Project Title: Genetic engineering of moth viruses for enhanced insecticidal efficacy

Report Type: Final Project Report

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Project Duration: 3 Year

Total Project Request for Year 1 Funding: $58,196
Total Project Request for Year 2 Funding: $60,000
Total Project Request for Year 3 Funding: $61,804

Other related/associated funding sources: Awarded
Funding Duration: 2024 - 2024
Amount: $20,000
Agency Name: Washington Commission on Integrated Pest Management
Notes: Application funded further exploratory research on viral control of codling moth, with specific objectives related to but not covered in this project. Project title: “Efficacy testing of novel viral pesticide CrpeNPV against codling moth and other agricultural pest insects”; there were no other related or associated funding sources for this project

WTFRC Collaborative Costs: None

Budget 1
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Item	2021	2022	2023
Salaries	$40,089.00	$41,425.00	$42,762.00
Benefits	$14,031.00	$14,499.00	$14,967.00
Wages			
Benefits			
RCA Room Rental			
Shipping			
Supplies	$4,076.00	$4,076.00	$4,075.00
Travel			
Plot Fees			
Miscellaneous			
Total	$58,196.00	$60,000.00	$61,804.00

Footnotes: Salaries and benefits are requested for a full-time GS-6 Lab Technician. Supplies are for molecular cloning, viral genotyping and DNA sequencing, cell culture and viral culture/purification.

OBJECTIVES

1) Develop genetic hybrids of CpGV that display increased efficacy in codling moth larvae.

It was initially proposed that admixtures of different strains of CpGV will be used to co-infect codling moth cell culture lines. However, in conversations with Prof. Johannes Jehle, from which the CpGV cell line (Cp14) was obtained, it was determined that this approach is not feasible. The efficiency of viral replication and speed of infection are very low in the CpGV cell line; this results in failure to product large amounts of virus from the cell line. Because of this, the proposed co-infection experiments will be carried out in codling moth larvae instead. Larvae will thus be exposed to admixtures of different strains, and efficacy trials will be conducted to screen for faster or more potent killing compared to baseline rates. Viral extracts will be made from larvae exposed to mixtures that display enhanced effectiveness, and will be genetically characterized to identify any genetic hybrids that may contain properties of the different virus strains combined in novel ways. Isolates of these hybrids will be cultivated, exposed to codling moth larvae and further screened for efficacy, with eventual applicability in both conventional and organic orchards. For authorized use in organic orchards intended products would be submitted to appropriate Material Review Organizations for official registration.

2) Genetically engineer CpGV to include the spider toxin, Hvt.

Standard molecular cloning and genetic engineering methods will be used to splice the spider toxin gene into the genome of a CpGV strain currently used for codling moth control. Genetically transformed viruses will be exposed to codling moth larvae and screened for efficacy. It is hypothesized that the presence of the spider toxin in CpGV will enrich the effectiveness of commercial formulations. Moreover, the presence of an additional virulence factor with a unique mode of action may serve as a safeguard against eventual development of resistance in codling moth populations. Eventual applicability would be sought for use in organic orchards. Use of this spider toxin has previously been patented, however the patent has expired, and the toxin may be used freely.

3) Co-infect codling moth larvae with CpGV and CrpeNPV.

The identification of a novel virus, CrpeNPV, that can infect codling moth provides new opportunities to explore enhanced formulations of viral control of codling moth utilizing both CpGV and CrpeNPV concurrently or in sequence during different seasonal generations. Fundamental research on coinfection of codling moth with CpGV and CrpeNPV is required. Cultivars of CpGV and CrpeNPV would be combined and exposed to codling moth larvae and then screened for efficacy. In addition to registration for organic use as described above in objective number one, appropriate measures will be taken as necessary for registration of use of CrpeNPV in USA for codling moth control.

SIGNIFICANT FINDINGS

- Dose-response and survival time assays with CrpeNPV against our colony codling moth insects, which was obtained from local orchards, demonstrated that the CrpeNPV virus was effective in killing local codling moth larvae.
- Compared to CpGV applied alone, CrpeNPV applied alone was less lethal than CpGV against our codling moth colony insects at low doses used to establish efficacy of the virus via dose-response analysis. However, at higher doses of CrpeNPV that approximate field application rates of CpGV, CrpeNPV was observed to be as equally efficacious as CpGV
- When CrpeNPV was combined with CpGV at a ratio of one-to-one, enhanced lethality was not observed compared to when either virus was applied individually; neither were inhibitory effects observed.

