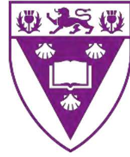

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Baculovirus synergism for improved management of false codling moth *Thaumatotibia leucotreta* Meyr. (Lepidoptera: Tortricidae)

A thesis submitted in fulfilment
of the requirements for the
degree of

MASTER OF SCIENCE

of

RHODES UNIVERSITY

by

David Graham Taylor

February 2021

Declaration

I, David Graham Taylor (g15t0120) hereby declare that the thesis submitted is my own work. It is being submitted for the degree of Master of Science at Rhodes University. It has not been previously submitted for assessment of any degree at any other university or other body, organisation outside of the university. February 2021

05 February 2021

Author's signature

Date

Abstract

Baculoviruses are an environmentally friendly and effective agent for managing lepidopteran pests. This includes the management of *Thaumatotibia leucotreta* (Meyrick) (Lepidoptera: Tortricidae), a serious pest of citrus in Southern Africa and a major threat to the South African citrus export industry. For more than 15 years, CrleGV-SA- based biopesticides have been used as part of an integrated pest management strategy for the control of *T. leucotreta* in citrus orchards in South Africa, under the names Cryptogran™ and Cryptex®. While these biopesticides have been effective during this period, there are some areas in which baculovirus use could potentially be improved. Baculoviruses are notoriously slow to kill in comparison to chemical-based pesticides, and lately, pest resistance to baculoviruses has become a major concern with the development of resistance by *Cydia pomonella* (Linnaeus) (Lepidoptera: Tortricidae) to its granulovirus occurring in the field in Europe. The consistent use of CrleGV-SA for more than 15 years in the field has raised concern that *T. leucotreta* could develop resistance to this virus, and has made it necessary to alter baculovirus-based management strategies to prevent this from occurring. A second baculovirus, Cryptophlebia peltastica nucleopolyhedrovirus (CrpeNPV), has recently been isolated and was shown to be effective against *T. leucotreta.* However, the interactions between CrleGV-SA and CrpeNPV are not yet understood and so it is important to test these interactions before both viruses are applied on the same orchards. Not only is it important to know whether these viruses could negatively impact each other, but it is also important to test whether they could interact synergistically. A synergistic interaction could not only provide a potential tool for the management of resistance, but it could also be exploited to improve baculovirus-based management of *T. leucotreta*.

In this study, a stock of CrleGV-SA was purified by glycerol gradient centrifugation from *T. leucotreta* cadavers, while a stock of CrpeNPV purified from *Cryptophlebia peltastica* (Meyrick) (Lepidoptera: Tortricidae) cadavers was provided by River Bioscience (Pty) Ltd. These stocks were screened for purity by a multiplex polymerase chain reaction (mPCR) protocol designed to detect CrleGV-SA and CrpeNPV. The occlusion body (OB) density was then calculated using darkfield microscopy and a counting chamber. Both stocks were shown to be pure within the limits of the mPCR protocol, and the CrleGV-SA and CrpeNPV stocks were calculated to contain 3.08×10^{11} OBs/mL and 1.92×10^{11} OBs/mL respectively

The first aspect of the interaction between CrleGV-SA and CrpeNPV that was investigated was the dose mortality, in terms of lethal concentration. This was calculated using 7-day surface-

dose biological assays for each virus and a 1:1 mixture of OBs of the two against *T. leucotreta* neonates. The lethal concentrations of each treatment required to kill 50 % of larvae (LC_{50}) and 90 % of larvae (LC_{90}) for each treatment were then calculated and compared using a probit regression. The mixed infection performed significantly better than either virus by itself, while each virus by itself did not differ significantly from the other. The LC_{50} for CrleGV-SA, CrpeNPV and the mixed infection were 1.53×10^4 OBs/mL, 1.15×10^4 OBs/mL and 4.38×10^3 OBs/mL respectively. The LC_{90} of CrleGV-SA, CrpeNPV and the mixed infection were calculated to be 4.10×10^5 OBs/mL, 1.05×10^5 OBs/mL, and 4.09×10^4 OBs/mL respectively.

The second aspect of the interaction between CrleGV-SA and CrpeNPV that was investigated was the speed of kill. A time-response biological assay protocol was created that allowed for effective observation of the larvae. This was then used to generate time-mortality data that were analysed by a logit regression function to calculate and compare the treatments at the time of 50 % larval mortality (LT_{50}) and the time of 90 % mortality (LT_{90}). Each virus by itself did not differ significantly from the other, while the mixed infection took significantly longer to kill 50 % and 90 % of the larvae, suggesting that there is competition for resources between viruses during the secondary, systemic phase of infection. The LT_{50} for CrleGV-SA, CrpeNPV and the mixed infection were 117.5 hours, 113.5 hours and 139.0 hours respectively. The LT_{90} for CrleGV-SA, CrpeNPV and the mixed infection were 153.2 hours, 159.3, and 193.4 hours respectively.

Finally, the composition of OBs recovered from the cadavers produced by the time-response biological assays were investigated by mPCR. A method for extracting gDNA from OBs in neonate-sized *T. leucotreta* larvae is described. The presence of CrpeNPV along with CrleGV-SA was noted in 4 out of 9 larvae inoculated with only CrleGV-SA. The presence of CrleGV-SA as well as CrpeNPV was noted in all but one larva inoculated with only CrpeNPV, and both CrleGV-SA and CrpeNPV were noted in all but one larva inoculated with a 1:1 mixture of the two, with one larva only being positive for CrleGV-SA. This suggests either stock contamination or the presence of covert infections of CrleGV-SA and CrpeNPV in the *T. leucotreta* population used in this study.

This is the second study to report an improved lethal concentration of a mixed infection of CrleGV-SA and CrpeNPV against *T. leucotreta* neonates, and the first study to report the slower speed of kill of a mixed infection of CrleGV-SA and CrpeNPV against *T. leucotreta* neonates. While the improved lethal concentration of the mixed infection is a promising step

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List of Abbreviations

× *g*	-	times gravity
°C	-	Degrees celsius
µL	-	microlitre
AcMNPV	-	Autographa californica multiple nucleopolyhedrovirus
AGE	-	Agarose gel electrophoresis
AgMNPV	-	Anticarsia gemmatalis multiple nucleopolyhedrovirus
AgseGV	-	Agrotis segetum granulovirus
AgseNPV	-	Agrotis segetum nucleopolyhedrovirus
ANOVA	-	analysis of variance
bp	-	base pair
BV	-	Budded virus
CE	-	Controlled environment
CfMNPV	-	Choristoneura fumiferana multiple nucleopolyhedrovirus
ChinNPV	-	Chrysodeixis includens nucleopolyhedrovirus
CpGV	-	Cydia pomonella granulovirus
CpGV-I12	-	Cydia pomonella granulovirus, Iranian isolate
CpGV-M	-	Cydia pomonella granulovirus, Mexican isolate
CpGV-R5	-	Cydia pomonella granulovirus, resistance-breaking isolate
CrleGV	-	Cryptophlebia leucotreta granulovirus
CrleGV-CV3	-	Cryptophlebia leucotreta granulovirus, Cape Verde isolate
CrleGV-SA	-	Cryptophlebia leucotreta granulovirus, South African isolate
CrpeNPV	-	Cryptophlebia pelstastica nucleopolyhedrovirus
CTAB	-	Cetramethyl amonium bromide
ddH2O	-	double distilled water
DNA	-	deoxyribonucleic acid
dsDNA	-	Double-strand deoxyribonucleic acid
dsRNA	-	Double stranded ribonucleic acid
egt	-	gene encoding ecdysteroid UDP-glucosyltransferase
EPA	-	Environmental Protection Agency

EpapGV	-	Epinotia aporema granulovirus
EPPO	-	European and Mediterranean Plant Protection Organisation
FCM	-	False codling moth
gDNA	-	genomic deoxyribonucleic acid
GMO	-	Genetically modified organism
GV	-	granulovirus
GVPs	-	Granulovirus capsule proteins
HearNPV	-	Helicoverpa armigera nucleopolyhedrovirus
HRF1	-	Host range factor 1
IPM	-	Integrated Pest Management
JH	-	juvenile hormone
kHz	-	Kilohertz
LC	-	Lethal concentration
LC50	-	Lethal concentration (50 %)
LC90	-	Lethal concentration (90 %)
LD	-	Lethal dose
LdMNPV	-	Lymantria dispar multiple nucleopolyhedrovirus
lef	-	Late expression factor
LT	-	Lethal time
LT50	-	Lethal time (50 %)
LT90	-	Lethal time (90 %)
Mabr	-	Mamestra brassicae nucleopolyhedrovirus
MNPV	-	Multiple nucleocapsid nucleopolyhedrovirus
mPCR	-	multiplex polymerase chain reaction
NPV	-	Nucleopolyhedrovirus
NTC	-	No-template control
OB	-	Occlusion body
PCR	-	Polymerase chain reaction
PhopGV	-	Phthorimaea operculella granulovirus
pif	-	*per os* infectivity factor
PlxyGV	-	Plutella xylostella granulovirus
PsunGV	-	Pseudaletia unipunctata granulovirus

qPCR	-	quantitative polymerase chain reaction
SDS	-	sodium dodecyl sulphate
SEM	-	Scanning electron microscopy
SeMNPV	-	Spodoptera exigua multiple nucleopolyhedrovirus
SfMNPV	-	Spodoptera frugiperda multiple nucleopolyhedrovirus
SNPs	-	Single nucleotide polymorphisms
SNPV	-	Single nucleocapside nucleopolyhedrovirus
SpliNPV	-	Spodoptera littoralis nucleopolyhedrovirus
T20	-	24-well trays containing 20 % diet plug thickness
TEM	-	Transmission electron microscopy
TnGV	-	Trichoplusia ni granulovirus
TnSNPV	-	Trichoplusia ni single nucleopolyhedrovirus
UV	-	ultraviolet
V	-	volts
v/v	-	volume per volume
V100	-	Vials containing 100 % diet plug thickness
V20	-	Vials containing 20 % diet plug thickness
V40	-	Vials containing 40 % diet plug thickness
w/v	-	weight per volume
XecnGV	-	Xestia c-nigrum granulovirus

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Chapter 1

Literature review

1.1 The citrus industry in South Africa

1.1.1 **The value of the citrus industry**

South Africa is the second biggest exporter of citrus after Spain, exporting almost 1.02 billion cartons of citrus in the 2019 season, translating to over 2 million tons, with the majority of that going to Europe (Citrus Growers Association, 2020). The value of the industry for the 2017/2018 growing season was over R19 billion (US$1.19 billion), or 25 % of the production value of the entire South African horticulture industry (Department of Agriculture, Forestry and Fisheries, 2018a). The industry requires a large work force to function, with fruit being picked and packed by hand and because of this it has been estimated that around a million South African households rely on the citrus industry to some degree (Department of Agriculture, Forestry and Fisheries, 2018a). The South African citrus industry has been built on exports and continues to rely on exports, with almost 80 % of all citrus produced in South Africa being exported. Oranges form the bulk of citrus production and exports in the industry at 70 million cartons exported, followed by lemons at 19 million cartons in the 2016/2017 growing season cartons (Department of Agriculture, Forestry and Fisheries, 2018b). Not only does the industry rely on the size of the international market to sell the citrus produced in South Africa, but the exported citrus typically fetches more than twice the price per ton than it does locally. Europe was the biggest importer of all citrus types except for soft citrus (United Kingdom) and lemons (Middle East) (Department of Agriculture, Forestry and Fisheries, 2018a), and it is therefore vital to identify and mitigate threats to the export industry.

1.1.2 **Threats to the citrus industry**

Two of the greatest threats to the industry, both in terms of production and ability to export fruit, are pathogens and pests, and they both affect the industry in similar ways. Firstly, damage caused to trees or fruit by pests or pathogens reduces total production of the industry, secondly, they are expensive to control and thirdly, they can threaten the export market (Moore et al., 2017). One of the most economically damaging citrus pests in South Africa is *Thaumatotibia leucotreta* (Meyrick) (Lepidoptera: Tortricidae). It is a pest that causes severe internal damage to fruit, leaving very little visible external damage (Daiber, 1979b; Daiber, 1979c). It has been

recorded in Israel where it has established, although this is the only known case of the species establishing outside of its natural range (Wysoki, 1986). There have been interceptions in California and Europe, but it has never established in either area (Moore, 2002). A shipment of fruit that are infested with *T. leucotreta* to areas outside of its natural range could result in its unintentional introduction into new areas, potentially threatening the agricultural industries in those areas (Moore, 2021). If fruit entering Europe are found to be infested with *T. leucotreta,* citrus exports to Europe may be halted entirely, incurring economic costs on the South African citrus industry (Malan et al., 2018; Moore, 2021). While lemons have been shown not to be hosts of *T. leucotreta,* oranges are highly susceptible to infestation by this pest (Moore et al., 2015a). Phytosanitary regulations put in place by certain countries prohibit the import of infested fruit, since this could result in the introduction of *T. leucotreta* to areas outside of its natural range and threaten the agricultural industries in those areas (Newton, 1990; Hofmeyr et al*.*, 2015; Hattingh et al., 2020). It is therefore critical that this pest is correctly managed.

1.2 The pest: *Thaumatotibia leucotreta*

Thaumatotibia leucotreta is a polyphagous pest endemic to Africa and some surrounding islands, specifically, Angola, Burkina Faso, Burundi, Cameroon, Cape Verde Islands, Congo, Eritrea, Ethiopia, Gambia, Ghana, Ivory Coast, Kenya, Madagascar, Malawi, Mali, Mauritius, Mozambique, Niger, Nigeria, Reunion Island Rwanda, Senegal, Sierra Leone, Somalia, South Africa, St Helena, Sudan, Swaziland, Tanzania, Togo, Uganda, Zambia and Zimbabwe (Figure 1.1). While it attacks multiple crops, it is of the greatest concern to the citrus industry in South Africa, where it threatens the survival of the export industry.

Figure 1.1. The known distribution of *Thaumatotibia leucotreta.* Red shading denotes the known native range, green shading denotes established populations outside of the native range (Moore, 2002; Wysoki, 1986).

The eggs (Figure 1.2A) are laid on the surface of fruit. While still new, they are cream coloured, changing to red as they mature and finally black just before they hatch (Daiber, 1979a). The theoretical developmental threshold for the eggs is 11.9 °C, but hatching has been observed down to 10.9 °C (Daiber, 1979a). Once hatched, the larvae (Figure 1.2B) penetrate the fruit, feed, and develop inside the fruit, until the 5th instar, when they leave the fruit and pupate in the soil. The feeding of the larvae causes severe internal damage to the fruit and results in them to falling to the ground. This phenomenon is known as premature fruit drop (Daiber, 1979b; Daiber, 1979c). Once in the soil, the larva spins a cocoon of silk and debris before pupating. The pupae (Figure 1.2C) are dark brown and covered in a chitinous layer with the sex of the pupae easily determined by examination for the presence of two small knobs on the rear, ventral

side of the abdomen, or by counting abdominal segments (Figure 1.3). Adults (Figure 1.2D) eclose after 4-33 days, depending on the season (Daiber, 1979c). The adult life span ranges from 13.7 days to 48.0 days depending on temperature and sex, while egg laying ranges from 5 eggs to 799 eggs influenced by temperature (Daiber, 1980). Fruits that ripen prematurely or have been damaged appear to be more attractive to *T. leucotreta* if the number of eggs laid per fruit is used as a measure of attractiveness (Newton, 1989). Although the activity of *T. leucotreta* in citrus orchards is highest in the summer months (December to February), they are present and active throughout the year (Begemann and Schoeman, 1999). The combination of its ability to severely impact citrus production and its recorded natural range not extending beyond sub-Saharan Africa make it one of the most important pests to manage effectively.

