data sets will be obtained across all life stages of codling moth. Individual transcriptomes will be generated for each life stage for comparison within codling moth and relative to similar data sets already published on record for other species. It is expected that unique life-stage expression profiles will be observed, with a mixture of genes that are expressed across most or all life stages, as well as genes that are expressed in one or few life stages. These datasets will be thoroughly analyzed relative to what is known in relevant scientific literature and body of knowledge to identify suitable gene targets for RNAi-mediated disruption of expression of vital genes across all life stages. Ideally, the most suitable gene targets will be specific to codling moth and few other species.

Potential Problems and Contingencies: State-of-the-art RNA-Seq methodologies and bioinformatic analyses will be utilized on biological samples taken from our internal codling moth colonies. There is thus a very low risk of substantial problems with this stage of the project. The high volume of data generated for each life-stage transcriptome may indeed be challenging to work with and efficiently analyze and parse out the most useful information. However, numerous optimized bioinformatic pipelines have been developed with which the lead scientists are experienced with, and bioinformaticians and computational scientists within our organization will be consulted with to ensure that best practices are followed. Assessment of the genetic diversity potential of targeted codling moth populations is essential to identify the best gene candidates for RNAi. Given that our laboratory may not contain representative genetic diversity of codling moth across Washington and the Pacific North West region due bottlenecking of genetic diversity and inbreeding rearing conditions, annual infusions into our colony have been made with wild codling moth from local orchards; these infusions will continue in the future.

Time-Plan: Transcriptome sequencing and analysis will be performed during the first six months of the project.

Objective 2. Conduct larval feeding bioassays with RNAi effectors combined with various feeding stimulators

Procedures: Candidate genes identified in the whole transcriptome datasets will be targeted for disruption by delivery of complementary dsRNA effector molecules via larval feeding. Genes will be targeted that are expressed in larval but also pupal, adult, and embryonic stages of life. For these candidate genes, gene-specific dsRNA will be generated synthetically, ordered from one of several commercial sources that supply dsRNA for RNAi experiments. dsRNA will also be generated

corresponding to a jellyfish gene to serve as a negative control to the experimental conditions. Additionally, dsRNA will be generated from template gDNA corresponding to a universal cellular housekeeping gene, inhibitor of apoptosis (IAP), known to be expressed throughout all life stages, and widely across all insects; RNAi against IAP has been shown to induce rapid mortality in a diversity of insects such as mosquitoes (Pridgeon et al., 2008) and plant bugs (Walker and Allen, 2011). Initial RNAi experiments will be conducted targeting disruption of IAP, as a positive control, in order to optimize protocols and methodology (RNAi against IAP would not be expected to serve as an eventual biopesticide target due to its widespread presence across insects and other domains of life such as fungi).

Initial feeding assays will be conducted via topical application of purified dsRNA solution to standard codling moth artificial diet (Wang et al., 2015). To control for effect of dsRNA feeding on insect mortality, control experiments will be performed through feeding of dsRNA targeting disruption of a selected jellyfish-specific gene that would not be present in the codling moth genome. Initially high concentrations of dsRNA will be applied to the food. For targeted genes that result in successful RNAi outcomes, lower concentrations of dsRNA will be assayed as well in order to assess minimum and optimal concentrations for eventual tree fruit trials. Individual neonate larvae will be placed in feeding chambers and allowed to feed unrestricted, while being monitored for growth, development, and mortality.

Throughout the course of the experiments, mortality, time of development, and size/growth will be measured during all life stages to evaluate persistence and effectiveness of RNAi beyond the larval stage. Furthermore, for genes which are observed to be disrupted by RNAi in codling moth feeding on dsRNA, new experiments will be performed in which larvae are given access to dsRNA admixtures that target multiple genes. This will be done to evaluate whether there is increased efficacy by targeting multiple genes for disruption simultaneously. For all experiments, sufficiently many insects will be assayed in order to be able to statistically demonstrate that increased mortality or development inhibition is due to the RNAi effect and not other experimental factors. Subsets of injected insects will be sampled for extraction of RNA and molecular assessment of target-gene disruption using standard qRT-PCR assay under experimental conditions of RNAi disruption versus controls.