RESULTS AND DISCUSSION

Administrative delays in hiring a dedicated technician persisted until the end of summer of the second year of the project (2022) resulting in substantial delays in launching this project. The process to initiate hiring on our side began during the first year of the project directly after the funding became available, and was finally resolved with the onboarding of the technician in August of 2022. Competency training

was provided to the technician during the autumn of 2022, and experimentation according to the objectives of the project have been ongoing continuously since then. Because of this, a No Cost Extension was sought and granted. However, due to the delay in the initiation of this project until autumn of 2022, more than a full year after the start date, current activities have been performed across a span of only two and a half years at time of filing these reports. Some of the experiments described below are yet incomplete, and currently ongoing. These experiments will be completed through the formal end date of the experiment at the end of April 2025, and if desired, an amended final report may be filed.

Objective 1. For Objective 1, the initial goal was to assess if combining different strains of the virus would result in greater efficacy in killing the codling moth larvae, as a result of recombination of genetic material between the different strains resulting in hybrid strains. If higher or faster larval mortality would be observed in assays where different viral strains were combined, then viral particles could be purified from larval hosts and genetically screened to determine if hybrid viral particles had formed. An alternative hypothesis would be that presence of different strains of virus co-infecting the same larvae could result in higher or lower rates or larvae mortality, simply due to the presence of the different strains in the same larval organisms even without viral hybridization events occurring.

For this objective we decided to focus our research on CpGV genome groups that are commercially available in the USA, namely genome group A (Cyd-X) and genome group E (ViroSoft). Baseline parameters were first established in our laboratory for the genome group E strain. For this, a dose-response curve was generated using five dosages on a ten-fold dilution series, compared to distilled water as a negative control. (Figure 1; Table 1) A clear dose-response effect was observed, with LC50 values of 9.43 x 10^5 and 2.11 x 10^5 at days 7 and 10 post infection exposure, respectively. At the highest dose (1 x 10^7 CpGV occlusion bodies (OBs) per mL), 100% larval mortality was observed by day 10, and 100% larval mortality was observed at the highest two dosages by day 14.