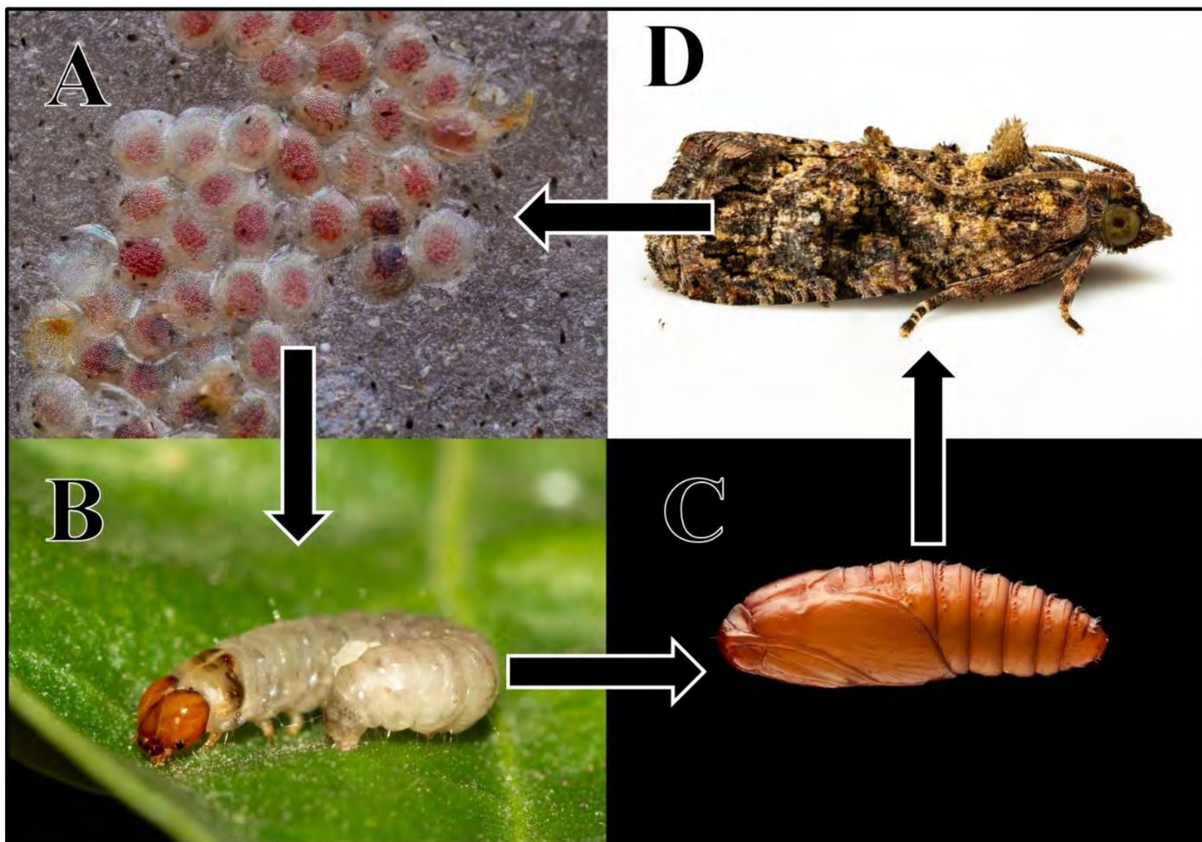

Figure 1.2. The stages of development of *T. leucotreta* (Daiber, 1979a; 1979b; 1979c, 1980). **A**: eggs, **B**: larva, **C**: pupa and **D**: adult. Not all the larval instars are included

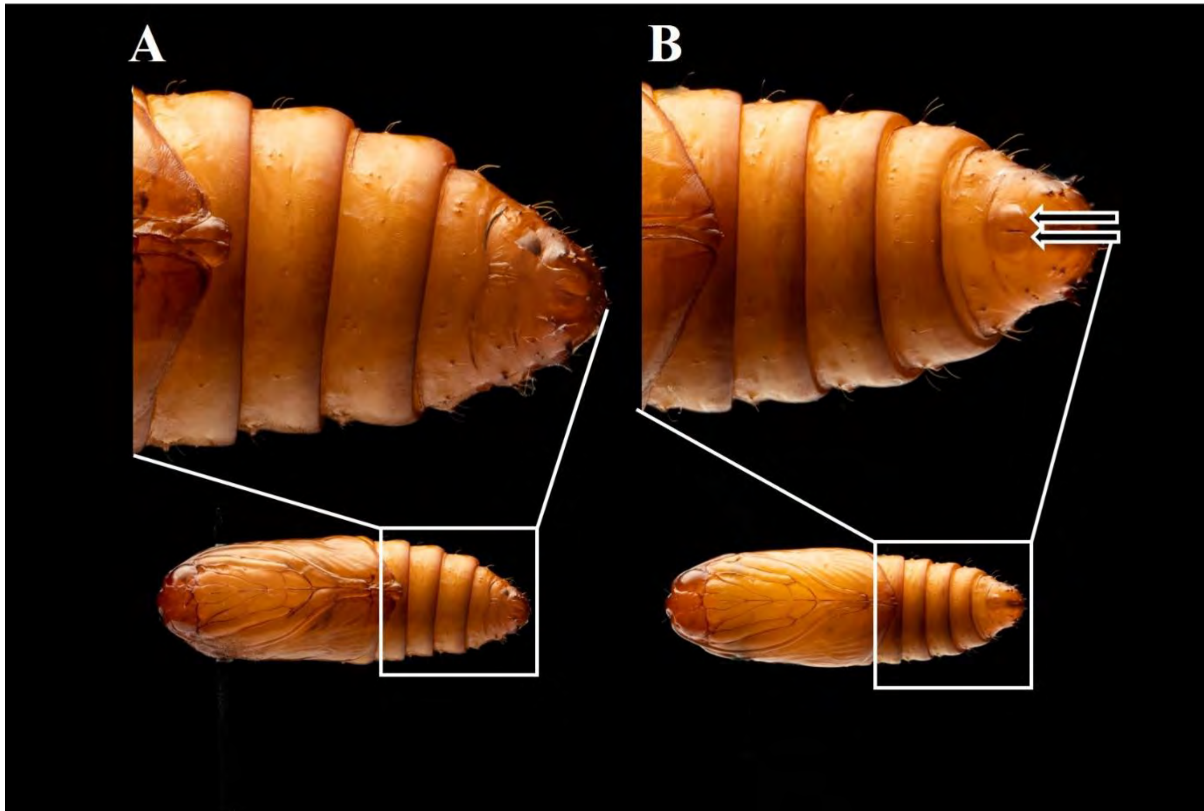

Figure 1.3. Male and female *T. leucotreta* pupae showing the features that distinguish between females (A) with 4 abdominal segments, lacking two knobs on the ventral side of the final segment, and males (B) with 5 abdominal segments with two knobs on the ventral side of the final segment. Arrows show the knobs present in male *T. leucotreta* pupae (Daiber, 1979c)

Europe and the United Kingdom are the biggest importers of highly susceptible citrus varieties (Department of Agriculture, Forestry and Fisheries, 2018b; Moore et al., 2015a). From the 1st of January 2018, the European Union added *T. leucotreta* to the European and Mediterranean Plant Protection Organisation (EPPO) A2 list, requiring that interception of *T. leucotreta* in imported goods be notifiable and that all imported goods require an extended cold-treatment period (EPPO, 2019). This has put more pressure on the South African citrus industry to manage *T. leucotreta* in citrus orchards very stringently.

1.3 Management of *Thaumatotibia leucotreta*

1.3.1 **Introduction to integrated pest management of *T. leucotreta***

Thaumatotibia leucotreta is currently controlled using an integrated pest management (IPM) programme (Moore, 2021). This system includes the use of chemical pesticides, pheromone traps, mating disruption sterile insect technique, orchard sanitation, post-harvest sanitation and

biological control, which includes entomopathogenic fungi, baculoviruses and parasitoids (Knox et al., 2015; Hofmeyr et al., 2016; Hatting et al., 2019; Malan et al., 2018; Moore, 2021). There is still a major focus on chemical control because the effects of chemical pesticides are detectable soon after application, making them an attractive choice for control of *T. leucotreta*. However, there is growing concern surrounding the side effects of chemical pesticide use, including health risks, ecological damage, and the development of resistance by insect pests (Georghiou, 1983; Yadav, 2010; Nicolopoulou-Stamati et al., 2016). Consequently, biological control is an attractive option to reduce the use of chemical pesticides while maintaining adequate management of pests. Baculoviruses are an important component within the suite of biological control options for the management of *T. leucotreta.*

1.3.2 **Baculovirus taxonomy and structure**

Baculoviruses are a group of dsDNA viruses forming the family *Baculoviridae*. Structurally, they consist of one or more nucleocapsids embedded in a matrix of either polyhedrin or granulin, forming an occlusion body (OB) (Figure 1.4). Until 2006, they were classified into two genera, nucleopolyhedroviruses (NPVs) and granuloviruses (GVs), with nucleopolyhedroviruses being further divided into those with single nucleocapsids (SNPVs) and those with multiple nucleocapsids (MNPVs), albeit not a taxonomic sub-grouping (Jehle et al., 2006). They have since been reclassified into four genera, based on phylogenetic analysis. The family is now split into *Alphabaculovirus, Betabaculovirus, Deltabaculovirus* and *Gammabaculovirus*. Alphabaculoviruses are NPVs that infect Lepidoptera, betabaculoviruses are GVs that infect Lepidoptera, gammabaculoviruses are NPVs that infect Hymenoptera, and deltabaculoviruses are NPVs that infect Diptera. NPVs are larger than GVs with an OB diameter of 0.4-3 µm compared to the 0.3-0.5 µm diameter of granuloviruses (Jehle et al., 2006).

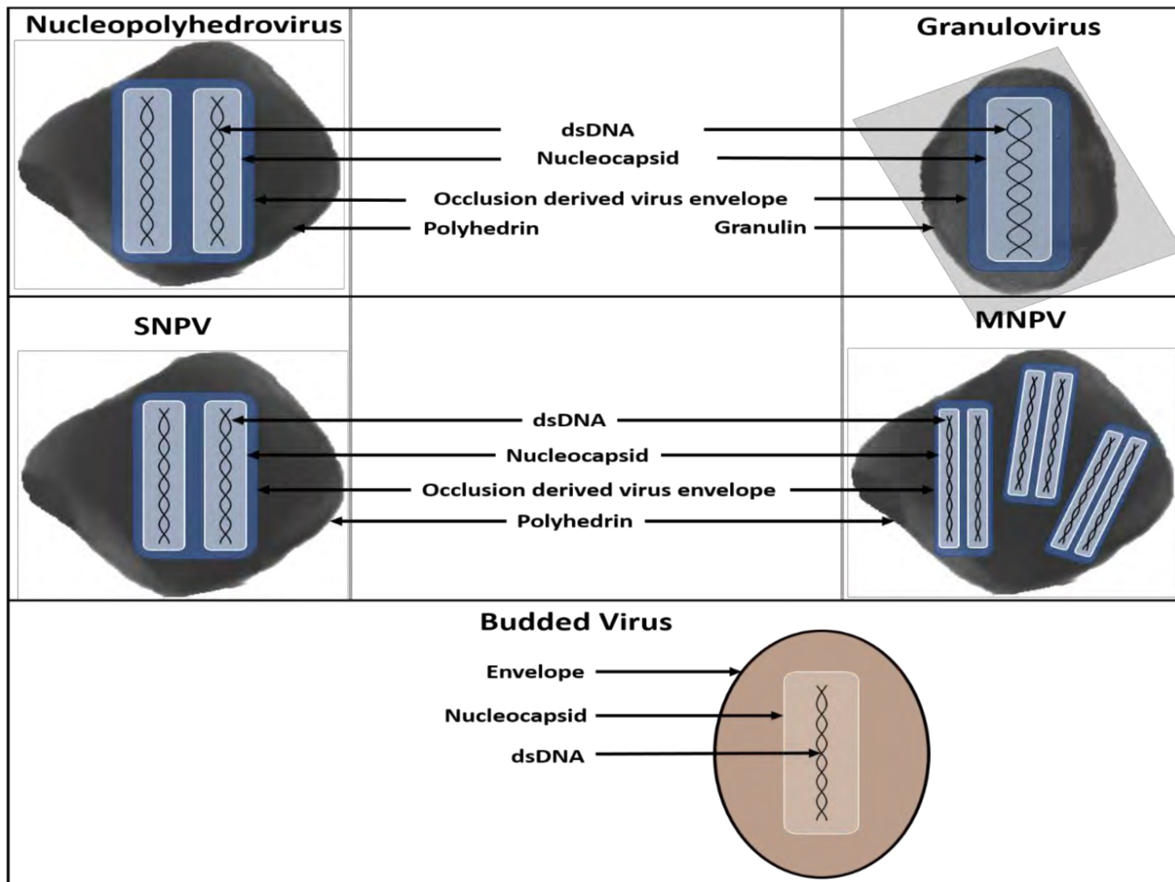

Figure 1.4. The diversity of baculovirus structure (adapted from Jukes, 2018)

1.3.3 **Typical baculovirus life cycle**

A diagram of the typical life cycle of baculoviruses is shown in Figure 1.5. Occlusion bodies are ingested by the larva and remain intact until they reach the alkaline environment of the midgut where the protein matrix dissolves, releasing the nucleocapsids. These then pass through the peritrophic membrane, often with the help of proteins embedded in the capsid membrane that disrupt the peritrophic membrane, creating pores large enough to allow the virions through (Hunter-Fujita et al., 1998; Rohrmann, 2019). The virions enter the midgut epithelial cells and migrate to the nucleus where they are replicated. They either leave the cells as OBs or get coated in the plasma membrane of the infected cell when they leave as budded viruses (BVs). Budded viruses can then infect most other cells of the larva, causing a secondary systemic infection. Both BVs and OBs are produced in both infection stages. The secondary infection causes the larva to liquify internally and eventually rupture, contaminating the immediate area with OBs which can then be consumed by other larvae, leading to horizontal transmission (Hunter-Fujita et al., 1998). The presence of the occluding protein makes the

viruses resistant to weathering and causes the viruses to target only feeding larvae, making baculoviruses an attractive option for biological control of lepidopteran pests.

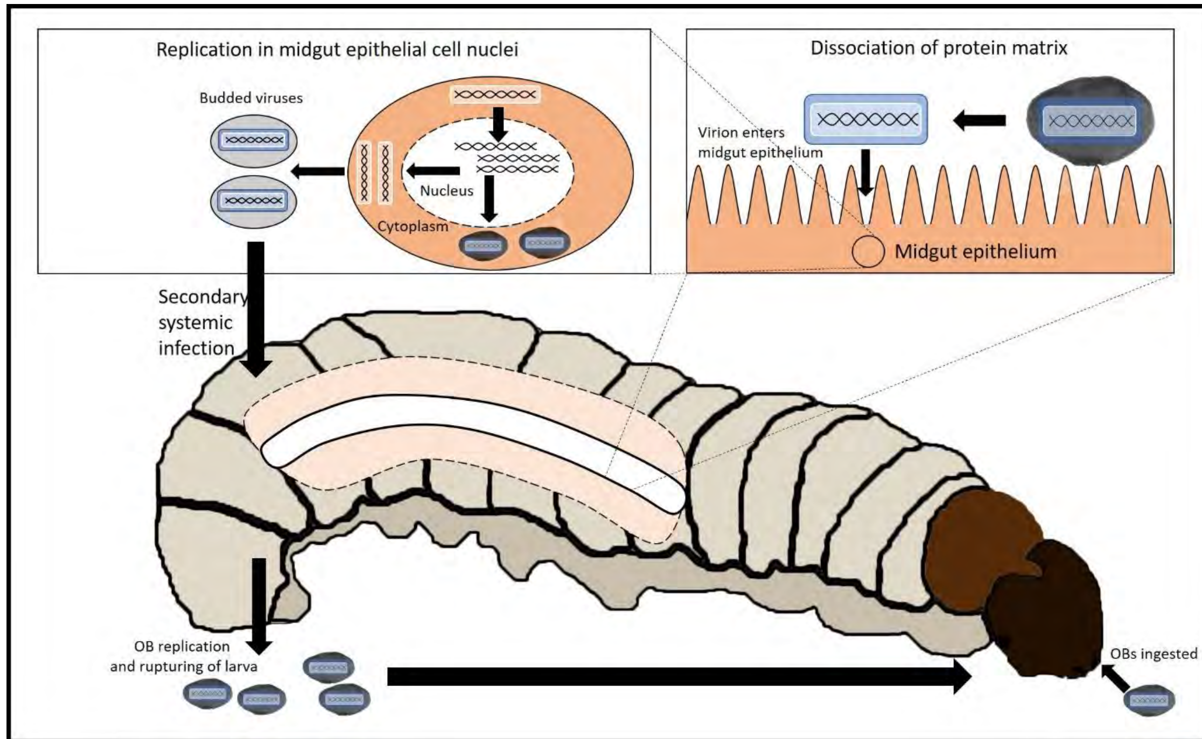

Figure 1.5. Typical baculovirus life cycle in a lepidopteran larva (adapted from Jukes, 2018).

1.3.3 **History of baculoviruses**

Baculovirus infection was first noted in *Bombyx mori* (Linnaeus) (Lepidoptera: Bombycidae) where the infection interfered with the production of silk, although the baculoviruses causing the disease were only described in the early 1800s (Nysten, 1808; Harrison and Hoover 2012). Use of baculoviruses as biopesticides only started in the 1970s (Starnes et al., 1993), with the first, Helicoverpa armigera nucleopolyhedrovirus (HearNPV) being registered for use by the American Environmental Protection Agency (EPA) in 1975 (Starnes et al., 1993). Since then, more than 600 baculoviruses have been characterised (Jehle et al., 2006) and many of the most economically important lepidopteran pests are managed in part using baculoviruses. Some of the most important pests that are managed with baculoviruses are *Cydia pomonella* (Linnaeus) (Lepidoptera: Tortricidae), a major pest of apples and pears, which is managed using Cydia pomonella granulovirus (CpGV) worldwide. Recent field resistance to the commercial strain of the virus has led to the discovery of new isolates, including one from South Africa (Motsoeneng et al., 2019). Baculoviruses also form part of the control programme of two species of *Helicoverpa* (Hardwick) (Lepidoptera: Noctuidae). *Helicoverpa armigera* (Hübner) is a polyphagous pest that feeds on many cultivated crops, including potatoes, legumes, fruits,

tobacco, and cotton (Haase et al., 2015). The NPV to which it is a host, Helicoverpa armigera nucleopolyhedrovirus (HearNPV) forms part of its control programme. There is a commercially produced biopesticide based on HearNPV that is produced in South Africa by River Bioscience (Pty) Ltd, called Helicovir™ (Knox et al., 2015). *Helicoverpa zea* (Boddie) has a similar host range to *H. armigera* and is managed, in part, with HearNPV (Forschler et al., 1992). The Plutella xylostella granulovirus (PlxyGV) is also used in the control programme of another major pest, *Plutella xylostella* (Linnaeus) (Lepidoptera Plutellidae), one of the main pests of cruciferous vegetables (Subramanian et al., 2010). In South Africa, this list of major pests controlled with baculoviruses can be expanded to include *T. leucotreta,* which has had the Cryptophlebia leucotreta granulovirus (CrleGV-SA) as part of its control programme for more than 15 years (Moore et al., 2015b; Malan et al., 2018).

1.3.5 **Baculovirus-based management of *T. leucotreta***

1.3.5.1 **Introduction to baculovirus -based control**

Baculovirus-based management strategies for *T. leucotreta* in South Africa are still in their infancy, despite 15 years of use. Only one baculovirus is currently registered for use against *T. leucotreta* on citrus, although a second, slightly more virulent baculovirus will be registered soon (Marsberg et al., 2017; Jukes and Van der Merwe, 2020; Moore, 2021). Baculovirus-based management of lepidopteran pests on citrus in South Africa is likely to become more common, more effective, and more diverse in the future.