Expected Results: Screening of the RNAi effect in insects via feeding dsRNA on artificial diet has been identified as an easy, effective and efficient way to assess large numbers of genes with assays resembling field conditions (Whyard et al., 2009). In codling moth it has been shown that feeding larvae with dsRNA can result in RNAi-mediated gene disruption and larval growth deficits (Wang et al., 2015), so it is expected that this approach will be successful. In experiments where RNAi is successful, disruption of target genes will result in increased mortality or developmental inhibition relative to control treatments. It is expected that there will be a correlation between RNAi phenotype (mortality or developmental inhibition) and reduction or elimination of mRNA of the targeted gene. Based upon the results of these experiments, genes that display mortality or developmental phenotypes correlate to disruption of their mRNA will be selected for further experimentation in Objective 3.

In the previous report on RNAi in codling moth, only larval-expressed genes were targeted via larval feeding on dsRNA (Wang et al., 2015). This objective expands upon those findings by examination of persistence of RNAi beyond the larval stage. While this has never before been examined in codling moth larvae, there is confidence that persistence of RNAi will be observed. In a closely related species of the same tortricid family of moths, the light brown apple month, *Epiphyas postvitanna*, it was observed that in larvae that were fed dsRNA effectors, the RNAi gene-disruption effect persisted for more than two weeks as the larvae progressed through the pupal and into the adult stage (Turner et al., 2006). Moreover, in codling moth injected with dsRNA in the pupal stage, RNAi-mediated gene disruption was observed into the adult stage (Wan et al., 2019).

Potential Problems and Contingencies:

While RNAi has been demonstrated to work in codling moth after delivery of dsRNA via larval feeding, these observations were limited to one gene in one published report from one laboratory, and for which no strong RNAi phenotype was observed. Further research is indeed necessary to optimize the

methodology related to target gene selection, dsRNA dosage, and duration of exposure, among other factors. If positive results are not immediately forthcoming, it may be necessary to confirm the RNAi effect via microinjection of dsRNA across all life stages, as RNAi via microinjections has also been recently reported for codling moth (Wan et al., 2019). This approach would be taken to confirm the efficacy of dsRNA molecules in inducing RNAi in codling moth in order to rule out insufficiency of supplied materials. The aforementioned IAP gene would be used as a control in this case. Embryonic injections of dsRNA would be performed using same methods as done for CRISPR experiments in codling moth (Garczynski et al., 2017). Larval, pupal and adult injections would be made into the midgut region as described for codling moth (Wan et al., 2019) and other insects (Walker et al., 2010, 2011).

It is well known that when attempting RNAi, not all genes may be disrupted equally, and some genes may not be disrupted at all. Furthermore, some targeted genes may not be disrupted sufficiently to result in a predicted phenotype, such as mortality in this case. Concordantly, for this project, candidate genes will be selected based upon the hypothesis that RNAi-mediated disruption of these genes will result in codling moth mortality or developmental inhibition, based upon what is generally known about the function of these genes. However, it is possible that even if RNAi mediated knockdown is achieved, there will not be increased/sufficient mortality observed. This may be expected due to biological complexities such as genetic redundancies (multiple genes provide similar functions) or species-specific gene functions in codling moth that diverge from hypothesized expectations. In consideration of these potential problems, multiple genes will be targeted for each life stage, and for each gene, multiple regions will be selected to serve as gDNA template to generate a diversity of dsRNA effector molecule types.

The optimal goal is to utilize RNAi to disrupt gene expression and induce mortality or arrested development in codling moth larvae before they enter the apple. This would be mediated through uptake of dsRNA molecules that codling moth larvae have ingested through feeding on leaf and other plant matter before entering the apple, as is the case for uptake of the codling moth granulovirus (Lacey et al., 2008). It has been remarked that while dsRNA sprayed as a biopesticide was as effective as spinosad in controlling damage by the CPB, it was nonetheless slower (Petek et al., 2020). It may be the case that RNAi may not be completely effective in preventing codling moth from entering the apple and causing initial damage to the fruit. It is thus proposed to target genes expressed in all stages of life. In this way, the RNAi effect will manifest itself over time during the generation it is applied to, resulting in increased mortality and reduced populations. In this way, codling moth damage will be reduced from one generation to the next across growing seasons.