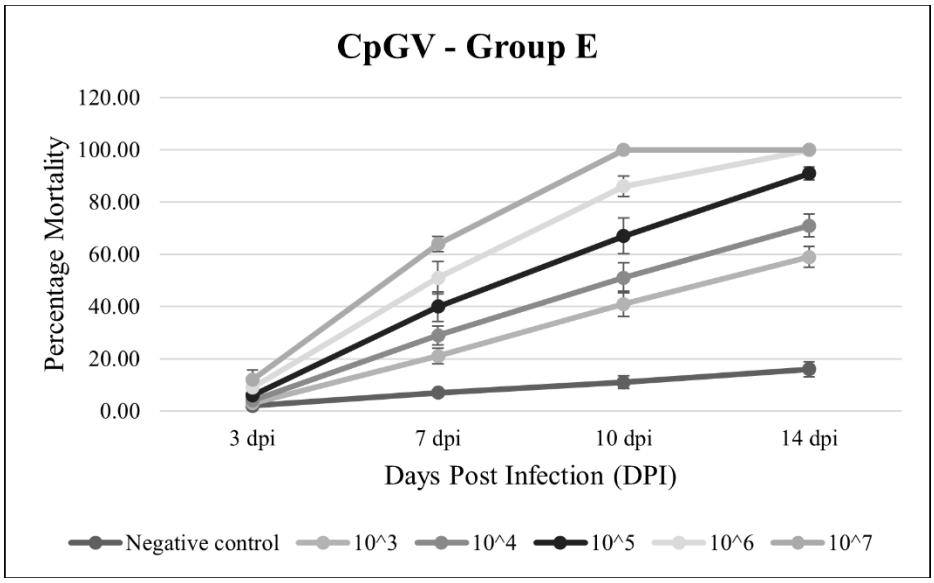

Figure 1. Dose-response mortality assay for codling moth neonate larvae exposed to CpGV - Group E. Twenty larvae tested per replicate (n=20), with five replicates for each treatment. 10 microliters of virus solution or distilled water control were applied at specified concentrations for all treatment groups at the specified dosage, ranging from 1 x 10^7 CpGV occlusion bodies (OB)/mL down to 1 x 10^3 OB/mL. Dilutions were made in autoclaved distilled water, which was also used as the negative control. Treatments were applied evenly to the surface of artificial diet in assay vials and allowed to dry for two hours, after which a single newly hatched neonate larvae was transferred to each assay vial. Larvae were scored for mortality on days 3, 7, 10 and 14 after initiation of experiments (dpi). Error bars indicate standard error values.

After establishing dose response baselines for CpGV, we decided it would be optimal to conduct further assays at concentrations comparable to field application rates based on input from stakeholders. Thus, samples of commercially available Cyd-X and Virosoft were obtained. Ten-fold dilution series were generated from these samples and five dosage steps were tested, such that doses were tested at Dose 1 - 10%, Dose 2 - 1%, Dose 3 - 0.1%, Dose 4 - 0.01%, Dose 5 - 0.001% of undiluted formulation. This dilution series was chosen considering that a standard field application rate is 3 oz of virus per 100 gallons of water per acre is equivalent to a dilution of approximately 0.023% dilution, which falls between Dose 3 and Dose 4.

At the higher dosage levels, substantially higher and faster mortality rates were observed compared to the lower dose-response dilution series (Figure 2). With the Group E, Virosoft product, even at the lowest dosages (0.001% of undiluted formulation) 45% mortality was apparent by day 3 post infection, and by 7 days post infection, nearly 100% mortality was observed across all dosages (Figure 2A). A different trend was observed when Group E (Virosoft) was combined with Group A (Cyd-X). 18-25% mortality was observed across all dosages after 3 days post-infection and 100% mortality was not observed for any and all dosages until by 10 days post-infection Figure 2B). Currently lacking in our datasets is Group A (Cyd-X) alone, which will be critical to the interpretation of the combined strains bioassay (Figure 2B). Bioassays with the higher dosages of Group A are currently ongoing and will be included in the final presentation.

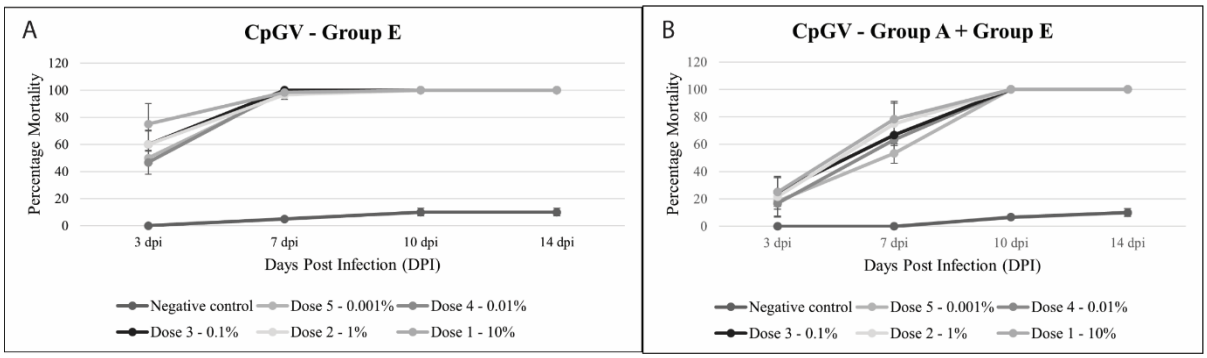

Figure 2. Dose-response mortality assay for codling moth neonate larvae exposed to CpGV – A) Group E. B) Group A+E. For each, twenty larvae tested per replicate (n=20), with three replicates for each treatment. 10 microliters of virus solution or distilled water control were applied at specified concentrations for all treatment groups at the specified dosage, ranging from Dose 1 - 10% of undiluted formulation to Dose 5 - 0.001% undiluted formulation, with 10-fold steps in between. Dilutions were made in autoclaved distilled water, which was also used as the negative control. For B) each dilution series was first generated, and then 1 mL of each strain was combined and applied to the larval diet, resulting in each strain being applied at half the dosage compared to when the strain was applied alone. Treatments were applied evenly to the surface of artificial diet in assay vials and allowed to dry for two hours, after which a single newly hatched neonate larvae was transferred to each assay vial. Larvae were scored for mortality on days 3, 7, 10 and 14 after initiation of experiments (dpi). Error bars indicate standard error values.