1.3.5.2 **Advantages of baculovirus control**

Baculoviruses stand to partially replace chemical-based management of *T. leucotreta*, and baculoviruses have some advantages over chemical pesticides. Their narrow host range ensures species-specific pest management, having no negative impact on other insect life including beneficial parasitoids (Moore et al., 2015b; Moore, 2021). This allows an indirect secondary advantage of allowing a healthy population of parasitoids and predators to accumulate, providing further, long-term management of *T. leucotreta*, as well as other pests (Moore, 2021). Baculoviruses can also co-evolve with their host, resulting in a smaller chance of resistance developing than with chemical pesticides. Their genetic diversity and ability to evolve also provides the opportunity to have multiple isolates, some of which may overcome resistance, should resistance to a certain isolate develop (Opoku-Debrah et al., 2016). Finally, there is the advantage of public perception. Export markets are largely driven by public perception, and public perception of chemical pesticides is becoming increasingly negative, especially in

Europe and the United Kingdom (Calliera et al., 2019; Matyjaszczyk et al., 2019; Moore, 2021). The European public is prepared to pay more for environmentally friendly produce that has no perceived health-risk associated with it (Cagalj et al., 2016). Baculovirus-based pest management can allow farmers to satisfy both criteria.

1.3.5.3 **Cryptophlebia leucotreta granulovirus**

Cryptophlebia leucotreta GV was first discovered in 1965 in a laboratory colony of *T.* (=*Cryptophlebia*) *leucotreta* in Cote d'Ivoire, and it was recorded that it could severely damage the colony (Angelini et al. 1965). It was later isolated from larvae of *T. leucotreta* on the Cape Verde islands and described by restriction profiles (Smith and Crooke, 1988; Jehle et al., 1992). Although the virus was potentially of great value to the South African citrus industry, the South African isolate was not extensively studied until the early 2000s and it was not available as a biopesticide until 2004 (Moore, 2002, Moore et al., 2015b). Restriction profiles showed the Cote d'Ivoire, Cape Verde and South African viruses to be distinct isolates (Jehle et al., 1992).

Two of the isolates have had their full genomes sequenced, the Cape Verde isolate CrleGV-CV3 (Lange and Jehle, 2003) and the South African isolate CrleGV-SA (van der Merwe et al., 2017). The South African isolate has been used in the field for more than 15 years and has remained stable over this period, with few, generally stable single nucleotide polymorphisms (SNPs) occurring between 2000 and 2015 (van der Merwe et al., 2017). Recent experience with the field development of resistance of *C. pomonella* to CpGV led to concerns about a similar situation with *T. leucotreta* and CrleGV-SA, resulting in a search for different CrleGV isolates. This culminated in the discovery of five distinct isolates within CrleGV-SA that can be taxonomically classified into two main groups, distinguishable by their restriction profiles and *egt* and *granulin* gene sequences (Opoku-Debrah et al., 2013).

The first commercial biopesticide containing CrleGV-SA, Cryptogran™ (River BioScience (Pty) Ltd.), was registered in 2004 and the second product, Cryptex® (Andermatt Biocontrol AG, Switzerland), was registered three years later (Moore et al., 2015b). Both products have been shown to be effective in field trials, being similarly effective to chemical control methods, sometimes exceeding the extent of control provided by chemical pesticides, particularly triflumuron (Moore et al., 2015b; Moore, 2021). This may be because of resistance to triflumuron (Hofmeyr and Pringle, 1998). At least two applications of CrleGV per season are recommended for maximum control as part of an IPM strategy (Moore et al., 2015b).

1.3.5.4 **Cryptophlebia peltastica nucleopolyhedrovirus**

Cryptophlebia peltastica nucleopolyhedrovirus (CrpeNPV) is a new single nucleocapsid alphabaculovirus described in 2016 that has been confirmed to infect at least three species: *Cryptophlebia peltastica* (Meyrick) (Lepidoptera: Tortricidae), *C. pomonella*, and *T. leucotreta*. It was isolated from infected larvae of *C. peltastica* while prospecting for a biocontrol agent for the same species and was shown to be more virulent against *T. leucotreta* than CrleGV-SA (Marsberg, 2016; Marsberg et al., 2017). Initial field trials have shown promise with CrpeNPV, reducing infestation of *T. leucotreta* by up to 96 % with some viral activity being recorded up to 12 weeks after application, a long period of persistence, making it a promising potential tool for the future management of *T. leucotreta* (Marsberg et al., 2017). It is in the process of being formulated as a commercial product for use against *T. leucotreta* (Marsberg et al., 2017; Hatting et al., 2019; Marsberg et al., 2018; Moore, 2021).

1.4 Challenges associated with baculovirus use

1.4.1 **Susceptibility to ultraviolet radiation**

Nucleic acids are susceptible to ultraviolet radiation, which causes the formation of thymine dimers (Durbeej and Eriksson, 2002), and thus, baculoviruses are susceptible to deactivation by ultraviolet light with more than 80% of their efficacy after 24 hours of sunlight lost due to genome-damage. An example of this was seen in HearNPV OBs, with a half-life as low as 5.8 hours in direct sunlight (Mcleod et al., 1977; Ignoffo et al., 1989), while a reduction in efficacy by exposure to UV light has also been noted in Spodoptera frugiperda nucleopolyhedrovirus (SfMNPV) (Behle and Popham, 2012). This severely limits their persistence in the field and is one of the greatest barriers to overcome for baculoviruses to form the basis of an effective biopesticide. By contrast, triflumuron maintains over 80 % of its efficacy after 75 days of weathering in the field, making it more persistent than baculovirus-based pesticides (Hofmeyr, 1984). The susceptibility of baculoviruses to ultraviolet (UV) radiation must be overcome if they are to compete with chemical pesticides.

1.4.2 **Pest resistance to baculoviruses**

Integrated pest management strategies that include biological control options are considered less susceptible to the development of resistance by their hosts because they co-evolve with their host (Eberle and Jehle, 2006; Dercole et al., 2010). While the theory works in some cases (Dercole et al., 2010), there are records of pests becoming resistant to biological control agents

(specifically microbial agents) through selection in laboratory experiments (Boots and Begon, 1993; Abot et al., 1996; Milks and Myers, 2000; Nakai et al., 2017); and, more importantly, in the field where certain biological control options are frequently used (Eberle and Jehle, 2006; Asser-Kaiser et al., 2007).

There had never been a case of resistance to a baculovirus developing in the field until the discovery of resistant *C. pomonella* populations in 2003-2004 in organic orchards in France and Germany (Fritsch et al., 2005, 2006; Sauphanor et al., 2006; Eberle and Jehle, 2006). The resistant moths were found in orchards where CpGV had been used regularly to control moths, where farmers had noticed that the virus was no longer providing adequate control (Eberle and Jehle, 2006). Further testing showed that the moths were resistant only to the Mexican isolate of the virus (CpGV-M), used in all commercial products up to that point and that resistance to this isolate was stable even without the selection pressure (Eberle et al., 2009; Berling et al., 2009). More recently, two new types of resistance appeared in some orchards toward resistance-breaking isolates (Jehle et al., 2017; Sauer et al., 2017). Additionally, the claim that type I resistance was stable without selection pressure has been brought into question by a recent laboratory-based study (Fan et al., 2019).

1.4.3 Speed of kill

While baculoviruses can be effective in the field, one of their weaknesses is the speed of kill. Speed of kill is the time between ingestion of a sufficient number of OBs to cause infection and death of the larva. Lethal time (LT) is the measurement of time required for a given percentage of a host population to succumb to the virus following inoculation. The accepted convention for pest control being the LT_{90} value, or the average time taken for a high dose of a control agent to kill 90 % of the target organism. The LT_{90} of baculoviruses, including both CrleGV-SA and CrpeNPV, typically exceeds three days after OB ingestion, giving the larvae three days to burrow into the fruit, cause internal damage and provide a pathway for pathogens into the fruit (Venette et al., 2003). This is not typically a problem with citrus, as it takes about 4 days for neonate larvae to penetrate the rind, and by this time the virus has taken hold and caused them to exit the fruit before they have caused permanent damage (Moore et al., 2011; Moore et al., 2015b). However, the slow speed of kill remains a problem for crops with thinner skins, such as avocados and grapes. This means that often the greatest effect of the virus is to prevent the pests from reaching sexual maturity, thereby reducing the population size of the future generations, but the cost is a potential loss of saleable crops in the short term (Moore

and Jukes, 2019). A secondary effect of the loss of a crop in the short term is that a perception that the viruses are ineffective can be cultivated, making farmers unwilling to use baculoviruses (Moore and Jukes, 2019). Increasing the speed of kill does not appear to affect the quantity of OBs produced per cadaver (Lei et al., 2019), but this may be dependent on the method used to improve speed of kill of a virus. Increasing the speed of kill of baculoviruses to further limit fruit damage by the generation of pests on which the virus is applied will make baculovirus-based pesticides a more effective and attractive choice for the management of lepidopteran pests.

1.5 Overcoming challenges of baculovirus-based pest management

1.5.1 **Bioprospecting**

Searching for new viruses or new isolates of known viruses can lead to the discovery of viruses with greater virulence. Genetic drift allows for multiple isolates of a single baculovirus species with differing phenotypes to develop, and isolates can be selected according to the desired phenotype. There can often be variation in the viral load, speed of kill, and virulence of the different isolates (Hodgson et al., 2001). New, more virulent isolates can be used to improve the efficacy of baculovirus-based biopesticides, or overcome problems like resistance (Berling et al., 2008). The value of having multiple isolates of a virus was realised when *C. pomonella* became resistant to the commercially available Mexican isolate of CpGV. This triggered a search for resistance-breaking isolates of CpGV that could be used as alternatives to CpGV-M, and resulted in the discovery of multiple isolates that were resistance-breaking. The most effective of these isolates, CpGV-I12, an isolate from Iran, was formulated into a new commercially available product called Madex I12 (Andermatt Biocontrol AG). A second product was also produced called Madex Plus (Andermatt biocontrol AG), using a different, unreported isolate (Zingg, 2008).

Another example of variation in virulence among different isolates of the same virus can be found with Chrysodeixis includens nucleopolyhedrovirus (ChinNPV). In a study done by Aguirre et al. (2019), 23 genetic isolates of ChinNPV were identified and their biological activity was compared in terms of virulence, speed of kill (specifically LT_{50}), and viral load per larva. While the different isolates did not vary significantly in their speed of kill, they did vary in their virulence and viral loads, potentially improving future biopesticides based on ChinNPV.

In recent years, the search for novel virus isolates that infect *T. leucotreta* has led to the discovery of CrpeNPV which is more virulent against *T. leucotreta* than CrleGV-SA, as well as the discovery of five new isolates of CrleGV-SA (Opoku-Debrah et al*.,* 2016, Marsberg et al., 2017). Isolation and characterisation of these isolates has broadened the tool kit for the management of *T. leucotreta*, providing isolates that may be used in the future to overcome resistance to baculoviruses, improve the speed of kill of baculovirus-based pesticides or improve their tolerance to ultraviolet radiation of baculovirus-based pesticides.

1.5.2 **Selection for UV resistance**

UV resistance can be overcome in three ways: the addition of UV-blocking compounds to the formulation (Killick, 1990; Dougherty et al., 1996; Zhu et al., 2013), the inclusion of a gene that encodes an enzyme to recognise and fix the pyrimidine dimers created by UV radiation into the baculovirus genome (Petrik et al., 2003) or the inclusion of a gene that makes the OB protein matrix more resistant to UV radiation (Li et al., 2015), and finally by artificial selection of a UV-resistant isolate of the baculovirus. The first two solutions have major drawbacks. The addition of UV-blocking compounds into the formulation makes the production of baculovirus-based pesticides more expensive, and the compounds must first be shown not to inactivate, or otherwise negatively impact the viruses. Genetic modification of the viruses to create a UV-resistant isolate is an elegant solution. However, there is severe mistrust of genetically modified organisms (GMOs) by the public (Malyska et al., 2016). Since public opinion drives the market, the use of GMOs on a crop would potentially negatively affect the marketability of the crop. Artificial selection for a UV-resistant trait in the virus could have the same result as genetic modification, but without the stigma. Initial experiments to select for UV-resistance in CrleGV-SA have had promising results, but the stability of the trait is not yet known (Mwanza, 2019).

1.5.3 **Increasing virulence by passage of a virus through a heterologous host**

Although most baculoviruses, especially granuloviruses, are limited to a single host, there are cases where a baculovirus can infect more than one species. When this occurs, the heterologous hosts are invariably very closely related, and virulence is reduced in the heterologous hosts when compared to the homologous host (Thiem and Cheng, 2009; Graillot et al., 2017; Moore and Jukes, 2019). In these cases, it has been shown that passaging the virus through a heterologous host can improve the virulence of that virus in the heterologous host, and this could potentially lead to the improvement of existing baculovirus-based biopesticides (Graillot

et al., 2017). The idea is that, while a virus may infect a heterologous host, it is not fully adapted to that host. Passaging through the heterologous host selects for traits that allow it to infect heterologous host more easily. This is the case with CpGV and *Grapholita molesta* (Busck) (Lepidoptera: Tortricidae), a heterologous host of CpGV. A mixture of CpGV isolates was passaged through *G. molesta* larvae and the LC_{90} improved 450-fold after just 12 generations (Graillot et al*.,* 2017). There is the potential to do a similar selection trial with CrpeNPV and *T. leucotreta,* since *T. leucotreta* is presumed to be the heterologous host of CrpeNPV.

1.5.4 **Genetic modification**

Genetic modification can be used to improve the speed of kill of baculoviruses by either the deletion or inactivation of genes that reduce the speed of kill, or by the addition of genes from other organisms that can increase the virulence of the virus. The *egt* gene encodes ecdysteroid UDP-glucosyltransferase, which prevents the insect from moulting. This allows the viral load to increase but reduces the speed of kill (O'Reilly and Miller, 1991). Fast-killing isolates of baculoviruses without the *egt* gene or with an inactivated *egt* gene produced between six and thirteen times fewer OBs per larva than isolates with a functioning *egt* gene, so while the increased speed of kill makes the virus a more efficient pesticide, it could potentially limit its natural spread in the field after application (Simón et al*.,* 2012; Moore and Jukes, 2019).

Alternatively, genes can be added to the genome of the virus to make them more virulent. A study by Burden et al. (2000) showed that the speed of kill of Autographa californica multiple nucleopolyhedrovirus (AcMNPV) could be increased without negatively impacting the virulence against *Trichoplusia ni* (Hübner) (Lepidoptera: Noctuidae) by including a gene derived from the genome of the mite *Pyemotes tritici* (LaGrèze-Fossat and Montagné) (Trombidiformes: Pyemotidae) that produced a paralytic toxin when expressed. While the toxin increased the speed of kill, it had the additional advantage of reducing the feeding of the larvae. In another example, a gene derived from the scorpion *Androctonus australis* (Linnaeus) (Scorpiones: Buthidae) that expressed an insect-specific neurotoxin was added to the genome of AcMNPV, significantly increasing the speed of kill. It should be noted that, although the venom produced by the scorpion is often fatal to humans, the component of its venom which was expressed in the recombinant baculoviruses was not harmful to mice, and so was probably not harmful to humans (Stewart et al., 1991). Additionally, both speed of kill and virulence can be increased by the expression of dsRNA that interferes with hormone-mediated gene expression. In a study done by Liu et al. (2019), HearNPV was modified to produce fragments

of dsRNA that interfered with genes in *H. armigera* related to juvenile hormone (JH), significantly improving both the lethal concentration and the speed of kill compared to the unmodified virus

Genetic modification has also been used to increase the host range of a baculovirus. A strain of AcMNPV that lacked the *p35* gene was modified with a cosmid containing sequences derived from Spodoptera exigua nucleopolyhedrovirus (SeMNPV). The recombinant virus could not only infect *Autographa californica* (Speyer) (Lepidoptera: Noctuidae) and *Spodoptera exigua* (Hübner) (Lepidoptera: Noctuidae), but it could also infect *Spodoptera litura* (Fabricius) (Lepidoptera: Noctuidae) which is outside of the host range of either parent virus (Wu et al., 2016).

A limitation of genetic modification is the public opinion of GMOs. Genetically modified organisms are mistrusted by the public and many law makers who legislate against their use based on misconceptions surrounding the use of genetic modification, and who are often pressured by public opinion into severely limiting its use (Malyska et al., 2016). The extent and stringency of legislation against GMOs varies from country to country, with America and South Africa being far more open to genetic modification in terms of aiding food production than Europe. While genetic modification could provide solutions to many of the problems associated with baculovirus use, anti-GMO legislation in Europe prevents these solutions from being implemented (Malyska et al., 2016).

1.5.5 **Baculovirus synergism**

Synergism is the interaction between two distinct entities with a result that is more than the sum of the parts. This means that when two agents are used simultaneously, their resulting effect will be statistically significantly greater than the more effective of the two agents by itself (Rummens, 1975). In the case of two or more viruses interacting synergistically, the effect could be that either a fewer total number of virus particles are required to kill the host than any of the viruses alone, or that a combination of viruses can kill the host quicker than any of the viruses alone. Synergistic interactions between baculoviruses in a lepidopteran host are not always limited to two viruses that can each infect the larva by themselves, rather, they often occur between a virus for which the insect is a host and a virus for which the insect is not the host (Biedma et al., 2015). The virus for which the insect is not the host provides something that makes the infection process of the infecting virus more efficient. This is usually a protein

that makes the peritrophic membrane more permeable to the virions, such as enhancin or Gp37 proteins (Roelvink et al., 1995; Li et al., 2003; Liu et al., 2011).