Time Plan: Experiments using RNAi against the IAP gene (positive control) and selected plant gene (negative control) will commence immediately at the start of the project in order to optimize the methodology; the IAP gene for codling moth has been identified in the published codling moth genome (Wan et al., 2019). Subsequently, target-gene RNAi experiments would be conducted as soon as ideal candidate genes are identified from the various life-stage transcriptomes. These experiments would be conducted from the middle of the first year of the project and onward until sufficiently effective target genes are identified and optimized for experimental field bioassays in Objective 3.

Objective 3. Perform controlled laboratory trials on efficacy of RNAi in neonate larvae and adults towards preventing codling moth damage in apples.

Procedures: For this objective, we will test RNAi efficacy using the best functioning candidate target genes that have been validated for gene disruption and codling moth mortality or developmental inhibition through the larval feeding assays in objective two. Target gene dsRNA will be synthesized and diluted in water to concentrations that have been observed to work in artificial diet RNAi assays. The codling moth behavioral modulator “Cidetrak – Da Mec” (Trécé Inc., Adair, Oklahoma) has been commercialized to affect codling moth larval and adult behavior through delaying location and entry of fruit. “Da Mec” will be mixed with dsRNA and tested in the lab to ensure that dsRNA is not degraded in the “Da Mec” solution. If the dsRNA remains intact, formulations will be made for spraying that

include tank mixtures of the dsRNA together with the "Da Mec" at appropriate concentrations. Additionally, larval feeding stimulants, such as monosodium glutamate (Pszczolkowski et al., 2002), trans-trans-l-anflnocyclobutane-l,3-dicarboxylic acid (Pszczolkowski and Brown, 2004) and L-aspartate (Pszczolkowski and Brown, 2014) will be tested in formulation with dsRNA alone or together with "Da Mec" in field experiments for efficacy in facilitated RNAi-mediated pest control. Initial trials with these materials would first be tested in the laboratory in controlled behavioral assays on apple leaf and fruit materials to measure the extent to which the various formulations elicit increased feeding behavior by codling moth larvae.

Within our experimental orchards, presence of codling moth will first be assessed with sticky traps baited with codlemone pheromone (Knight et al., 2002). Then, at the onset of codling moth activity, formulation spraying regiments will be implemented with validated mixtures of target-gene dsRNA, "Da Mec" and/or aforementioned feeding stimulants. Initially, dsRNA will be tested at highest dose observed to be effective in artificial diet feeding assays. Randomized block trial replicates will be utilized with respect to different treatment conditions plus no-dsRNA treatment controls. After each flight period, degree of damage to apples will be assessed and compared across each block trial with appropriate statistical measurements employed to assess effectiveness of dsRNA treatments in reducing or preventing codling moth damage to apple fruit.

Expected Results: If this approach is successful, it is expected that there will be reduced codling moth damage to apple fruit in experimental blocks treated with target-gene dsRNA versus controls. At this stage the efficacy of dsRNA in killing codling moth larvae or otherwise disrupting their development will have been validated in laboratory assays. As such, in properly replicated and controlled field block trials, any reductions in codling moth damage to fruit may be attributed to the RNAi effect