It must be noted that in Figure 2B, each virus was combined and presented to the codling moth larvae each at half the dosage compared to presentation of Group E alone (Figure 2A). For these experiments, dilution series for each strain were generated separately, and then at each dosage, 1 mL of each were combined and then applied to the larval diet. However, this 50% reduction in dosage of each strain by itself should not be expected to result in the differences observed for treatment with Group E alone versus the combination of Group A and Group E, considering that in Group E alone the 100% larval mortality was observed by day 3 of the experiment and the dilution series across all five dosages spans more than a 50% difference. It must be mentioned that the experiments conducted in Figure 2A and 2B were conducted at the same time of the year with the same dilution series aliquots for Group E, so differences may not be attributed to sampling differences. Previously published findings that Group E CpGV (R5 Strain) is more potent at killing susceptible codling moth larvae than a

50%/50% mixture of Group A and E strains (Graillot et al., 2016) mirror results presented here. However, this study reported only LC50 values and scored mortality at 7 days post infection and did not examine speed of kill effects.

Pending analysis of data for bioassays conducted with Group A alone, the delayed mortality observed when Groups A and E were combined may be attributable to a form of interference that is known to occur in instances of co-infection (Du et al., 2022) whereby different virus types compete for primacy of infection and suppress the infection of other virus types; this phenomenon has been observed to occur even between different strains of the same species of virus. Co-infection of codling moth by different strains of CpGV has been examined elsewhere. Alternatively, it was reported that a CpGV mutant displayed deficits in viral replication, and these deficits resulted in competitive disadvantages during co-infection of the mutant and wild-type versions of the virus (Elmenofy et al., 2015). Whether interference or differences in replication efficacy or something else entirely accounts for the differences in viral efficacy in codling moth larvae subjected to Group E virus versus the combination of Groups A and E, increased mortality rates were not observed when combining the different viral strain types, thus the possibility of hybridization to form more lethal virus types was excluded.

Objective 2. For Objective 2, the general goal was to genetically engineer a version of the CpGV Group A genome that has been modified in the laboratory to be amenable to genetic engineering, including the insertion of foreign genes (Hilton et al., 2008). The specific goal was to incorporate an insect-specific spider toxin, known as *Hvt*, into the CpGV Group A genome to introduce a secondary mode of action during viral infections, which could result in enhanced lethality and also serve as a safeguard against development of resistance. For this line of experiment, an artificial bacmid construct was designed that would ultimately enable expression of the *Hvt* spider-toxin in codling moth larvae that have been infected with the genetically engineered CpGV virus (Figure 3). Production of this construct was commercially outsourced, and was generated and provided to us during the final year of the project. At which time, we undertook efforts to transfer the *Hvt* transgene construct from the standard cloning bacmid into to the CpGV Bacmid so that codling moth larval infection experiments could be conducted to produce the genetically modified CpGV-*Hvt* virus and assess effects of the *Hvt* toxin. These transfer experiments were conducted using a commercially available bacmid transformation kit that allows transfer of genetic material from cloning bacmids to end-use bacmids, such as the CpGV Bacmid. Protocols were followed as described in previous reports on genetic engineering of the CpGV Bacmid (Hilton et al., 2008, Gebhardt et al., 2014). Despite our efforts, at present time of writing, we have not been able to recover genetically engineered CpGV bacmid.

Figure 3. Expression construct for the Hvt toxin gene. Relevant features include the Hvt spider-toxin gene open reading frame (ORF) positioned downstream of the *Drosophila melanogaster* (Dmel) heat shock protein 70 (hsp70) gene expression driver, which includes the regulatory region and promoter sequences. The Dmel hsp70 promoter has previously been shown to drive constitutive gene expression across different insects including moths (Uhlírová et al., 2002).