1.5.5.1 ***In Vitro* baculovirus interactions**

Cell lines have allowed research to be conducted to better understand synergistic interactions between two baculoviruses. In a study done by McClintock and Dougherty (1987), a cell line derived from *Lymantria dispar* (Linnaeus) (Lepidoptera: Lymantriidae) (Ld652Y) (Disney and McCarthy, 1985) was initially infected with Lymantria dispar multiple nucleopolyhedrovirus (LdMNPV) and later superinfected with AcMNPV. The Ld652Y cell line is not permissive to AcMNPV, but the interaction between LdMNPV and AcMNPV allowed AcMNPV to replicate in the cell line, producing large numbers of AcMNPV BVs, but reducing the production of LdMNPV BV. This interaction was further investigated to determine how LdMNPV assists AcMNPV infection of the non-permissive cell line. It was found that without LdMNPV being present, DNA replication of AcMNPV did occur in the cells, but proteins were not expressed, suggesting that a trans-acting factor was provided by LdMNPV that allowed AcMNPV infection. The trans-acting factor was later discovered to be a 25.7 kDa polypeptide that was named host range factor 1 (HRF1) (Cheng and Lynn, 2009). The promotion of coinfection by the provision of a trans-acting factor by one of the two viruses has also been noted in coinfections of Sf21 cells derived from *Spodoptera frugiperda* (Smith) (Lepidoptera: Noctuidae) by SeMNPV and AcMNPV. In this case, AcMNPV provides the trans-acting factor that allows SeMNPV to infect the Sf21 cell line (Yanase et al., 1998).

In another cell line derived from *Choristoneura fumiferana* (Clemens) (Lepidoptera: Tortricidae) named CF-203 (Sohi et al., 1993), infection by AcMNPV is prevented by apoptosis. This can be prevented by first infecting the cells with Choristoneura fumiferana multiple nucleopolyhedrovirus (CfMNPV), after which infection by AcMNPV occurred and AcMNPV OBs were produced. This suggests that CfMNPV prevents the apoptosis caused by AcMNPV, allowing for the replication of AcMNPV (Cheng and Lynn, 2009).

1.5.5.2 ***In Vivo* baculovirus interactions**

It is often the case that more than one virus can be discovered in, and extracted from, a single host. These viral mixtures are usually two isolates of the same virus, with one isolate having a

deletion that prevents it from infecting its host *per os*, and one isolate that can infect the host *per os.* An example of this phenomenon can be found in both *C. fumiferana* and *S. frugiperda*. The viruses with the deletions can infect the tissues in the hemocoel, but not the midgut epithelial cells and are known as defective strains. The strain without the deletion can infect the midgut epithelial cells and allows the defective strain through the midgut into the hemocoel. The deletion of the defective strain means that the genome is smaller and replication can occur faster, speeding up the infection and causing the viral load to increase faster (Cheng and Lynn, 2009).

Synergism has also been observed between different baculovirus species as early as 1970. Pseudaletia unipunctata granulovirus (PsunGV) and a nucleopolyhedrovirus (unspecified in the study) extracted from infected *Pseudaletia unipunctata* (Haworth) (Lepidoptera: Noctuidae) larvae were fed to *P. unipunctata* larvae in mixed infections. It was found that proteins embedded in the protein matrix of the PsunGV OBs aided the passage of the nucleopolyhedrovirus through the peritrophic membrane, speeding up the primary infection. This effect was counteracted in the secondary infection of the hemocoel where the replication of the NPV limited the replication of the PsunGV, and larval mortality was reduced compared to a single PsunGV infection (Tanada and Hukuhara, 1971). This could potentially be remedied by deactivating the PsunGV particles before adding them into the NPV formulation. Another study showed a synergistic interaction between AcMNPV and Trichoplusia ni single nucleopolyhedrovirus (TnSNPV) in mixed infections of *T. ni* larvae. The authors suggest that the improved potency of the mixture is due to TnSNPV providing an enhancin protein that allows for a more efficient primary infection by AcMNPV (Lara-Reyna et al., 2003). The interaction between Anticarsia gemmatalis multiple nucleopolyhedrovirus (AngeMNPV) and Epinotia aporema granulovirus (EpapGV) is similar to the two previous cases mentioned. AngeMNPV infects the host *Anticarsia gemmatalis* (Hübner) (Lepidoptera: Noctuidae) and EpapGV provides the Gp37 protein which allows AngeMNPV to pass through the peritrophic membrane more efficiently. When the viruses were mixed the LC_{50} was decreased significantly and the speed of kill was increased significantly, despite the fact that no infection by EpapGV ever occurred in *A. gemmatalis* larvae (Biedma et al*.,* 2015). A similar result occurs when Spodoptera littoralis nucleopolyhedrovirus (SpliNPV) and Xestia c-nigrum granulovirus (XecnGV) are mixed and used to infect *S. litura*. The SpliNPV infects the host, while the XecnGV provides *enhancin*, making the peritrophic membrane more permeable to SpliNPV and making the process of primary infection more efficient (Guo et al., 2007).

The addition of Gp37 to SeMNPV and AcMNPV significantly increased their efficacies against *S. exigua* as well as increasing the efficacy of *Bacillus thuriniensis* (Liu et al*.,* 2011). This scenario has been replicated with Mamestra brassicae nucleopolyhedrovirus (MabrNPV) and proteins derived from XecnGV. In this study the proteins involved were not mentioned by name, they were simply described as alkaline-soluble proteins of granulovirus capsules (GVPs). It was found that adding 10 µg/mL of GVPs to a MabrNPV formulation increased the infection rate in *Mamestra brassicae* (Linnaeus) (Lepidoptera: Noctuidae) significantly in the field (Goto et al*.,* 2015).

Multiple isolates of a single virus species can often interact synergistically. For example, Phthorimaea operculella granulovirus (PhopGV) is used to control both *Phthorimaea operculella* (Zeller) (Lepidoptera: Gelechiidae) and *Tecia solanovora* (Povolný) (Lepidoptera: Gelechiidae), both major pests of potatoes. Different isolates showed varying virulence for the two species. The most virulent isolates for each species were passaged through that species to cause them to adapt to the species in question and were then mixed. The mixed infections were up to 23.6 times more effective against *P. operculella* and up to 4.9 times more effective against *T. solanovora* (Espinel-Correal et al., 2012). The authors suggested that the isolates could be mixed in a single pesticide to target both pests simultaneously. Graillot et al. (2014) showed that mixing a novel isolate of CpGV, CpGV-R5, with the isolate to which *C. pomonella* was resistant, CpGV-M, allowed the CpGV-M to infect and kill the resistant larvae at a similar rate to non-resistant larvae. This could have implications in the future, should *T. leucotreta* become resistant to either CrleGV-SA or CrpeNPV.

1.6 Motivation, aims and objectives

1.6.1 **Motivation**

CrleGV-SA has been used as part of an IPM programme for the control of *T. leucotreta* in citrus orchards for more than 15 years (Moore et al., 2015b). The use of CrleGV-SA as a biopesticide has some problems that have not yet been addressed, namely, the slow speed of kill relative to chemical pesticides, and the risk of the development of resistance due to long-term, repeated application. Both issues could potentially be addressed by exploiting synergistic relationships between baculoviruses infecting the same host. Both CrleGV-SA and CrpeNPV can infect *T. leucotreta,* and preliminary studies have shown that mixing the two viruses results in greater virulence in *T. leucotreta* than either virus alone (Jukes et al., 2017).

While it is known that CrleGV-SA and CrpeNPV have a synergistic interaction in terms of dose-mortality in *T. leucotreta*, it is not yet clear how simultaneous infection by both viruses will affect the speed of kill and it is unknown how the ratio of each virus changes throughout the infection. If it is found that simultaneous infection by both viruses causes a faster speed of kill as well as having increased virulence, baculovirus-based biopesticides for the management of *T. leucotreta* could be improved to be faster acting and cheaper to produce, due to the lower concentration that would be required. Any changes in the ratio of the two viruses throughout the infection cycle have potential implications for production of the baculovirus-based biopesticides.

1.6.2 **Aims and objectives**

The overall aim of this study was to assess the overall effects of interactions between the two viruses that infect *T. leucotreta* by dose-response bioassays of virus mixtures and time-response bioassays of virus mixtures on *T. leucotreta* neonates. The first objective was to determine the virulence of each virus against *T. leucotreta*. The second objective was to determine and compare the virulence of a mixture of the viruses with the virulence of each virus by itself. The third objective was to determine the speed of kill of each virus against *T. leucotreta* neonates. The fourth objective was to determine and compare the speed of kill of the mixture with that of each virus by itself. The final objective was to determine which viruses were present, and in what relative quantities, in the cadavers after each treatment.

1.7 Overview of chapters

Chapter 2 describes the purification, enumeration and molecular screening of stocks of CrleGV-SA and CrpeNPV OBs. This was achieved for CrleGV-SA by crude OB extraction from *T. leucotreta* cadavers, followed by glycerol gradient centrifugation. A stock of CrpeNPV OBs purified from *C. peltastica* cadavers was supplied by River Bioscience (Pty) Ltd. The density of OBs of each stock was enumerated using darkfield microscopy and a counting chamber. Finally, a 1:1 (OB:OB) stock of CrleGV-SA and CrpeNPV was created.

Chapter 3 reports and compares the biological activity of each virus by itself, as well as a 1:1 mixture of the two in terms of lethal concentration against *T. leucotreta* neonates. This was achieved using surface-dose biological assays. The biological activity of each virus by itself and a mixed infection in terms of speed of kill is reported in **Chaper 4**. This was achieved using a surface-dose time-response biological assay protocol.

A technique for extracting DNA from OBs in neonate-sized *T. leucotreta* cadavers was developed in **Chapter 5**, after which the composition of baculoviruses in cadavers produced by the time-response biological assays was determined by using mPCR.

Finally, **Chapter 6** is a general discussion of the results reported in the previous chapters, with an emphasis placed on the potential reasons for the results obtained, as well as the implications these results may have for the South African citrus industry. The chapter also outlines potential future research that this study has made possible.

Chapter 2

Purification, screening and enumeration of CrleGV-SA and CrpeNPV stocks

2.1 Introduction

This chapter describes and discusses the acquisition of baculovirus stocks, the processes by which baculovirus stocks were screened, and the implications of the screening results for later experiments.

Baculoviruses can be obtained from cadavers of larvae that have died of a baculovirus infection (Grzywacz et al., 2004). An infection occurs either because the larva is stressed, thus leaving it vulnerable to a covert infection becoming overt, or because the larva consumed OBs of a baculovirus to which it was a host (Hughes et al., 1993; Opoku-Debrah et al., 2013). Larvae that have died of baculovirus infection can be identified by their milky appearance in comparison to healthy larvae and their flaccidity (Hunter-Fujita et al., 1998). When their membrane is ruptured, a milky haemolymph spills out. The haemolymph contains millions to billions of OBs and these can be purified by centrifugation through a sucrose or glycerol gradient (Grzywacz et al., 2004). Purifying by glycerol gradient provides a stock that is sufficiently pure for transmission electron microscopy, and thus this method was used. This is the first step in identifying whether a sample contains the desired virus (GV or NPV), since the morphological differences between GVs and NPVs can be seen.

The final step in screening the virus stocks is to use a PCR protocol to confirm the presence of the desired virus in the stock and to show that other viruses are either absent or in a concentration lower than is detectable by the PCR method. This can be done using a multiplex polymerase chain reaction (mPCR) protocol. This is a common and accepted way of screening baculovirus stocks where multiple baculoviruses may be present (Wennman and Jehle, 2014; Manzán et al., 2008). Only one known NPV infects *C. peltastica*, and only one GV species is known to be present in *T. leucotreta* populations (Moore, 2002; Opoku-Debrah et al., 2013; Marsberg, 2016; Jukes, 2018), and thus it is possible to test the purity of a CrleGV-SA homogenate simply by confirming the presence of one GV-specific DNA sequence and confirming the absence of one NPV-specific DNA sequence. Screening of the NPV stock can

be done in a similar way and a multiplex PCR protocol was developed by Jukes (2018) for the detection of CrleGV-SA and CrpeNPV in mixed infections. The assay uses two sets of primers, the first of which creates a 187 bp amplicon from the NPV-specific *pif-1* region, while the other set of primers creates a 378 bp amplicon from the GV-specific *lef-4* region. When the amplicons undergo electrophoresis the sizes of the amplicons are sufficiently different so that there is adequate separation between the bands when both viruses are present. The limitation is that the protocol used here is only effective down to a gDNA concentration of 3 pg/µL for CrpeNPV and between 50 pg/µL and 500 pg/µL for CrleGV-SA (Jackson, 2018), meaning that covert infections are not necessarily detectible using this method.

Enumeration of baculoviruses can be done in one of three ways: counting OBs in a representative sample of the stock using darkfield microscopy and a haemocytometer (Dhladhla et al., 2018; Jukes, 2018), using a quantitative polymerase chain reaction (qPCR) protocol (Dhladhla et al., 2018; Jukes, 2018), or by scanning electron microscopy (SEM) (Dhladhla et al., 2018). The qPCR is most useful when a baculovirus concentration must be calculated in a mixed infection where darkfield microscopy would not allow for the differentiation between the different viruses (Jukes, 2018; Wennman and Jehle, 2014; Zwart et al., 2008). The SEM and darkfield microscopy techniques both produce comparable and consistent results (Dhladhla et al., 2018). Scanning electron microscopy has the advantage of having a higher resolution than darkfield microscopy and thus baculoviruses can be distinguished from debris in an impure sample, although the results do not differ significantly from those of darkfield microscopy and preparation of samples is more complicated for SEM than for darkfield microscopy (Dhladhla et al., 2018), and thus the darkfield microscopy method was chosen.

The first objective of this chapter was to purify OBs from cadavers of infected *T. leucotreta* larvae by glycerol gradient centrifugation. The second objective was to visualise a sample of each stock by transmission electron microscopy as a first step of stock screening. The third objective was to extract DNA from each sample and perform a multiplex PCR protocol on each stock to confirm the relative purity of the extracted OBs. Once relative purity had been confirmed, the fourth objective was to enumerate each stock using a haemocytometer and darkfield microscopy.

2.2 Materials and Methods

2.2.1 **Crude extraction of OBs**

A stock of CrpeNPV OBs was provided by River Bioscience (Pty) Ltd. and so only a stock of CrleGV-SA OBs needed to be purified. Cadavers of infected *T. leucotreta* larvae were provided by River Bioscience (Pty) Ltd., and these were used for the OB extraction. The protocol used for the crude extraction was taken from Marsberg (2016), which was originally adapted from Grzywacz et al. (2004). Larvae were crushed and homogenised in a 1.5 mL microcentrifuge tube with 1 mL of 0.1 % (v/v) sodium dodecyl sulphate (SDS) solution, after which the solution was vortexed for 2 minutes. The solution was then centrifuged at 100 × *g* for 10-20 seconds. The supernatant was pipetted into a new centrifuge tube and another 1 mL of 1.0 % (v/v) SDS solution was added to the pellet. The pellet was re-suspended and then centrifuged at 100 × *g* for 10-20 seconds. The supernatant was added to the previously collected supernatant and the pellet was discarded. The supernatant was then centrifuged at 2500 × *g* for 5 minutes to pellet out the OBs, after which the supernatant was discarded and the pelleted OBs were re-suspended in 1 mL of ddH_2O to wash them. Once again, the suspension was centrifuged at 2500 × *g* for 5 minutes and the pellet was resuspended in 200 µL of ddH_2O.

2.2.2 **Purification by glycerol gradient centrifugation**

A glycerol gradient centrifugation protocol adapted from Marsberg (2016), originally adapted from Grzywacz et al. (2004) was used to purify the crude extraction to create a pure virus stock. A glycerol gradient with a range of 30 % to 80 % (v/v) was created in a centrifuge tube. Each layer had a volume of 3 mL. The crudely purified OB suspension was then pipetted on top of the gradient, and the tube was centrifuged at 12 100 × *g* for 15 minutes. The band containing the OBs at the point of meeting of the 50 % and 60 % layers was pipetted into a clean microcentrifuge tube containing 1 mL of ddH_2O. The tube was then centrifuged at 12 100 × *g* for 15 minutes and the supernatant was discarded. Once again, the pellet was resuspended in 1 mL of ddH_2O and centrifuged at 12 100 × *g* for 15 minutes. The supernatant was discarded and the pellet was resuspended in 200 µL of ddH_2O and stored at -20 °C.

2.2.3 **Transmission electron microscopy**

Carbon grids for transmission electron microscopy were prepared using the protocol described by Jukes (2018), originally adapted from methods described by Abdulkadir et al. (2013) and Opoku-Debrah et al. (2013). Five microlitres of purified OBs were pipetted onto the carbon

grid and allowed to stand for 60 seconds, after which the excess liquid was drawn off using filter paper. To stain the OBs, 5 µL of 1 % uranyl acetate was pipetted onto the grid and allowed to stand for 30 seconds. The excess uranyl acetate was drawn off using filter paper and the grids were then allowed to dry. The OBs were viewed using a Zeiss Libra 120 transmission electron microscope (Zeiss, Germany) and the images produced were analysed using Mega View (G2) Olympus analysis software (Olympus, Japan) to capture the images and calculate the size of the OBs in the images. Images were stored on a compact disk and the grids were stored in a grid case at the ambient temperature of the laboratory.

2.2.4 **Molecular screening of virus stocks**

2.2.4.1 **DNA Extraction**

DNA was extracted from each stock using the Zymo Research Quick-DNA™ Miniprep kit (Zymo Research, USA) using the protocol provided with the kit.

2.2.4.2 **Polymerase chain reaction**

A polymerase chain reaction (PCR) was set up to amplify the CrpeNPV *pif-1* region and CrleGV-SA *lef-4* region with each sample containing 12.5 µL of *Taq* ReadyMix HotStart PCR Kit (KAPA Biosystems, USA), 1 µL 10 µM of either CpNPV-pif1F/R or ClGV-L4F/R oligonucleotide solution (Table 2.1) and 2 µL of gDNA solution. A positive control for the *pif-1* region was set up using gDNA from CrpeNPV that had previously been confirmed by BLAST analysis and a positive control for the *lef-4* region was set up using gDNA from CrleGV-SA. A negative control for each region received nuclease free H_2O in the place of gDNA. Nuclease free H_2O was then added to each vial to increase the total volume of each vial to 25 µL. The PCR cycle consisted of an initial 3-minute denaturing period at 95 °C. After denaturation, the samples underwent 30 cycles of 95 °C for 30 s, 55 °C for 30 s, and 72 °C for 45 s. Following the 30 cycles, an elongation period at 72 °C for 1 minute was used to finish the PCR reaction.