Potential Problems and Contingencies: The most considerable potential problem is that things do not always work in the field as they do in the laboratory, for any number of reasons. Environmental exposure of dsRNA is a primary concern. Preliminary experiments will be conducted during the first two years of the experiment, in which dsRNA formulations with and without external feeding elicitors are sprayed on controlled apple leaf and fruit material. In subsequent days and weeks, samples will be taken to assess persistence of presence of dsRNA. It may be necessary to utilize biodegradable nanoparticle encapsulators, such as "BioClay" (Mitter et al., 2017). Based upon this information, it may be necessary to make one or more sprays of dsRNA formulations during each flight season to ensure maximum efficacy against codling moth larvae. Experimental trials testing sequential spraying regiments of the formulations onto apple leaf and fruit preparations in the laboratory may be utilized to assess optimal conditions for inducing larval mortality or developmental inhibition. Finally, while it is aimed to identify target genes by which RNAi induces complete mortality in the larval stage, RNAi efficiency or time-frame of activity may be reduced under field conditions. As such, larval mortality or developmental inhibition may be delayed beyond entry of larvae into the apple. Under these conditions, initial RNAi efficacy may be observed via observations of reduction in apple damage during the first flight treatment but would instead manifest through reduced codling moth populations across generations and field seasons. As such, it would be necessary to continue experimentation and assessments beyond the three-year scope of this proposal.

Time Plan: Formulations with IAP dsRNA, "Da Mec, and the feeding stimulants will be made and tested in the laboratory during years one and two to assess viability of the approach of combining these compounds with synthetic dsRNA without degradation of dsRNA. Preliminary assessments of dsRNA longevity in field conditions will also be made during the first two years to better inform spraying conditions during the eventual third year experiments. If RNAi is proven to be functional in laboratory assays, the field trial experiments in Objective 3 will be conducted during the third year during the times where codling moth larvae and adults are behaviorally active.

RESULTS AND DISCUSSION

For Objective 1, whole transcriptome RNA-sequencing has been conducted on neonate and fifth instar whole larvae, as well as hibernaculum-stage overwintering larvae to facilitate identification of

candidate genes for the canonical long double-stranded RNA (dsRNA) RNAi pathway. Initial targets have been identified, including the IAP gene, which has severed as a "model" gene for RNAi in other insects (Pridgeon et al., 2008; Walker and Allen, 2011), and also chitin synthase genes, *chitin synthase A* (CHSA) and *chitin synthase B* (CHSB). CHSA has recently been demonstrated to be a good RNAi target with a larval mortality phenotype across multiple Lepidoptera Families (Rana et al., 2020), though it has not been examined as an RNAi effector in any Tortricidae. Additional targets have been identified for genes that are known in other species to be involved in sex determination.

In addition to whole transcriptome messenger-RNA (mRNA) sequencing, small RNA transcriptome sequencing has been conducted on neonate, third instar and fifth instar larvae to identify "trigger" sequences that would direct effector dsRNA molecules into an alternative RNAi cellular pathway, known as the piRNA pathway (Flynt 2021). This approach has been pursued in collaboration with Dr. Alex Flynt (University of Mississippi) after initial observations of no long dsRNA mediated RNAi phenotype, as described below with regards to Objective 2.

For Objective 2, thorough experimental feeding assays attempting RNAi against codling moth larvae by exposing the larvae to dsRNA targeting the IAP and CHSA genes and compared to saline buffer (in which the dsRNA is diluted) and dsRNA of the non-insect jellyfish gene, green fluorescent protein (GFP). Several different approaches have been taken including: 1) overlaying a standard large dose of target gene dsRNA (500 ng/µL) once on top of the larval diet; 2) applying two large doses on top of the larval diet several days apart (as reported in Wang et al., 2015) 3) mixing in a lower dose (final mixed concentration at 50 ng/µL) of target gene dsRNA directly into the larval diet; 4) testing neonate larvae; 5) testing 3rd or 5th instar larvae that were first reared on untreated larval diet and then transferred to dsRNA treated diet. For all of these approaches taken, at least 20-50 larvae were tested per replicate, with 2-3 replicates tested per condition. Regardless of the approach taken, however, no mortality effect was observed when IAP or CHSA were targeted compared to the saline buffer and GFP dsRNA controls.

Lack of a mortality phenotype may occur for several reasons. It is possible that the genes targeted (or sequence regions of these genes) are not good targets in codling moth. This is possible, however unlikely. Both genes have proven to be effective long dsRNA targets for RNAi in other species. Furthermore, dsRNA has been generated sufficiently long to generate a breadth of cleaved small RNA molecules across the length of the gene to initiate the sequence-specific mRNA degradation pathway, and in the case of CHSA, the dsRNA was derived from the exact region of the gene used effectively in RNAi assays against several other moth species (Rana et al., 2020).