Objective 3. For Objective 3, the goal was to investigate effects of co-infections of CpGV and CrpeNPV on codling moth larvae. After establishing baseline parameters for CpGV (Genome Group E) and CrpeNPV, co-infection experiments have been conducted with these two specimen. Initial assays were first conducted with CrpeNPV alone, to generate a dose-response curve, as with CpGV (Group E) using the same five dosages on a ten-fold dilution series, compared to distilled water as a negative control. For CrpeNPV infections alone, maximum larval mortality was observed to be 81% for the highest dose (1 x 10^7 OBs/mL) on day 14 (Figure 4A). Compared to infections with CpGV (Group E) for which 100% larval mortality was observed with the highest dose as early as day 10 (Figure 1), overall larval mortality effects caused by CrpeNPV were lower than larval mortality effects caused by CpGV (Group E) at all dosages and time-points examined. These observations are consistent with results obtained in the initial report on efficacy of CrpeNPV against codling moth (Wennmann et al., 2019), which compared efficacy of CrpeNPV against codling moth to CpGV strains representative of genome groups A and B, but not E. When both viruses were combined at equal ratios, mortality rates were lower early in the period of infection, 3 dpi and 7 dpi, more similar to when CrpeNPV was presented alone; later in the infection, 10 dpi and 14 dpi, larval mortality rates were higher, more similar to when CpGV was presented alone (Figure 4B, Table 1). It must be noted that for these co-infections, since dilution series formulations for each virus were combined at each dosage, the effective dosage of each virus was 50% of the dosage when either virus was presented alone. These results are similar to findings of a previous report, in which it was observed that co-infection of cutworm larvae (*Agrotis segetum*) with both an NPV (AgseNPV-B) and a GV (AgseGV) did not result in changes in mortality rates when either virus was presented alone (Wennmann et al., 2015). These findings may be attributable to the principle of super-infection exclusion, by which infection of individual cells by one virus precludes simultaneous infection by other viruses (Beperet et al., 2014).

Table 1. Codling moth percent larval mortality range across all viral dosages at each time-point days post infection (dpi), from lowest to highest dosage

	3 dpi	**7 dpi**	**10 dpi**	**14 dpi**
CpGV (Group E)	3% - 12%	21% - 64%	41% - 100%	59% - 100%
CrpeNPV	2% - 3%	10% - 22%	22% - 51%	48% - 81%
CpGV (E) + CrepeNPV	0% - 7%	5% - 29%	37% - 81%	65% - 100%