Table 2.1. Primer design for the *pif-1* region of CrpeNPV and the *lef-4* region of CrleGV-SA (Jukes, 2018).

Name	Length	Sequence (5'-3')	Amplicon size (bp)	Tm
CpNPV-pif1F	20	ATCGGGATGGTTGGTCAAGT	187	59
CpNPV-pif1R	20	CAACGCATGTATTCGTCCGT		59
ClGV-L4F	21	TTCGCTTTCTAAACCGCTGTC	378	59.2
ClGV-L4R	22	AGGATGACGTTCCTAATGACGG		59.9

2.2.4.3 **Agarose gel electrophoresis**

Electrophoresis of the amplicons was performed through a 1 % (w/v) agarose gel prepared with 100 mL 1 × TAE buffer (40mM Tris-acetate, 20mM acetic acid, 1mM Ethylenediaminetetraacetic acid) and 1 g of agarose gel powder. Four microlitres of ethidium bromide was added to the gel as a stain. The initial DNA extractions and the amplicons were run through the gel at 100 V for 35 minutes alongside a GeneRuler 1kb ladder (Thermo Fisher, USA). The gel was then visualised under UV light using a ChemiDoc™ XRS+ (Bio-Rad, USA) with images being processed and captured using the associated Image Lab™ software (Bio-Rad, USA).

2.2.5 **Occlusion body enumeration of virus stocks**

A 10 µL sample of each of the two virus stocks was diluted to a 5 × dilution in sterile dH_2O. This was then diluted a further 5 × in 0.07 % (v/v) SDS solution, bringing the suspension to a 25 × dilution. The samples were then sonicated at 60 kHz for a total of 60 seconds each, to separate the clumps of OBs into single OBs suspended in solution. The SDS was added to prevent the clumps from reforming. The 60 seconds was divided into four rounds of 15 seconds to prevent overheating of the samples. The sonicated suspensions were then further diluted to make 1:50, 1:60, 1:70 and 1:80 dilutions of the sonicated suspension (1:1250 to 1:2000 suspension of the original virus stock). A 0.02 mm Thoma bacterial counting chamber (Hawksley Helber Bacteria 1 cell Thoma Z30000) and cover slip were cleaned with ethanol and a lens cloth. Five microlitres of the most dilute suspension was then transferred into the counting chamber and was covered with the cover slip, before being observed at 400 × magnification by darkfield microscopy, with the OBs visible as points of light. Of the main 4 × 4 grid, each corner square and one of the centre squares were counted. The entire grid of each

square was counted. Before counting commenced, each dilution was tested starting from the most dilute and working upward to find the dilution that resulted in there being between 6 and 9 OBs per square of the grid, or between 96 and 144 OBs for each large square of the counting chamber. Once this had been achieved, the corresponding dilutions were used for counting. Counting was done three times, using a new 5 μL for each count. The concentration was calculated using Equation 2.1. A stock containing equal numbers of OBs of CrleGV-SA and CrpeNPV was created by using Equation 2.2. This brought the final concentration of the stock for the mixed infections to the same as that of the pure virus stock with the lower concentration.

Equation 2.1. Equation for determining the concentration of OBs using a counting chamber.

$$Concentration\ (OBs\ per\ mL) = (D\ \times X) \div (N\ \times V)$$

Where D = dilution factor, *X* = number of OBs counted, N = number of sub-squares counted and V = volume (mL).

Equation 2.2. Equation used to calculate a 1:1 mixture of different OBs from stock solutions of different concentrations.

$$Vh = Vl\left(\frac{Cl}{Ch}\right) + Vw$$

Where *Vh* = Volume of stock with the higher OB concentration, *Vl* = Volume of stock with the lower concentration, *Ch* = Concentration of the stock with the higher concentration, *Cl* = concentration of the stock with the lower concentration and *Vw* = volume of water (*Vl*–*Vh*).

2.3 Results

2.3.1 **Transmission electron microscopy**

The grid prepared from the CrpeNPV stock showed that the sample contained OBs with an external morphology that was congruent with the external morphology of NPVs (Figure 2.1), being between 739 nm and 1304 nm in diameter (n = 22). The grid prepared from the CrleGV-SA stock contained OBs that exhibited external morphology congruent with GVs (Figure 2.2), being roughly oval-shaped with a width of between 187 nm and 232 nm and a length of between 329 and 400 nm (n = 18).

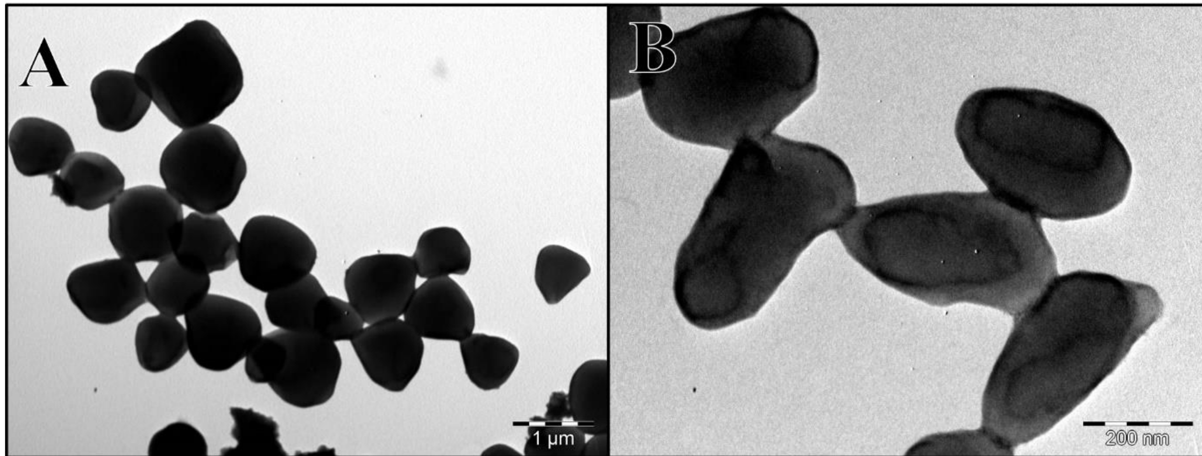

Figure 2.1. Transmission electron micrograph of A: CrpeNPV and B: CrleGV-SA, showing the external morphologies of both viruses.

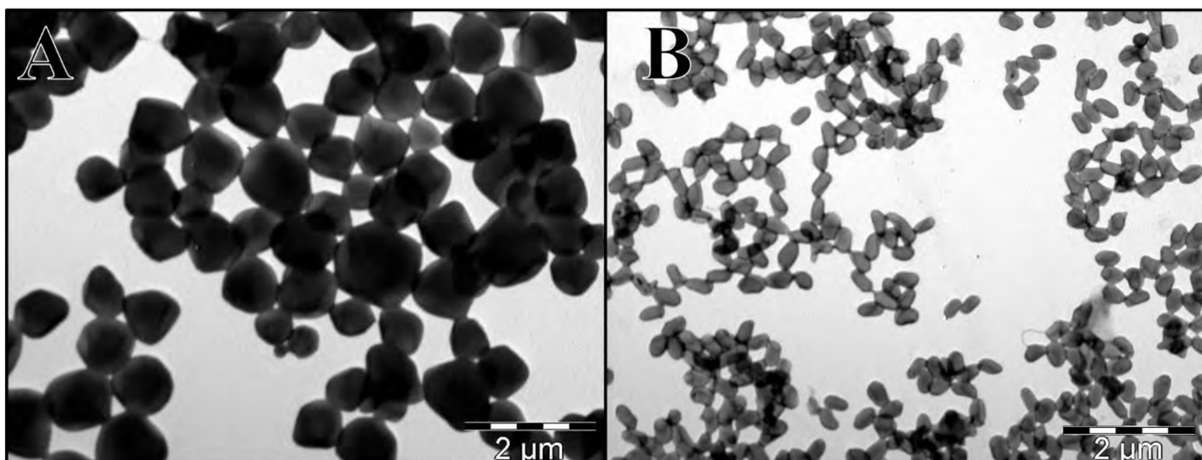

Figure 2.2. Transmission electron micrograph of A: CrpeNPV and B: CrleGV-SA, demonstrating the size difference between the two species.

2.3.2 **Molecular screening of virus stocks**

Following agarose gel electrophoresis (AGE), the DNA extractions from both CrpeNPV and CrleGV-SA stocks produced bands of greater than 10 000 bp (Figure 2.3A). The *pif-1* PCR product of CrpeNPV created a band of 170-200 bp while the *pif-1* PCR product of CrleGV-SA showed no sequence had been amplified. The -*pif-1* positive control showed a PCR product of 170-200 bp and the *pif-1* negative control showed that no sequence had been amplified. The *lef-4* amplicon of CrleGV-SA was 350-450 bp while the *lef-4* reaction of CrpeNPV produced no amplicon. The positive control of *lef-4* was shown to have a sequence length of 350-450 bp. The negative control of *lef-4* produced no bands after AGE (Figure 2.3B).

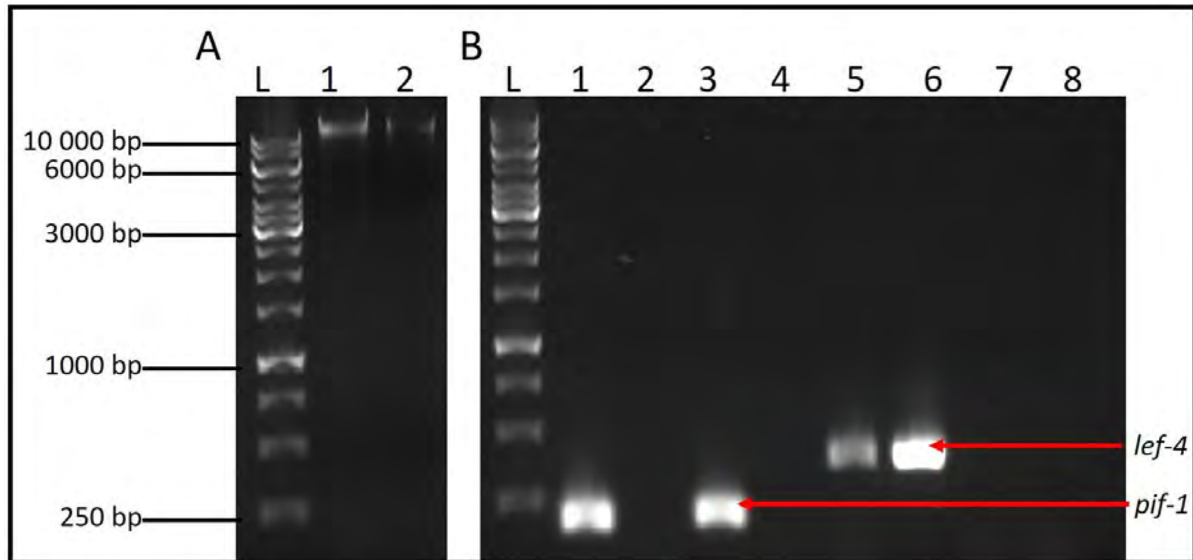

Figure 2.3. AGE of the initial screening of the CrleGV-SA and CrpeNPV virus stocks. The abbreviation bp denotes base pairs. Gel A, Lane L: Generuler 1kb ladder. Lane 1: Genomic DNA from the CrpeNPV stock. Lane 2: Genomic DNA from the CrleGV-SA stock. Gel B, Lane L: GeneRuler 1kb ladder. Lane 1: CrpeNPV *pif-1*. Lane 2: CrleGV-SA *pif-1*. Lane 3: *pif-1* positive control. Lane 4: *pif-1* negative control. Lane 5: CrleGV-SA *lef-4*. Lane 6: *lef-4* positive control. Lane 7: CrpeNPV *lef-4*. Lane 8: *lef-4* negative control.

2.3.3 **Occlusion body enumeration of virus stocks**

The stock of CrleGV-SA was found to contain 3.08×10^{11} OBs/mL and the stock of CrpeNPV was found to contain 1.92×10^{11} OBs/mL. The stock of the mixed OBs was created to contain 1.92×10^{11} OBs/mL where 50 % of the OBs were CrleGV-SA and 50 % of the OBs were CrpeNPV.

2.4 Discussion

The OBs of CrleGV-SA were extracted from cadavers of larvae that showed symptoms of baculovirus infection after being inoculated with the CrleGV-SA, and were purified using a glycerol gradient centrifugation protocol. The CrpeNPV OBs were provided by River Bioscience (Pty) Ltd. Confirmation of the presence of OBs was done using transmission electron microscopy, and the stocks were then screened to confirm that there was no contamination in either virus stock. Finally, enumeration of the virus stocks was done by counting OBs in a haemocytometer using darkfield microscopy.

The TEM results were consistent with the external morphology described by Jehle et al. (2006). The CrpeNPV virus stock contained OBs with a diameter of between 739 nm and 1304 nm,

falling in the size range of alphabaculoviruses described by Jehle et al. (2006) of between 150 nm and 3000 nm. While the shape of the OBs in the CrpeNPV stock was uneven, they were roughly as wide as they were long. Once again, this was consistent with the description of alphabaculovirus morphology in Jehle et al. (2006) and is also consistent with the morphology of CrpeNPV as described by Marsberg (2016). The shape of the OBs observed in the CrleGV-SA stock was different to the those of the CrpeNPV stock, being smaller and longer than they were wide. This shape is consistent with the description of the shape of betabaculoviruses given by Jehle et al. (2006) and is also consistent with the shape described by Moore (2002). The sizes of the OBs in the CrleGV-SA stock were smaller than those in the CrpeNPV stock, and consistent with the size described by Whitlock (1980), although they were larger than those described by Moore (2002), who reported a length of approximately 215 nm.

The AGE showed that the *pif-1* amplicon was slightly shorter than 200 bp, and was therefore consistent with the 187 bp reported by Jukes (2018) while the *lef-4* amplicon was slightly shorter than 400 bp, also being consistent with the length reported by Jukes (2018). While the amplicons were not sequenced, they were of the expected size, which suggests that each stock contained the expected virus, and was not contaminated. These results are limited by the sensitivity of the mPCR assay (Jackson, 2018), but they show as far as is possible that the stocks of each virus were pure. The test is more sensitive to CrpeNPV than to CrleGV, therefore the purity of a stock of CrleGV-SA can be confirmed to a higher degree than a stock of CrpeNPV.

The concentrations of 3.08×10^{11} OBs/mL and 1.92×10^{11} OBs/mL for the CrleGV-SA and CrpeNPV stocks respectively, provided a sufficient number of OBs to perform all of the experiments required for this study. There needed to be enough OBs to work with the LC_{90} concentrations for each virus against *T. leucotreta* neonates. The highest reported LC_{90} for each of these viruses against *T. leucotreta* neonates was reported by Jukes (2018) at 3×10^6 OBs/mL for CrleGV-SA and 2.75×10^6 OBs/mL for CrpeNPV. This means that at the concentrations for each stock were sufficient for the experiments to continue.

This chapter described the purification, enumeration and screening of the stocks of CrleGV-SA and CrpeNPV to be used in this study. The stocks were found to contain the desired OBs in sufficient quantities to continue with the study, and the stocks were shown not to be cross-contaminated. The next chapter details the process of quantifying the LC_{50} and LC_{90} for each virus and a mixture of the two viruses, as well as comparing the LC_{50} and LC_{90} of the mixed

infection with those of single infections to determine whether CrleGV-SA and CrpeNPV interact synergistically in mixed infections.

Chapter 3

Evaluation and comparison of the lethal concentrations of single and mixed infections of CrleGV-SA and CrpeNPV

3.1 Introduction

Since the overall aim of this study was to determine the relative virulence of a mixture of CrleGV-SA and CrpeNPV to *T. leucotreta,* the first objective was to determine the virulence of each virus in isolation, and to use these values as a benchmark to which the virulence of a mixture of the two viruses could be compared. Virulence of a pathogen can be experimentally determined by using biological assays to generate data that can be used to create mortality curves (Cory and Bishop, 1997). Mortality curves can be compared to evaluate the relative virulence of multiple treatments and to calculate doses at which a certain percentage of a population is killed. These can be calculated as doses or concentrations, depending on how the virus was administered, where dose denotes the number of OBs ingested while concentration is the concentration of OBs administered. In the case of dose being used, any point along the curve will be expressed as a LD_x value (L = Lethal, D = Dose, x = percentage of the population that is killed) or in the case of concentration, a LC_x value (C = concentration). Methods for performing dose-response biological assays to establish the virulence of baculoviruses against lepidopterans are diverse, but they can be classified into three types: droplet feeding, diet incorporation and surface-dose methods.

The three method types differ in the way that the virus is fed to the larvae. Droplet feeding involves starving the larvae and then feeding them a small droplet of water with the virus mixed in. Each larva must consume a droplet in its entirety, after which the larvae are placed on a food source containing no virus and allowed to feed (Pereira-da-Conceicoa et al., 2012). The rate of larval mortality is recorded after a certain time. The main advantage of this method is that the number of OBs consumed by each larva can be controlled more precisely than with alternative methods. This potentially results in an accurate impression of the true virulence of the baculovirus against the chosen lepidopteran species and can be used to calculate lethal dose rather than lethal concentration. However, the disadvantages are that the starvation of the larvae could cause excess stress. It can also be a difficult method to perform on very small larvae such as *T. leucotreta* neonates.