A further cause of no RNAi-mediated phenotype could be attributed to the phenomena of reduced long-dsRNA cellular transport and processing, which has observed in some lepidopteran species, resulting in poor RNAi response in these species (Shukla et al., 2016). The piRNA pathway is one potential alternative to the long-dsRNA pathway that may be viable for inducing RNAi in codling moth, as there are indications that this pathway can be a viable approach to induce RNAi effect in lepidopteran species (Flynt, 2021).

As mentioned above, for Objective 1, an alternative RNA-sequencing approach, aimed at sequencing only small RNAs has been conducted, to identity trigger sequences that activate the piRNA-mediated RNAi pathway. As such, small RNA transcriptomes have been generated across several codling moth larval stages, and several different piRNA trigger sequences have been identified. Double stranded RNA sequences have been generated for IAP and CHSA as well as GFP (as a negative control), each with two different piRNA trigger sequences as leaders. dsRNA feeding experiments as described above with 500 ng/µL applied on top of larval diet and allowed to dry before placing larvae, or alternatively mixing in a lower dose of dsRNA directly into the larval diet. As of yet, no mortality effect has been observed for the target genes compared to negative controls.

Whether long dsRNA or the piRNA pathway have been used to induce RNAi, it has been reported that some insect species may be insensitive to RNAi uptake through feeding due to degradation of the dsRNA effectors in the oral track (Allen and Walker, 2012) or midgut (Luo et al., 2013). In codling moth larvae, this may be tested for through injection of dsRNA effectors directly into the

hemolymph of third to fifth instar larvae. Alternatively, embryonic injections targeting RNAi pathways with dsRNA molecules has also been reported to be efficacious in codling moth, with phenotypes subsequently observed in larval hatchlings (Pospíšilová et al., 2023).

As such, embryonic injection experiments have been initiated targeting CHSA and CHSB with long dsRNA molecules. Preliminary results indicate reduced neonate hatching and larval survival among larvae that do hatch, compared to saline and GFP injected controls. A full analysis of these experiments will be presented in the final report. Furthermore, additional injection and feeding experiments will be conducted during the final year with IAP and genes involved in sex determination and juvenile hormone pathways, which are respectively known to affect embryonic and larval development. If the injection experiments are determined to be fully successful in inducing an RNAi-mediated mortality phenotype, while the feeding experiments are not, it may indicate that larval feeding of neither long dsRNA nor piRNA trigger constructs are viable approaches to trigger the RNAi effect in codling moth larvae.

An additional approach that is being considered to overcome potential limitations related to either uptake of degradation of dsRNA delivered to codling moth via feeding involves the usage of nanoparticle materials to protect and deliver the dsRNA effector molecules to the cell. Such nanoparticles have been demonstrated to be effective for inducing RNAi in another lepidopteran species, the black cutworm moth, *Agrotis ypsilon* (Li et al., 2019). We have recently initiated discussions with Dr. Jinlong Han of Colorado State University, who is currently using this approach to induce RNAi in leafhoppers, to determine the best approach for incorporating nanoparticles into our RNAi experiments for temperate tree fruit insects including the codling moth.

In new trials conducted this year, similar mortality levels were observed for CHSA and GFP above levels observed for the control water treatment. Although it appears that mortality was slightly higher in larvae treated with CHSA dsRNA versus those treated with GFP dsRNA, high standard deviation values across these two treatment groups indicates statistical insignificance. These results suggest that non-specific effects of the dsRNA solutions themselves may be causing mortality as opposed to activation of RNAi specific pathways (Figure 1). More recently, however, research progress has been stalled due to an apparent infection of our codling moth research colony with the codling moth granulosis virus (CpGV). This infection is likely the result of cross-contamination from our ongoing CpGV research conducted elsewhere in our research facilities. Subsequently, the CpGV colony infection has resulted in high mortality in all RNAi assays, whether larvae are fed artificial diet treated with target gene dsRNA, control GFP dsRNA, or control water. Current efforts are underway to cure our colony through careful, focused individual insect rearing, and enhanced cleanliness protocols, across multiple generations to eliminate sick individuals and improve the overall health of the colony. We aim to resume experimentation in the coming months.