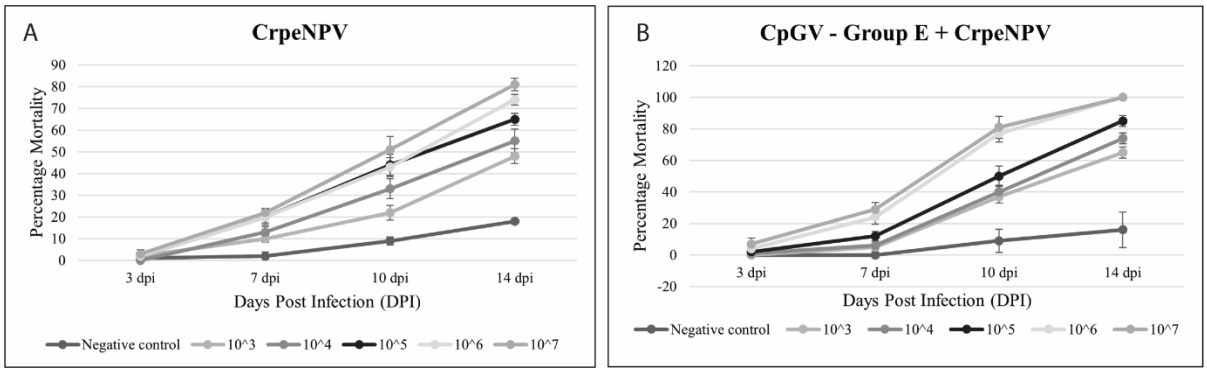

Figure 4. Dose-response mortality assay for codling moth neonate larvae exposed to A) CrpeNPV or B) CpGV – Group E and CrpeNPV. In either condition, twenty larvae tested per replicate (n=20), with five replicates for each treatment. 10 microliters of virus solution or distilled water control were applied at specified concentrations for all treatment groups, ranging from 1 x 10^7 CpGV occlusion bodies (OB)/mL down to 1 x 10^3 OB/mL. Dilutions were made in autoclaved distilled water, which was also used as the negative control. For B, 1 mL of each virus at same concentration was combined. Treatments were applied evenly to the surface of artificial diet in assay vials and allowed to dry for two hours, after which a single newly hatched neonate larvae was transferred to each assay vial. Larvae were scored for mortality on days 3, 7, 10 and 14 after initiation of experiments (dpi). Error bars indicate standard error values.

Next, we examined higher dosages of CrpeNPV and CrpeNPV combined with CpGV – Group E that reflected field application rates of CpGV, which are typically applied at 3 oz per 100 gallons of water per acre. The CrpeNPV virus was supplied to us at a reported concentration of 1 x 10^{11} OBs per mL. This is comparable to OB concentrations for commercial formulations of CpGV, which may range from 1 x 10^{10} to 1 x 10^{14} OBs per mL, or even higher. Regardless, a 10-fold dilution series was established, and five dosage steps were tested, such that doses were tested at Dose 1 – 1 x 10^{10}, Dose 2 - 1 x 10^{9}, Dose 3 - 1 x 10^{8}, Dose 4 - 1 x 10^{7}, Dose 5 - 1 x 10^{6} of undiluted sample. At these higher dosages, larval mortality rate averaged ranged from 70% to 85% at three days post infection, and by seven days post infection, 100% larval mortality was observed across all dosage applications (Figure 5A). These findings are comparable to results when CpGV – Group E was applied across a dilution series spanning the field application rate equivalents (Figure 2A). Similarly, when these higher dilution series dosages of CrpeNPV and CpGV – Group E were combined at a one to one ratio, similar larval mortality rates were observed, with average mortality spanning 51.67% to 85% across all dosages at three days post infection, and by seven days post infection 100% larval mortality was observed for all dosages. These findings suggest that when CrpeNPV is applied at similarly high viral loads as commercial formulations of CpGV, similar levels of codling moth larval mortality may be achieved.
In a recent publication, it was shown that CpGV (either Group A or Group E) and CrpeNPV may both co-infect single codling moth larvae individuals. However, CrpeNPV was not able to facilitate replication of CpGV – Group A in codling moth larvae displaying Type I resistance that were resistant to CpGV – Group A (Hinsberger et al., 2021). This stands in contrast to the ability of CpGV – Group E to facilitate replication of CpGV - Group A in Type I resistant larvae. In another report, CrpeNPV was shown to effectively kill codling moth larvae that were resistant to CpGV (Wennmann et al., 2019), for Type I, Type II and Type III resistant populations. Thus, at least for Type I resistant codling moth larvae, CrpeNPV may effectively kill the larvae, but would not facilitate co-infection with the CpGV – Group A virus. However, presentation of both CrpeNPV and CpGV, either at the same time or in rotation may serve to preclude development of resistance to either virus in susceptible codling moth populations due to the fact that these viruses represent different genera of baculoviruses.