The second method is diet incorporation, which involves mixing the virus into the diet and then allowing the larvae to feed. This method can be used with very small larvae, but it is difficult to quantify the number of OBs consumed accurately, since the number of OBs consumed increases the more the larva feeds unless a small quantity of diet with a known number of OBs is given and the larva consumes it in its entirety (Grzywacz et al., 2004). Additionally, the method cannot be used when the diet is cooked, since baculoviruses begin to become inactivated above 36℃ and heat above 70℃ results in rapid inactivation (Chang et al., 1998). Finally, surface dose biological assays involve the application of the virus to the surface of diet, after which the larvae are allowed to feed. This method is useful for larvae that typically burrow into their food source, since they will take in a single dose of virus as they burrow through the surface. The number of OBs consumed by each larva cannot be precisely determined and so the dose-response must be determined in terms of concentration rather than number of OBs consumed. Cooked diet can be used for this method. There is the additional advantage of the method being the most similar out of the three to the application of pesticides in the field, and so it can be considered the most appropriate current method for the initial stages of the development of biopesticides. Additionally, this method has been used extensively to test baculoviruses against *T. leucotreta* neonates, providing a substantial background on which this study could be built (Moore et al., 2011; Opoku-Debrah et al., 2016; Jukes et al., 2017). For these reasons, the surface dose method was used for this study.

The purpose of this study was to determine whether virulence can be improved by a mixture of CrleGV-SA and CrpeNPV, as opposed to either of these viruses in isolation. Synergism between baculovirus isolates has gained increasing interest as a potential way to improve the efficacy of biopesticides (Jukes et al., 2017; Graillot et al., 2016). The development of resistance to CpGV-M in the field in France and Germany has renewed interest in baculovirus synergism as a potential tool for combatting resistance to baculoviruses (Graillot et al., 2016). Cases of synergism in mixed infections between baculovirus species have been recorded in a handful of cases, and there are even fewer reports of interactions between alphabaculoviruses and betabaculoviruses in mixed infections (Tanada and Hukuhara, 1971; Lara-Reyna et al., 2003; Jukes et al., 2017). It is an underexplored aspect of baculovirus biology although there is evidence dating back to 1971 that synergism between alphabaculoviruses and betabaculoviruses does occur, when synergistic interaction was reported between PsunGV and an unspecified alphabaculovirus in *P. unipunctata* larvae (Tanada and Hukuhara, 1971). Similarly, a synergistic interaction between AngeMNPV and EpapGV has been reported in *A.*

gemmatalis larvae, where proteins provided by EpapGV allowed for AngeMNPV to pass through the peritrophic membrane more easily and thus result in a more efficient infection (Biedma et al., 2015). More recently, Jukes et al. (2017) reported that CrleGV-SA and CrpeNPV were more virulent in a mixed infection than either virus by itself in larvae of *T. leucotreta.* This study adds to the work reported by Jukes et al. (2017), by confirming the trend reported and by including a different mixture of the two viruses.

The first objective reported in this chapter was to establish the LC_{50} and LC_{90} of each virus by itself against *T. leucotreta* neonates using dose-response biological assays. The next objective was to establish the LC_{50} and LC_{90} of a mixture of CrleGV-SA and CrpeNPV against *T. leucotreta* neonates. The final objective was to compare the LC_{50} and LC_{90} of the mixture to those of each virus in isolation to determine whether virulence could be increased by mixing the two viruses.

3.2 Materials and Methods

3.2.1 **Acquisition of neonates**

Eggs of *T. leucotreta* laid on wax paper were cut to fit into petri dishes and washed for 15 seconds in 1 % (w/v) sodium hypochlorite and rinsed in sterile water. The sheets were allowed to dry in a laminar flow cabinet after which they were placed in petri dishes, sealed with Parafilm and left in a Controlled Environment (CE) room at 27℃ until the eggs hatched.

3.2.2 **Surface-dose biological assays**

Methods for the surface-dose biological assays were adapted from Moore et al. (2011), Marsberg (2016) and Jukes (2018). Seven-day surface dose biological assays were done in 24-well trays. Two hundred and fifty grams of artificial FCM diet powder (Moore et al., 2014) was mixed with 270 mL distilled water and baked at 200℃ for 30 minutes in a glass dish of 31 cm × 20 cm × 6 cm, the top of which was covered with a layer of aluminium foil. Once baked, the diet was transferred into the wells of the 24-well trays. This was done inside a laminar flow cabinet. A 10 mL syringe with the end cut off was used to cut the diet to fit the wells and the piston of the syringe was used to compress the diet into the bottom of the wells. Each well received 100 µL of viral dilution and a single concentration was used per tray, with one tray receiving sterile, deionised water as a control. The concentrations of OBs for each virus are shown in Table 3.1. Viral doses were prepared using the method of serial dilution shown in Figure 3.1. For CrleGV-SA, the highest dose was 2×10^5 OBs/mL stepping down in increments

of 5 × dilution to 64 OBs/mL. For CrpeNPV and the 1:1 mixed treatment (stock preparation discussed in section 2.2.5), the highest dose was 3×10^5 OBs/mL (1.5×10^5 OBs/mL CrleGV-SA, 1.5×10^5 OBs/mL CrpeNPV) stepping down in increments of 3 × dilution to the lowest dose of 1.235×10^3 OBs/mL. Once the doses had been applied, the trays were allowed to dry in the laminar flow cabinet, after which neonate *T. leucotreta* larvae were transferred onto the surface of the diet, using a fine paintbrush to prevent physical damage to the larvae. The trays were then covered with a glass sheet, cut to fit underneath the lid of the 24-well trays, which prevented the larvae from moving between the wells or escaping the trays entirely. Trays were sealed with Parafilm™ (Bemis Company Inc., Wisconsin) and left in a CE room for seven days at 27℃ after which the larval mortality in each tray was recorded. Larvae were recorded as dead when they failed to respond to being touched with a sterile toothpick. This entire process was conducted three times.

Table 3.1. Concentrations of OBs used in the surface-dose biological assays (OBs/mL).

	Concentration (OBs/mL)	
	CrleGV-SA	CrpeNPV and Mixed Infection
Dose 1	2.00×10^5	3.00×10^5
Dose 2	4.00×10^4	1.00×10^5
Dose 3	8.00×10^3	3.33×10^4
Dose 4	1.60×10^3	1.11×10^4
Dose 5	3.20×10^2	3.70×10^3
Dose 6	6.40×10^1	1.24×10^3
Control	0	0

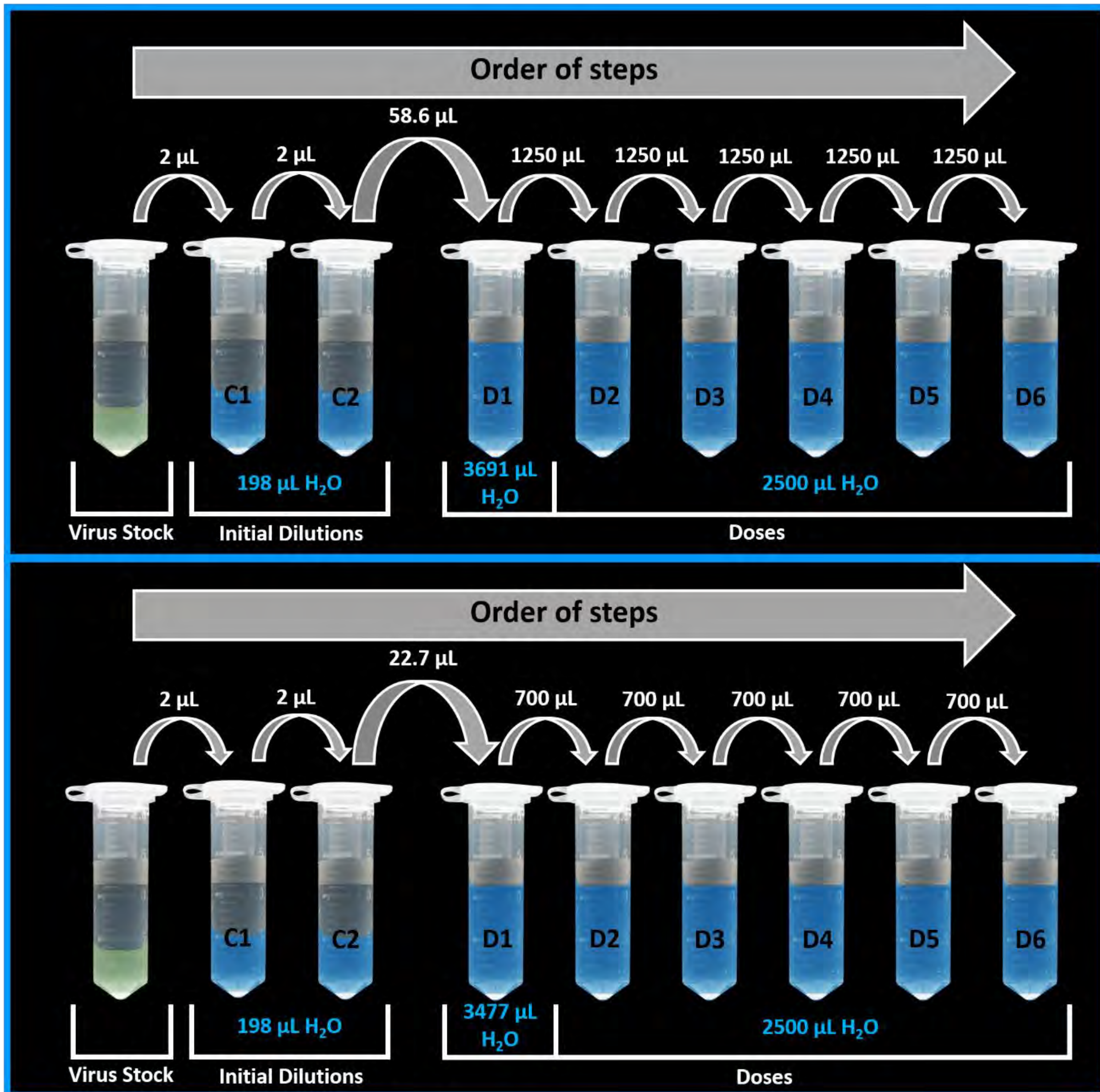

Figure 3.1 Serial dilution method used to produce the doses used. The same concentrations were used for CrpeNPV and the CrpeNPV + CrleGV-SA mixture (top), while different doses were used for CrleGV-SA by itself (bottom). C1: 1/100 dilution, C2 $1/1 \times 10^4$ dilution. D1: dose 1 (highest concentration) with decreasing concentrations denoted by higher dose numbers to D6 (lowest concentration). Final concentrations of each dose are shown in Table 3.1. (Adapted from Marsberg, 2016).

3.2.3 **Statistical analysis**

The data were analysed and corrected for control mortality using Abbott's Correction (Abbott, 1925), after which a probit analysis was performed in RStudio version 1.2.1335 © 2009-2019

RStudio, Inc. using the "drc" (Ritz et al., 2015) and "Hmisc" (Harrell et al., 2019) packages to determine two semi-lethal doses (LC_{50} and LC_{90}) of each viral treatment against *T. leucotreta*.

The mortality curves were compared using an ANOVA. The "EDcomp" function in the "drc" package was then used to determine which treatments differed from each other significantly at both the level of LC_{50} and LC_{90}.

3.3 Results

The purpose of the experiment was to determine whether virulence of a viral treatment to *T. leucotreta* neonates could be increased by using a mixed infection. CrleGV-SA was the least virulent of the three treatments, with the LC_{50} and LC_{90} doses being 1.53×10^4 and 4.10×10^5 OBs/mL respectively (Tables 3.2, 3.3). CrpeNPV was the second most virulent treatment with the LC_{50} and LC_{90} doses being 1.15×10^4 and 1.05×10^5 OBs/mL respectively (Tables 3.2, 3.3). The most virulent treatment was the 1:1 mixture of CrleGV-SA and CrpeNPV with the LC_{50} and LC_{90} values being 4.38×10^3 and 4.19×10^4 OBs/mL respectively (Tables 3.2, 3.3).

Table 3.2. Percentage larval mortality for each dose-response biological assay replicate.

	Mortality (%)		
	CrleGV-SA		
Dose (OBs/mL)	Rep 1 (n = 24)	Rep 2 (n = 24)	Rep 3 (n = 24)
0	8.33	8.33	0.00
6.40×10^1	8.33	4.17	8.33
3.20×10^2	12.50	8.33	16.67
1.60×10^3	25.00	29.17	20.83
8.00×10^3	37.50	37.50	33.33
4.00×10^4	62.50	70.83	62.50
2.00×10^5	91.67	95.83	79.17
	CrpeNPV		
0	12.50	4.17	4.17
1.24×10^3	16.67	20.83	12.50
3.70×10^3	50.00	41.67	20.83
1.11×10^4	50.00	54.17	37.50
3.33×10^4	79.17	83.33	29.17

1.00×10^5	100.00	100.00	87.50
3.00×10^5	100.00	100.00	95.83
	Mixed infection		
0	8.33	4.17	0.00
1.24×10^3	33.33	41.67	12.50
3.70×10^3	62.50	41.67	12.50
1.11×10^4	79.17	87.50	66.67
3.33×10^4	91.67	95.83	79.17
1.00×10^5	95.83	95.83	95.83
3.00×10^5	100.00	95.83	100.00

The ANOVA revealed that there was at least one significant difference among the treatments (Df = 4, LR = 52.87, $p < 0.0001$). The EDcomp function revealed that the mixed infection had a significantly lower LC_{50} than both CrleGV-SA (t = 2.91, p = 0.0036) and CrpeNPV (t = -8.40, $p < 0.0001$). However, the LC_{50} values for CrleGV-SA and CrpeNPV did not differ significantly from each other (t = 1.06, p = 0.288). The LC_{90} value of the mixed infection was also significantly lower than the LC_{90} values of both CrleGV-SA (t = 2.07, p = 0.038) and CrpeNPV (t = -5.08, $p < 0.0001$), but the LC_{90} values of CrleGV-SA and CrpeNPV did not differ significantly (t = 1.60, p = 0.110).

Table 3.3. Lethal concentrations of CrleGV, CrpeNPV and the mixed inoculation against *T. leucotreta* for seven-day surface dose biological assays. SE = Standard error.

	CrleGV		**CrpeNPV**		**50/50 GVNPV**	
	Concentration (OBs/mL)	SE	Concentration (OBs/mL)	SE	Concentration (OBs/mL)	SE
LC_{50}	1.53×10^4	3.021×10^3	1.15×10^4	1.46×10^3	4.38×10^3	6.36×10^2
LC_{90}	4.10×10^5	1.57×10^5	1.05×10^5	2.22×10^4	4.19×10^4	8.53×10^3

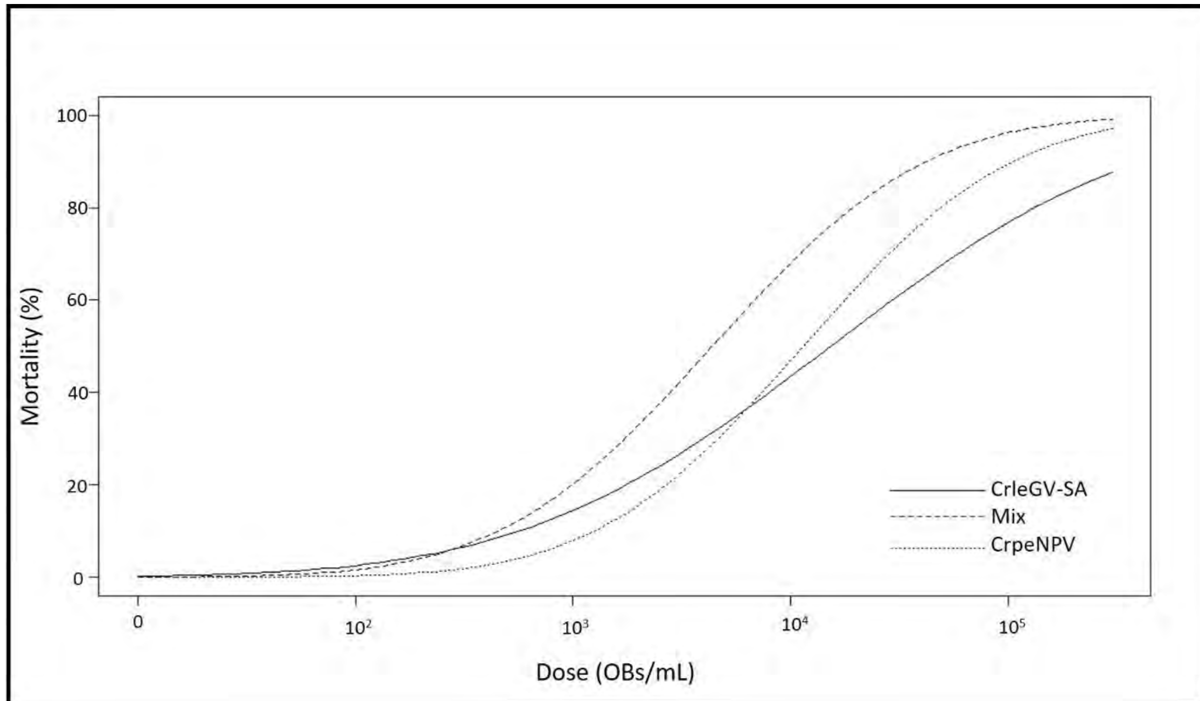

Figure 3.2. The dose-mortality curves of CrleGV-SA, CrpeNPV and the mixed inoculation.

3.4 Discussion

This chapter focused on the relative virulence of a mixed infection of CrleGV-SA and CrpeNPV on *T. leucotreta* larvae. This was achieved using biological assays, where the virus was administered using the surface-dose method, chosen because it best resembles the way the virus would be administered in the field and it also best resembles the way in which the larvae are likely to feed on the virus in the field. A probit regression model was used to determine the LC_{50} and LC_{90} values of each treatment. The treatments were then analysed by comparing the LC_{50} and LC_{90} values as a measurement of virulence.