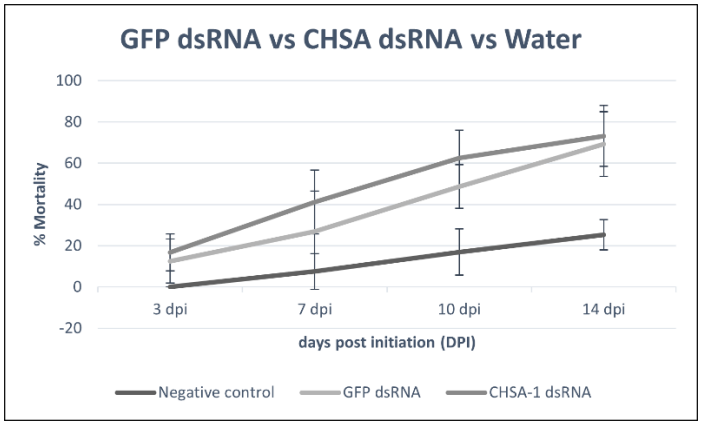

Figure 1 – Comparison of codling moth larvae exposed to artificial diet treated with water (negative control), GFP dsRNA (negative control), or CHSA-1 dsRNA (experimental treatment). For dsRNA treatments, 10 microliters of dsRNA at a concentration of 500 nanograms per microliter were overlaid on to artificial codling moth larval diet and after allowing the

solution to dry, a single neonate larvae was placed on top of the diet. For water treatment, four replicates were performed, for GFP dsRNA treatment, eight replicates were performed for CHSA-1 dsRNA treatment, fourteen replicates were performed; for all replicates, twenty individual codling moth larvae were tested. Error bars indicate standard deviation values. Larval mortality was assessed by assessment of larval motility.

REFERENCES

Allen ML and Walker III WB, 2012. Saliva of Lygus lineolaris digests double stranded ribonucleic acids. *Journal of insect physiology*, *58*(3), pp.391-396.

Flynt AS, 2021. Insecticidal RNA interference, thinking beyond long dsRNA. *Pest Management Science*, *77*(5), pp.2179-2187.

Garczynski SF, Martin JA, Griset M, Willett LS, Cooper WR, Swisher KD and Unruh TR, 2017. CRISPR/Cas9 editing of the codling moth (Lepidoptera: Tortricidae) CpomOR1 gene affects egg production and viability. *Journal of economic entomology*, *110*(4), pp.1847-1855.

Knight AL, Larson D. and Christianson B, 2002. Flight tunnel and field evaluations of sticky traps for monitoring codling moth (Lepidoptera: Tortricidae) in sex pheromone-treated orchards. *Journal of the Entomological Society of British Columbia*, *99*, pp.107-116.

Li J, Qian J, Xu Y, Yan S, Shen J, and Yin M, 2019. A facile-synthesized star polycation constructed as a highly efficient gene vector in pest management. *ACS Sustainable Chemistry & Engineering*, *7*(6), pp.6316-6322.

Luo Y, Wang X, Wang X, Yu D, Chen B, and Kang L, 2013. Differential responses of migratory locusts to systemic RNA interference via double-stranded RNA injection and feeding. *Insect molecular biology*, *22*(5), pp.574-583.

Mitter N, Worrall EA, Robinson KE, Li P, Jain RG, Taochy C et al, 2017. Clay nanosheets for topical delivery of RNAi for sustained protection against plant viruses. *Nature plants*, *3*(2), pp.1-10.

Petek M, Coll A, Ferenc R, Razinger J and Gruden K, 2020. Validating the potential of double-stranded RNA targeting Colorado potato beetle mesh gene in laboratory and field trials. *Frontiers in Plant Science*, *11*, p.1250.