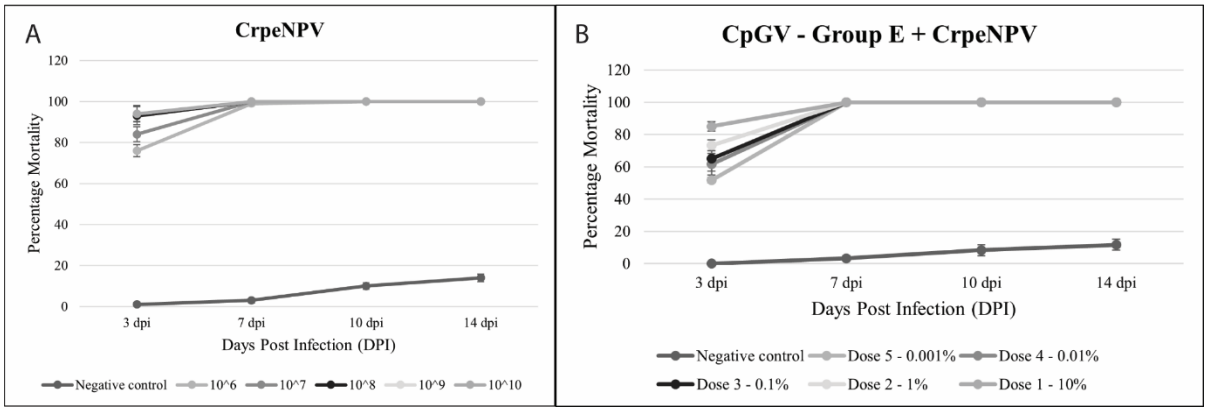

Figure 5. Dose-response mortality assay for codling moth neonate larvae exposed to field application equivalent dosages of A) CrpeNPV or B) CpGV – Group E and CrpeNPV. In either condition, twenty larvae tested per replicate (n=20), with five replicates for each treatment. 10 microliters of virus solution or distilled water control were applied at specified concentrations for all treatment groups, For A) ranging from 1 x 10^{10} CpGV occlusion bodies (OB)/mL down to 1 x 10^{6} OB/mL. Dilutions were made in autoclaved distilled water, which was also used as the negative control. For B) 1 mL of each virus at same concentration was combined. Treatments were applied evenly to the surface of artificial diet in assay vials and allowed to dry for two hours, after which a single newly hatched neonate larvae was transferred to each assay vial. Larvae were scored for mortality on days 3, 7, 10 and 14 after initiation of experiments (dpi). Error bars indicate standard error values.

Finally, survival time analyses were conducted, in which larval mortality rates were measured twice daily across 3 dpi through 7 dpi for both CpGV (Group E) and CrpeNPV presented individually (Figure 6); results for survival time CpGV and CrpeNPV co-infection assays are pending. For these assays, a single concentration was assessed for each virus, 3.7 x 10^2 OB/mL. This dosage, which is lower than the range of concentrations applied in our dose-response assays, was chosen to best assess how/when the course of infection causes larval mortality. This was done because differences in larval mortality rates for CpGV (Group E) and CrpeNPV were already apparent by 7 dpi at all higher doses tested (1 x 10^3 OB/mL through 1 x 10^7 OB/mL). For the dosage tested, a marked difference in survival rates was observed during the early phase of the infection, from 96 to 120 hours post infection (4 to 5 days after initiation of the experiment). These results are consistent with observations from the dose-response assays conducted at higher concentrations. Subsequently, we observed similar survival time rates for both CpGV (Group E) and CrpeNPV, with a substantial decrease in survival occurring between days 5 and 6 (from 126 to 144 hours post infection), compared to the no-treatment control in which only autoclaved distilled water was applied to the larval diet. These results suggest that infections by both viruses have similar time-course metrics with apparent higher lethality to codling moth larvae caused by CpGV.