Larvae of *T. leucotreta* are cryptic feeders. This means that they burrow into the fruit, often leaving no visible damage, and then feed inside the fruit for the rest of the larval phase (Daiber, 1979b). The only practical way to introduce the virus to the larvae in the field is by targeting the neonates as they attempt to burrow into the fruit. This can be achieved by applying OBs to the surface of the fruit before the larvae enter the fruit so that in the process of burrowing into the fruit, the larva inadvertently consumes OBs. Using a surface-dose protocol for performing dose-response bioassays was therefore the most comparable to the way in which the larvae would come into contact with the virus in the field.

The LC_{50} and LC_{90} values calculated for CrleGV-SA and CrpeNPV against neonates differ from those of previous studies. When compared to the values reported by Marsberg (2016), the LC_{50} and LC_{90} values for CrpeNPV were 5.2 × lower and 9 × lower, respectively, than those reported in this study. However, the reported LC_{50} and LC_{90} values of these two viruses against *T. leucotreta* neonates are not consistent, since another study by Jukes et al. (2017) reported the median lethal concentrations of CrleGV-SA and CrpeNPV to be 1.4 × higher and 6.1 × higher respectively than the results reported in this study (Table 3.4). The same trend was noted when comparing the LC_{90} values reported by Jukes et al. (2017) with the LC_{90} values reported in this study, being 13 × higher and 6 × higher for CrleGV-SA and CrpeNPV respectively. The LC_{50} for CrleGV-SA reported here falls within the range of results reported by Opoku-Debrah et al. (2016), with values between 1.89×10^5 OBs/mL and 7.10×10^5 OBs/mL for different isolates of CrleGV. The raw data from Jukes et al. (2017) and Marsberg (2016) were available, and so the calculations were performed again using the same statistical methods as were used in this study. Of the differences noted, the LC_{50} for CrpeNPV reported by Jukes et al. (2017) was significantly higher and the LC_{90} for CrpeNPV reported by Marsberg (2016) was significantly lower than the results reported in this study (Table 3.5). The LC_{90} for CrleGV-SA reported by Jukes et al. (2017) was significantly higher than that reported in this study. Variation in calculated lethal concentrations among studies is not uncommon and could be attributed to a number of factors including colony health, variation in the handling of larvae, variation in the enumeration of the virus stock, a difference in environmental conditions or differences in the way the lethal doses were calculated (Lacey and Kaya, 2013). This means that in future studies where lethal concentrations must be known at the beginning of a study, it is important for biological assays to be done by the person performing the study rather than using values from the literature. Because biological assays for each virus were performed for this study before the mixed infection was investigated, despite the lethal concentrations being available in the literature, a comparison between the virulence of each virus by itself and the mixed infection could reasonably be made.

Table 3.4. Re-analysis of surface-dose biological assay data for CrleGV-SA from Jukes et al. (2017) and comparison with results from this study. Significant differences in the results in this study are denoted by *** ($p < 0.0001$). Larvae per dose per replicate = 24. Replicates = 3.

Study	Lethal Concentration	Concentration (OBs/mL)	t value	p value
Jukes et al., 2017	LC_{50}	2.13×10^4	0.899	3.68×10^{-1}
	LC_{90}	$5.42\times 10^{6***}$	-4.72	2.33×10^{-9}

Table 3.5. Re-analysis of surface-dose biological assay data for CrpeNPV from Jukes et al. (2017) and Marsberg (2016) and comparison with results from this study. Significant differences in the results in this study are denoted by ** ($p < 0.001$), **** ($p < 0.0001$). Larvae per dose per replicate = 48. Replicates = 3 (Marsberg, 2016), Larvae per dose per replicate = 24. Replicates = 3 (Jukes et al., 2017).

Study	Lethal Concentration	Concentration (OBs/mL)	t value	p value
Marsberg, 2016	LC_{50}	$2.20 \times 10^{3***}$	4.23	2.35×10^{-5}
	LC_{90}	9.68×10^4	1.64	1.02×10^{-1}
Jukes et al., 2017	LC_{50}	$7.05 \times 10^{4**}$	3.43	6.10×10^{-4}
	LC_{90}	6.28×10^5	1.94	5.28×10^{-2}

The LC_{50} of the mixed infection was 2.3 × and 2.8 × lower than those of CrpeNPV and CrleGV-SA, and these differences were significant in both cases. When the LC_{90} values were compared, the mixed infection had an LC_{90} that was 5.1 × lower than the LC_{90} of CrpeNPV and 13 × lower than CrleGV-SA, although only the former difference was statistically significant. It was therefore established that a 1:1 mixture of OBs of CrleGV-SA and CrpeNPV was more virulent than either virus by itself at the 50 % mortality point, and more virulent than CrpeNPV by itself at the 90 % mortality point. It has been established in a previous study that CrpeNPV is significantly more virulent against *T. leucotrata* than CrleGV-SA (Marsberg et al., 2017). However, Jukes et al. (2017) found no significant difference between the two. While the difference in virulence was reported by Marsberg et al. (2017), it is important to note that the values were reported as lethal concentration. Since CrpeNPV has multiple virions per OB whereas CrleGV-SA only has one virion per OB, CrpeNPV may not be more virulent than

CrleGV-SA against *T. leucotreta.* This study found no significant difference between the virulence of CrleGV-SA and CrpeNPV, although the relatively large variance in the CrleGV-SA data may account for this. A previous study by Jukes et al. (2017) reported that mixtures of CrleGV-SA and CrpeNPV resulted in lower LC_{50} and LC_{90} values than either virus by itself, although the OB proportions of the mixed infection was different to that used in this study. However, the results calculated here are similar to those reported by Jukes et al. (2017), confirming that the interaction between CrleGV-SA and CrpeNPV in a mixed infection can be synergistic. This is the only other study that has investigated the interactions between these two viruses and so there are no more results to which these can be compared. However, other studies have investigated mixed baculovirus infections and found synergistic interactions between other baculoviruses. Tanada and Hukuhara, (1971) reported that that the virulence of an unspecified NPV in *P. unipunctata* could be increased by the addition of PsunGV OBs. They showed that this was due to a factor embedded in the protein matrix of PsunGV rather than being caused by the virion, and that this factor aided the primary stage of infection by the unspecified NPV. In the secondary stage of infection, the replication of PsunGV was limited by the NPV, decreasing the mortality caused by the secondary infection. This effect could potentially be removed by inactivating the added PsunGV. Another study showed that the LC_{50} of AngeMNPV against *Anticarsia gemmatalis* larvae could be almost halved by the addition of EpapGV, even though *A. gemmatalis* is not a host of EpapGV (Biedma et al., 2015). Yet another study reported synergism between AcMNPV and TnSNPV as well as between AcMNPV and Trichoplusia ni granulovirus (TnGV) in *T. ni* larvae, where the addition of TnSNPV decreased the LC_{50} by 8-fold, and the addition of TnGV increased the LC_{50} by 10.7-fold compared to AcMNPV alone (Lara-Reyna et al., 2003). The increase in virulence between CrleGV-SA and CrpeNPV in mixed infections in *T. leucotreta* larvae recorded in this study adds to the list of baculoviruses known to interact synergistically, showing that there is potential to improve the baculovirus-based biopesticides for the control of *T. leucotreta* in the future.

This chapter reported the biological activity in terms of dose-response for CrleGV-SA, CrpeNPV and a 1:1 mixture of both on *T. leucotreta* neonates. A combination of the two viruses resulted in a significant improvement in virulence, suggesting that CrleGV-SA and CrpeNPV interact synergistically. This means that both agents could be used in an area without reducing the mortality rate caused by either virus, and using both simultaneously could potentially

improve control of *T. leucotreta*. The next chapter describes and compares the speed of kill of CrleGV-SA, CrpeNPV and a 1:1 mixture of the two on *T. leucotreta* neonates.

Chapter 4

Evaluation and comparison of the speed of kill of single and mixed infections of CrleGV-SA and CrpeNPV

4.1 Introduction

In chapters 2 and 3, the virulence of pure viruses as well as a mixture of the two viruses and adapted methods to be used in this chapter to determine the speed of kill were described. The concentrations of each treatment that killed 50 % and 90 % of the larvae (LC_{50} and LC_{90} respectively) were established. These values could then be used to establish and compare the speeds of kill of CrleGV-SA, CrpeNPV and a 1:1 combination of the two.

Baculovirus synergism between CrleGV-SA and CrpeNPV has previously only been measured and compared in terms of dose-response by Jukes (2018), who found that mixtures of CrleGV-SA and CrpeNPV were more virulent than either virus alone, with a CrpeNPV-dominant mixture being more effective than a CrleGV-SA-dominant mixture. In chapter 3, it was shown that a mixture of the two viruses had a significantly lower lethal concentration than either virus alone. Since the improvement in the lethal concentration of a mixed infection has been established, it was important to investigate the speed of kill. While dose-response is a good measure of viral interaction, from a practical agricultural perspective, the speed of kill is very important, since this could affect the crop loss in the short-term (Moore and Jukes, 2019). Speed of kill has always been a limitation of pest control using baculoviruses, since the time from ingestion of OBs to larval death is typically more than 72 hours (Moore et al., 2011), where chemical pesticides typically kill faster. Time between application of a pesticide and mortality of the pest is time in which fruit can be damaged by the pest, resulting in monetary loss (Moore et al., 2011). Speed of kill is determined experimentally using time-response bioassays, where organisms are exposed to a high sublethal dose (typically LC_{90}/LD_{90} or higher) and the proportion of organisms killed over time is measured (Farrar and Ridgeway, 1998). A logit model can be used to determine the probability of death of an organism or the percentage of a population killed after a certain time interval post-exposure by calculating the points along the curve (Van Beek and Hughes, 1998). Results are reported as LT_x where LT =

“Lethal Time” (time after exposure) and x = the percentage of the population mortality in that time post-exposure.

Time-response bioassays are similar to dose-response bioassays in their design. There are the same three major types of time-response bioassays as dose-response (droplet feeding, diet incorporation, and surface-dose) with the same advantages and limitations, all having been discussed in chapter 3 (Boots and Begon, 1994; De León and Ibarra, 1995). Once again, a surface-dose protocol was chosen here, firstly, because a surface-dose protocol was used to determine the LC_{90} dose required for the time-response experiments, and secondly, because surface-dose best mimics the application of the virus in the field (Moore et al., 2015b). Neonates were chosen because they are the instar targeted in the field (Moore et al., 2011; Moore et al., 2015b).

This study used surface-dose time-response bioassays to determine the killing speed of CrleGV-SA, CrpeNPV and a mixture of both against *T. leucotreta* neonates. The data were then analysed further to determine whether the synergistic interaction between CrleGV-SA and CrpeNPV resulted in a significantly improved speed of kill.

4.2 Materials and Methods

4.2.1 **General methodology: optimisation of the time-response biological assay protocol**

The methods for time-response biological assays from Marsberg (2016) were modified for more effective monitoring of the larvae. Initial trial attempts using the methods described by Marsberg (2016) indicated that the larvae could not be observed at each time interval, due to there being excess diet. Survival and ease of observation were therefore compared using both 24-well plates and glass vials of 23 mm × 50 mm where different quantities of diet were used. For each replicate, 60 vials with cottonwool plugs and one glass lid were autoclaved, and one 24-well tray was washed in tap water containing 1 % (w/v) sodium hypochlorite. The glass vials were split into three sets of 20, where set one received the full quantity of artificial diet, set two received 40 % of the full quantity or artificial diet and set 3 received 20 % of the full quantity of artificial diet, where the full quantity was a single plug of diet prepared as described in section 3.2.2. Twenty of the wells of the 24-well tray received 20 % (diet plug thickness) of the full quantity of artificial diet. To prepare the diet for the vials receiving the full quantity, 250 g of artificial diet powder was mixed with 300 mL of dH_2O in a Pyrex baking dish, covered with a layer of aluminium foil and baked at 200 °C for 30 minutes. The top of each vial was

then stamped once into the diet and the diet was compressed into the bottom of the vial using the piston of a 10 mL syringe surface-sterilised with 70 % (v/v) EtOH (Figure 4.1). To prepare artificial diet for the vials receiving 40 % and 20 % of the full quantity of diet, and for the 24-well tray, 50 g of dry diet was placed in a Pyrex tray with 100 mL of deionised water and spread evenly over the bottom of the tray. The tray was then covered with a layer of aluminium foil and baked at 200 °C for 20 minutes.

The vials receiving the full diet quantity were stamped once each into the tray containing 250 g of diet, and the diet plug was then compressed into the bottom of the vial using the piston of a 10 mL syringe (Figure 4.1). The vials receiving 20 % of the full diet quantity were stamped once into the tray containing 50 g of diet and the vials receiving 40 % of the diet were stamped twice into the same tray. The diet was then compressed into the bottom of each vial as before. To transfer diet into the wells of the 24-well tray, a syringe with the end cut off (Figure 4.1) was used to stamp out diet from the tray containing 50 g of diet and the piston was then used to push the diet out of the tube and into each well. Diet was compressed into the bottom of each well using the piston of the syringe. Of the 24 wells available, 20 were used. Each vial and well then received a single *T. leucotreta* neonate which was transferred using a fine paintbrush to avoid causing physical harm

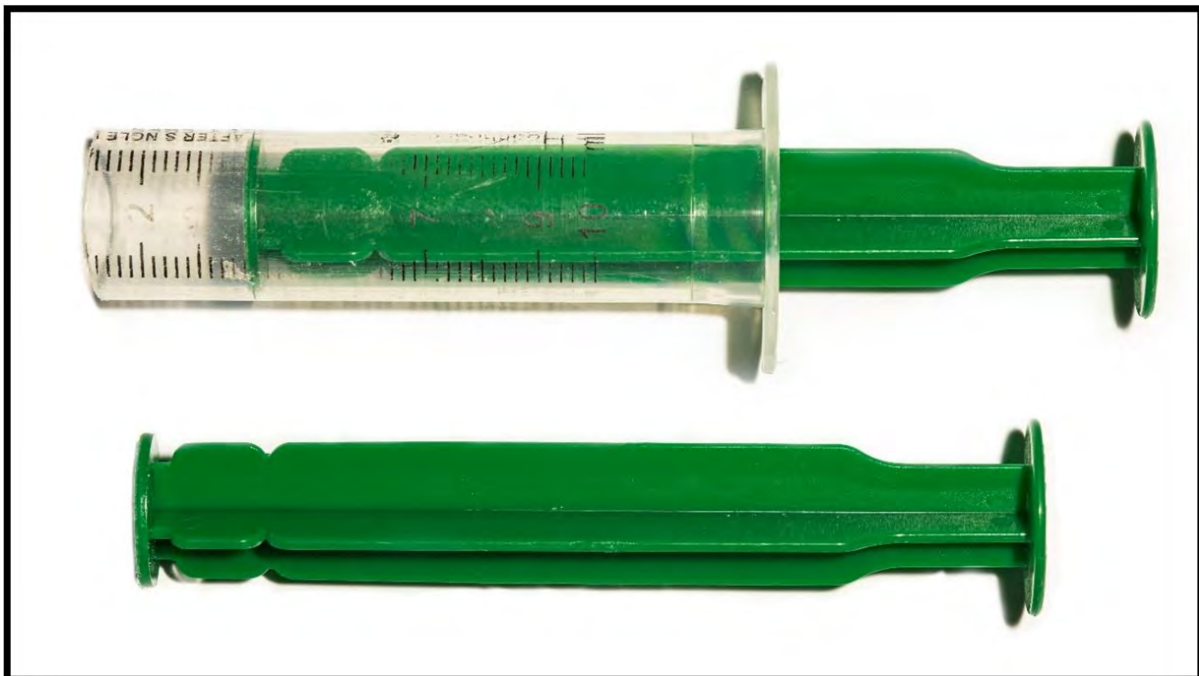

Figure 4.1. Modified 10 mL syringe used for cutting diet (top) and syringe piston used for compressing diet into wells and vials.

4.2.2 **Acquisition of neonates**

Sheets of wax paper on which *T. leucotreta* eggs had been laid were acquired from the Department of Entomology, Rhodes University, which has a captive colony. The sheets of paper were placed in petri dishes with a small amount of tissue paper in a corner that had been made wet with sterile deionised water. Each tray was sealed with Parafilm™ and stored in a CE room at 27℃ until the eggs hatched. Neonates were then used in the experiment within 8 hours of hatching.

4.2.3 **Time-response biological assays**

The methods for the time-response bioassays were adapted from Marsberg (2016) and altered to maximise the percentage of larvae that could be observed at each time interval (see sections 4.2.1 and 4.3.1). Fifty grams of artificial diet was mixed with 100 mL of deionised water in a glass baking tray with dimensions of 31 cm × 20cm × 6cm. The tray was covered with aluminium foil and the diet was then baked at 200 ℃ for 20 minutes. The diet was then stamped into sterile glass vials with an internal diameter of 23 mm and a height of 40 mm. The top surface of the diet of fifty vials was then treated with 437 µL of the concentration at which 90 % of larvae are killed (LC_{90}) of the chosen viral treatment and allowed to dry. The other fifty vials were treated with 437 µL of sterile deionised water. A single *T. leucotreta* neonate larva was transferred to the surface of the diet in each vial using a fine paintbrush to avoid damaging the larva. The vials were then closed with a plug of sterile cotton wool. Each vial was inspected for dead larvae for the first time after 16 hours, after which they were inspected every 8 hours until more than 95 % of the virus-treated larvae had died. The vials were inspected under a dissecting microscope at 0.8 × magnification. If a larva could not be located, its burrow was carefully opened with a sterile toothpick. This experiment was done in triplicate for each treatment (CrleGV-SA, CrpeNPV and a 1:1 mixture of the two). Larvae that died in the first 48 hours were counted as having died from handling error and so were eliminated from the pool. Larvae that did not die and showed no sign of infection were also eliminated, as they have no relevance in time-response experiments (Farrar and Ridgeway, 1998).