Pospíšilová K, Van't Hof AE, Yoshido A, Kružíková R, Visser S, Zrzavá M, et al, 2023. Masculinizer gene controls male sex determination in the codling moth, Cydia pomonella. *Insect biochemistry and molecular biology*, *160*, p.103991.

Pridgeon JW, Zhao L, Becnel JJ, Strickman DA, Clark GG and Linthicum KJ, 2008. Topically applied AaeIAP1 double-stranded RNA kills female adults of Aedes aegypti. *Journal of medical entomology*, *45*(3), pp.414-420.

Pszczolkowski MA, Matos, LF, Zahand A and Brown JJ, 2002. Effect of monosodium glutamate on apple leaf consumption by codling moth larvae. *Entomologia experimentalis et applicata*, *103*(1), pp.91-98.

Pszczolkowski MA and Brown, JJ, 2004. Enhancement of spinosad toxicity to Cydia pomonella neonates by monosodium glutamate receptor agonist. *Phytoparasitica*, *32*(4), pp.342-350.

Pszczolkowski MA and Brown JJ, 2014. Enhancement of insecticides against codling moth (Lepidoptera: Tortricidae) with l-aspartate in laboratory and field experiments. *Journal of economic entomology*, *107*(3), pp.1163-1171.

Rana S, Rajurkar AB, Kumar KK and Mohankumar S, 2020. Comparative analysis of Chitin SynthaseA dsRNA mediated RNA interference for management of crop pests of different families of Lepidoptera. *Frontiers in Plant Science*, *11*, p.427.

Shukla JN, Kalsi M, Sethi A, Narva KE, Fishilevich E, Singh S, et al, 2016. Reduced stability and intracellular transport of dsRNA contribute to poor RNAi response in lepidopteran insects. *RNA biology*, *13*(7), pp.656-669.

Turner CT, Davy MW, MacDiarmid RM, Plummer KM, Birch NP and Newcomb RD, 2006. RNA interference in the light brown apple moth, Epiphyas postvittana (Walker) induced by double-stranded RNA feeding. *Insect molecular biology*, *15*(3), pp.383-391.

Walker WB and Allen ML, 2010. Expression and RNA interference of salivary polygalacturonase genes in the tarnished plant bug, Lygus lineolaris. *Journal of Insect Science*, *10*(1), p.173.

Walker III WB and Allen ML, 2011. RNA interference-mediated knockdown of IAP in Lygus lineolaris induces mortality in adult and pre-adult life stages. *Entomologia Experimentalis et Applicata*, *138*(2), pp.83-92.

Walker III WB, Gonzalez F, Garczynski SF and Witzgall P, 2016. The chemosensory receptors of codling moth Cydia pomonella–expression in larvae and adults. *Scientific Reports*, *6*(1), p.23518.

Walker III WB, Roy A, Anderson P, Schlyter F, Hansson BS and Larsson MC, 2019. Transcriptome analysis of gene families involved in chemosensory function in Spodoptera littoralis (Lepidoptera: Noctuidae). *BMC genomics*, *20*(1), p.428.

Walker III WB, Cattaneo AM, Stout JL, Evans ML and Garczynski SF, 2023. Chemosensory receptor expression in the abdomen tip of the female codling moth, Cydia pomonella L.(Lepidoptera: Tortricidae). *Insects*, *14*(12), p.948.

Wan F, Yin C, Tang R., Chen M., Wu Q., Huang C., Qian W., et al, 2019. A chromosome-level genome assembly of Cydia pomonella provides insights into chemical ecology and insecticide resistance. *Nature Communications*, *10*(1), p.4237.

Wang J, Gu L, Ireland S, Garczynski SF and Knipple DC, 2015. Phenotypic screen for RNAi effects in the codling moth Cydia pomonella. *Gene*, *572*(2), pp.184-190.

Whyard S, Singh AD and Wong S, 2009. Ingested double-stranded RNAs can act as species-specific insecticides. *Insect biochemistry and molecular biology*, *39*(11), pp.824-832.