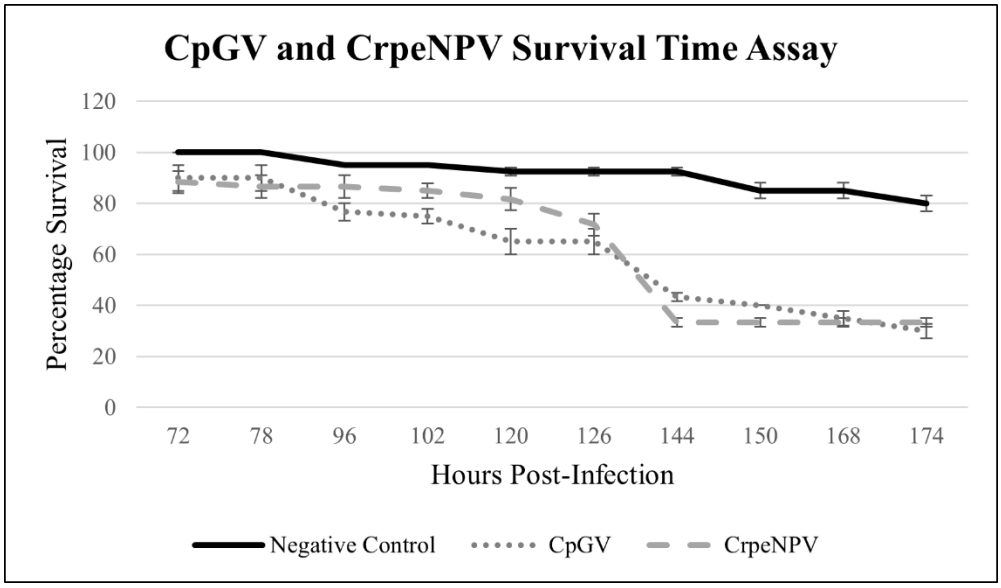

Figure 6. Survival time assays for codling moth neonate larvae exposed to CpGV (Group E) or CrpeNPV. Twenty larvae tested per replicate (n=20), with three replicates for each treatment. 10 microliters of virus mixture or distilled water control were applied at specified concentrations for all treatment groups, at a single concentration of 3.7 x 10^2 occlusion bodies (OB)/mL. The dilutions for each virus was made in autoclaved distilled water, which was also used as the negative control. Treatments were applied evenly to the surface of artificial diet in assay vials and allowed to dry for two hours, after which a single newly hatched neonate larvae was transferred to each assay vial. Larvae were scored for mortality twice daily 72 hours through 174 hours after initiation of experiments (hpi). Error bars indicate standard error values.

In closing, it must be mentioned that these findings in this report reflect laboratory assays conducted with virus formulations applied to artificial larval diet. A next step would be to provide CrpeNPV sprayed on apple tree cuttings after which neonate larvae would be permitted access and damage to apples would be assessed. Currently we do not have appropriate permissions to conduct trials of any kind with CrpeNPV outside of our certified quarantine laboratory, which limits our research with this virus. Accordingly, CrpeNPV is not currently approved for usage in the USA. However, that may not be the case at some point in time in the future.

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Executive Summary

Project Title: Genetic engineering of moth viruses for enhanced insecticidal efficacy

Keywords: CpGV, CrpeNPV, codling moth, biological control, virus

Abstract: The codling moth granulovirus (CpGV) has been used for decades, primarily in organic apple orchards, to control codling moth by killing larval hatchings before or shortly after they enter the apple. However, in recent years, the first scientific report of codling moth resistant to CpGV in the USA, and indeed right here in Washington State, was published. Since then, concerns and suspicions have been mounting that the extent of CpGV-resistant codling moth in Washington is greater and more widespread than the published record indicates. Novel approaches and solutions for pest management of codling moth, especially in organic tree fruit orchards, are thus needed. To address this issue, we proposed a three-pronged approach to research ways to improve codling moth management based on the use of insect viruses. First, we sought to investigate the hypothesis that novel CpGV genotypes generated through coinfection of codling moth larvae by mixed CpGV genotypes may result in higher or faster rates of larval mortality. We approached this with larval co-infection studies with two commercially available CpGV formulations, each representing a different strain of the virus. In our studies, we did not observe enhanced rates of larval lethality when both strains of CpGV were simultaneously presented to the larvae, compared to presentation of either strain alone. Second, we sought to genetically engineer a modifiable version of the CpGV genome to include an insect-specific spider toxin. This would be done to introduce an additional mode of action directly into the virus as a means to promote enhanced lethality and/or counter-mechanisms to the development of resistance in virus-susceptible codling moth populations. Currently, our efforts to generate genetically engineered clones of the CpGV genome have been unsuccessful. This endeavor remains a work in progress. Third, we sought to explore further the efficacy of a novel baculovirus, known as CrpeNPV, against codling moth larvae. CrpeNPV was discovered in South Africa in the litchi moth, which is closely related to the codling moth, and subsequently reported to be able to kill codling moth larvae, including those that were resistant to CpGV strains. We confirmed dose-response efficacy of CrpeNPV against locally sourced codling moth and demonstrated that CrpeNPV is equally effective as CpGV in killing codling moth larvae at comparable field application rates of the virus. Finally, when CrpeNPV was combined with CpGV we did not observe enhanced larval mortality compared to when either virus was presented alone, though inhibitory effects were not observed either. Currently, to our knowledge, the CrpeNPV is not approved for usage in the USA as a biopesticide. If that status changes, it may be considered in future applications as part of effective codling moth management strategies, including management of resistance to CpGV.