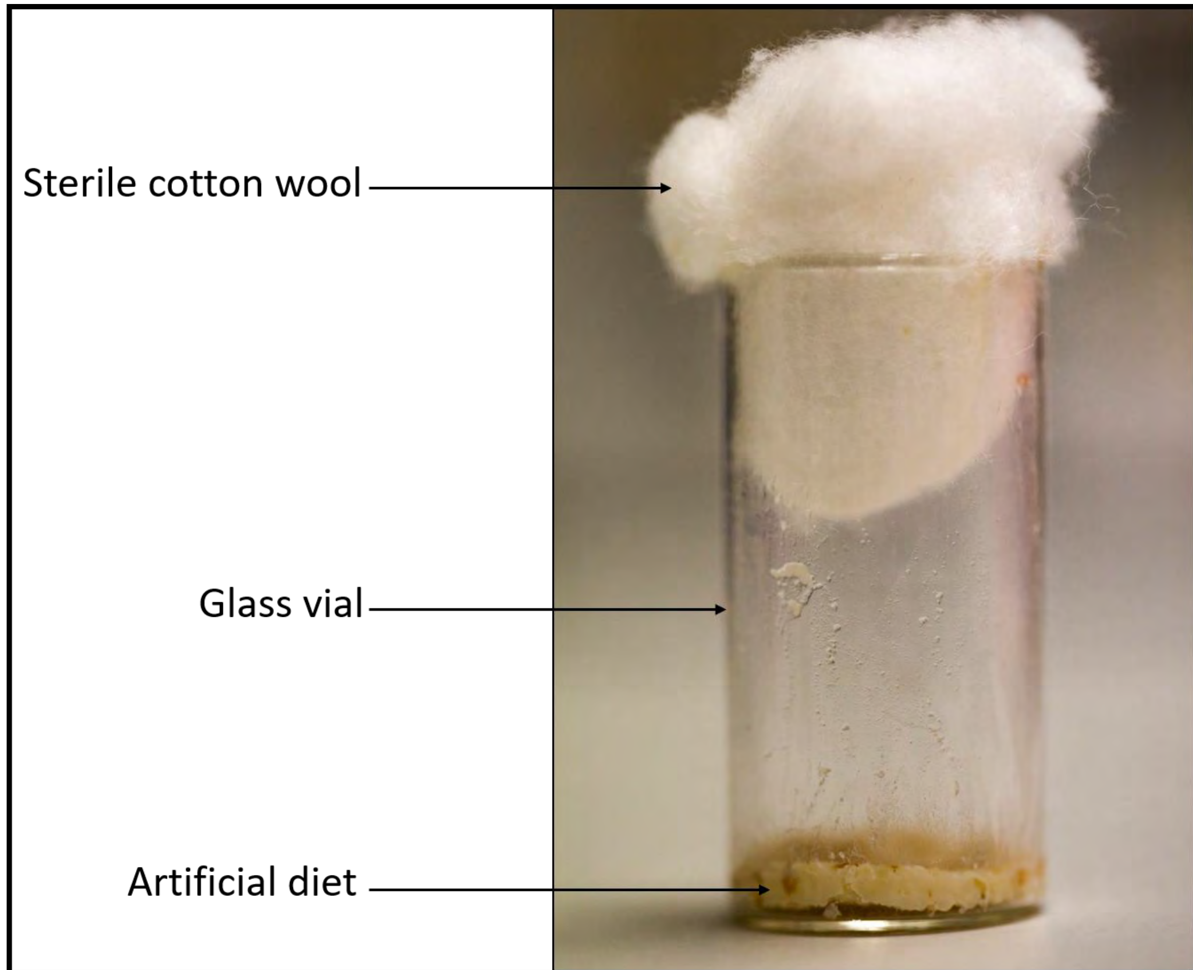

Figure 4.2. A vial complete with cotton wool and diet used in the time-response bioassays.

4.2.4 **Statistical analysis**

The data were analysed in RStudio using the packages "ecotox" (Hlina et al., 2019) to perform the logit analysis, "dplyr" (Wickham et al., 2019a), "tidyr" (Wickham and Henry, 2019), "tidyverse" (Wickham et al., 2019b), "tibble" (Müller and Wickham, 2019) to restructure and tidy the data, and "pacman" (Rinker and Kurkiewicz, 2017), "cowplot" (Wilke, 2020), "janitor" (Firke, 2020) and "ggplot2" (Wickham, 2016) to create figures from the analysed data. Cumulative mortality data were analysed using a logit generalised linear model. To test whether time and treatment were significant predictors of mortality, a Wald's chi squared test with type ii sum of squares was performed. Once time and treatment had been established as significant predictors of mortality, the LT_{50} and LT_{90} values were calculated from the logit model. The 95 % confidence intervals for the LT_{50} and LT_{90} values were plotted to show whether there were significant differences in the LT_{50} and LT_{90} values among the different treatments.

4.3 Results

4.3.1 **General methodology: optimisation of the time-response biological assay protocol**

Vials containing 20 % of the full quantity of diet (V20) provided the best opportunity to observe larvae, with the lowest mean percentage of larvae observed being 80 % at 28 hours and no obvious difference in larval health (Figure 4.3). Other diet quantities resulted in a lower percentage of larvae being observed, with the lowest mean percentages for 24-well trays (T20), vials containing 40 % of the full quantity of diet (V40) and vials containing the full quantity of diet (V100) being 45 %, 40 % and 55 % respectively (Figure 4.3). The mean percentage of larvae successfully observed across all time points for V20, T20, V40 and V100 were 89 %, 60 %, 59 % and 65 % respectively. There was no indication that the diet quantity affected the overall health of the larvae.

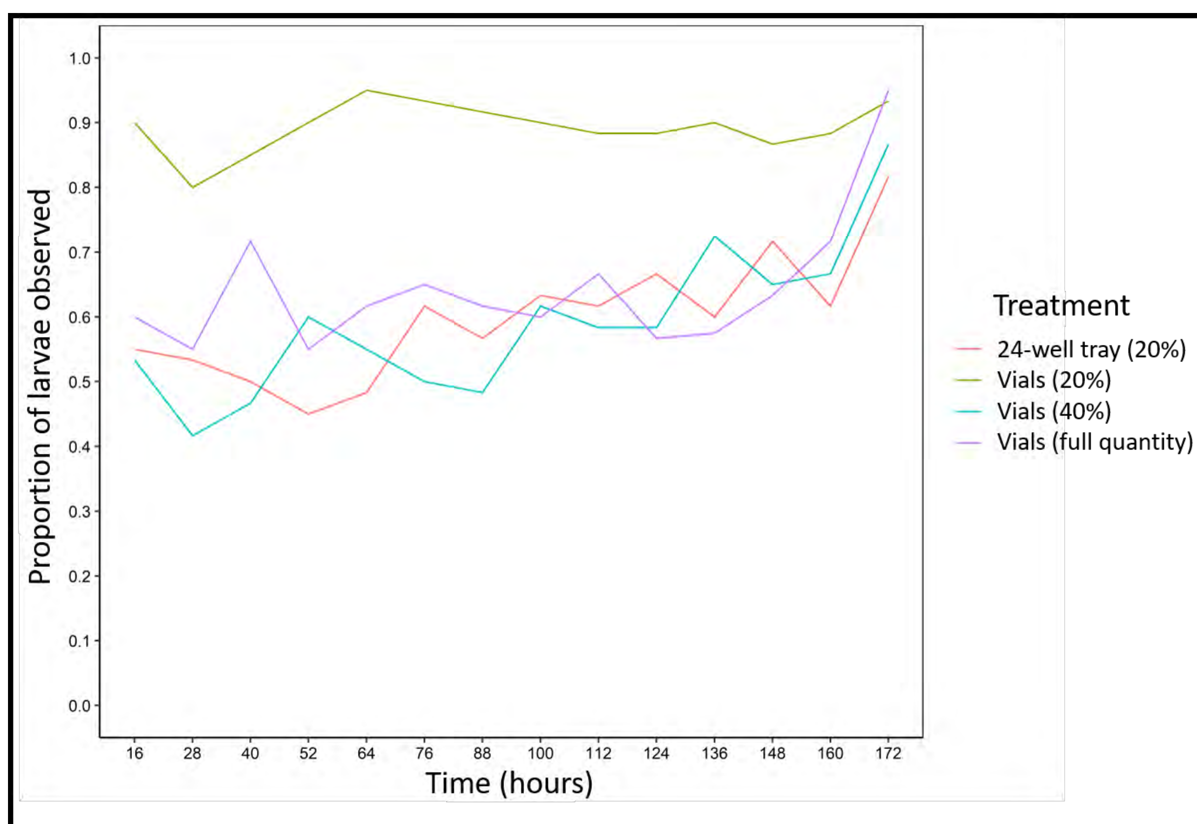

Figure 4.3. Proportion of larvae observed at each time interval. Red denotes the 24-well tray containing 20 % diet plug thickness, green denotes vials containing 20 % of the full quantity of diet, cyan denotes vials containing 40 % of the full quantity of diet, and purple denotes vials containing the full quantity of diet as a control.

4.3.2 **Time-response biological assays**

The purpose of this experiment was to determine and compare the LT_{50} and LT_{90} of CrleGV-SA, CrpeNPV and a 1:1 mixture of CrleGV-SA and CrpeNPV OBs when used on *T. leucotreta* neonates. Surface-dose time-response biological assays were performed on *T. leucotreta* using the LC_{90} values reported in chapter 3 (Table 4.1). A logit GLM was then used to calculate and compare the LT_{50} and LT_{90} values among the three treatments. Time-mortality curves (Figure 4.4) and confidence intervals (Figure 4.5) were then plotted.

Table 4.1. Total mortality of larvae in the time-response biological assays.

Virus	Replicate	Number of larvae	Total mortality (%)	Mean mortality (%)
CrleGV-SA	1	50	66	
	2	50	92	82
	3	50	88	
CrpeNPV	1	50	90	
	2	50	80	89
	3	50	97	
Mix	1	50	84	
	2	50	84	88
	3	50	96	

A Wald's chi squared test with type ii sum of squares revealed that both time (χ^2 = 578.98, df = 1, P < 0.001) and treatment (χ^2 = 16.28, df = 2, P < 0.001) were significant predictors of mortality.

Table 4.2. Percentage *T. leucotreta* larval mortality over time after being treated with the LC_{90} of each viral treatment.

Time (hours)	Mortality (%)								
	CrleGV-SA			CrpeNPV			Mixed Infection		
	rep 1 n=33	rep 2 n=46	rep 3 n=44	rep 1 n=45	rep 2 n=40	rep 3 n=58	rep 1 n=42	rep 2 n=42	rep 3 n=48
16	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
24	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
32	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0

40	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
48	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
56	0.0	0.0	0.0	2.2	10.0	3.4	0.0	0.0	0.0
64	0.0	0.0	0.0	4.4	15.0	5.2	0.0	0.0	0.0
72	0.0	2.2	4.5	6.7	15.0	5.2	0.0	0.0	0.0
80	0.0	4.3	6.8	8.9	17.5	10.3	2.4	0.0	0.0
88	24.2	6.5	11.4	13.3	20.0	10.3	7.1	4.8	4.2
96	51.5	10.9	13.6	26.7	30.0	13.8	7.1	7.1	10.4
104	63.6	17.4	13.6	33.3	35.0	20.7	7.1	21.4	16.7
112	72.7	32.6	34.1	46.7	42.5	27.6	7.1	35.7	31.3
120	84.8	39.1	40.9	57.8	60.0	60.3	7.1	47.6	41.7
128	84.8	47.8	43.2	57.8	67.5	70.7	11.9	52.4	52.1
136	93.9	47.8	59.1	66.7	80.0	79.3	16.7	59.5	64.6
144	100.0	69.6	72.7	77.8	87.5	87.9	19.0	64.3	70.8
152	100.0	73.9	95.5	84.4	92.5	93.1	31.0	71.4	75.0
160	100.0	80.4	100.0	84.4	92.5	93.1	38.1	78.6	83.3
168	100.0	95.7	100.0	91.1	95.0	93.1	50.0	81.0	89.6
176	100.0	100.0	100.0	95.6	100.0	93.1	59.5	88.1	89.6
184	100.0	100.0	100.0	97.8	100.0	93.1	64.3	95.2	95.8
192	100.0	100.0	100.0	97.8	100.0	93.1	69.0	100.0	100.0
200	100.0	100.0	100.0	97.8	100.0	94.8	71.4	100.0	100.0
208	100.0	100.0	100.0	100.0	100.0	98.3	76.2	100.0	100.0
216	100.0	100.0	100.0	100.0	100.0	100.0	81.0	100.0	100.0
224	100.0	100.0	100.0	100.0	100.0	100.0	85.7	100.0	100.0
232	100.0	100.0	100.0	100.0	100.0	100.0	92.9	100.0	100.0
240	100.0	100.0	100.0	100.0	100.0	100.0	95.2	100.0	100.0
248	100.0	100.0	100.0	100.0	100.0	100.0	97.6	100.0	100.0
256	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0
264	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0
272	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0
280	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0

The logit regression model (Table 4.2) showed that virus with the fastest speed of kill at the 50 % mortality point was CrpeNPV with a LT_{50} of 113.5 hours, followed closely by CrleGV-SA with a LT_{50} of 117.5 hours, although this difference was not statistically significant (Figure 4.5). At the point of 90 % mortality, the fastest-killing treatment was CrleGV-SA with a LT_{90} of 153.2 hours, followed closely by CrpeNPV with a LT_{90} of 159.3 hours, although once again, this difference was not statistically significant (Figure 4.5). The slowest-killing treatment at both the points of 50 % mortality and 90 % mortality was the mixture, with a LT_{50} of 139.0 hours and a LT_{90} of 193.4 hours. Both of these times are statistically significantly longer than both the LT_{50} and LT_{90} of CrleGV-SA, and the LT_{50} and LT_{90} of CrpeNPV (Figure 4.5).

Table 4.3. Logit regression data for all time-response treatments on *T. leucotreta* neonates.

Logit regression data								
		Slope	**SE**	***P*-value**	**Chi-Squared**	**intercept**	**SE**	***P*-value**
CrleGV-SA	1	25.29	2.236	<0.0001	14.02	-50.63	4.507	<0.0001
	2	19.7	1.174	<0.0001	26.72	-41.44	2.493	<0.0001
	3	21.2	1.356	<0.0001	45.59	-44.22	2.853	<0.0001
CrpeNPV	1	14.76	0.818	<0.0001	12.02	-30.46	1.717	<0.0001
	2	13.76	0.809	<0.0001	41.31	-27.91	1.673	<0.0001
	3	16.27	0.823	<0.0001	43.53	-33.62	1.724	<0.0001
Mixed Infection	1	15.81	0.889	<0.0001	35	-35.15	1.994	<0.0001
	2	18.19	1.094	<0.0001	22.3	-38.34	2.332	<0.0001
	3	19.73	1.151	<0.0001	13.06	-41.52	2.445	<0.0001

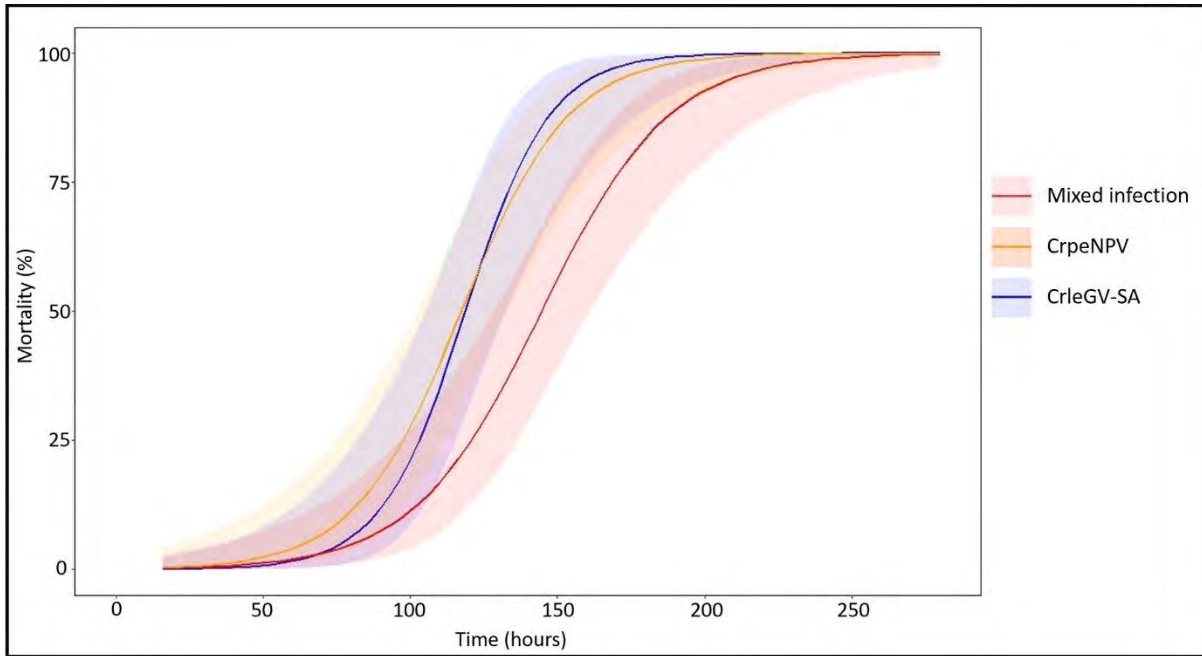

Figure 4.4. Time-mortality curves of CrleGV-SA (blue), CrpeNPV (yellow) and a mixed infection of CrleGV-SA and CrpeNPV (red) on *T. leucotreta* neonates. Shaded areas denote standard error.

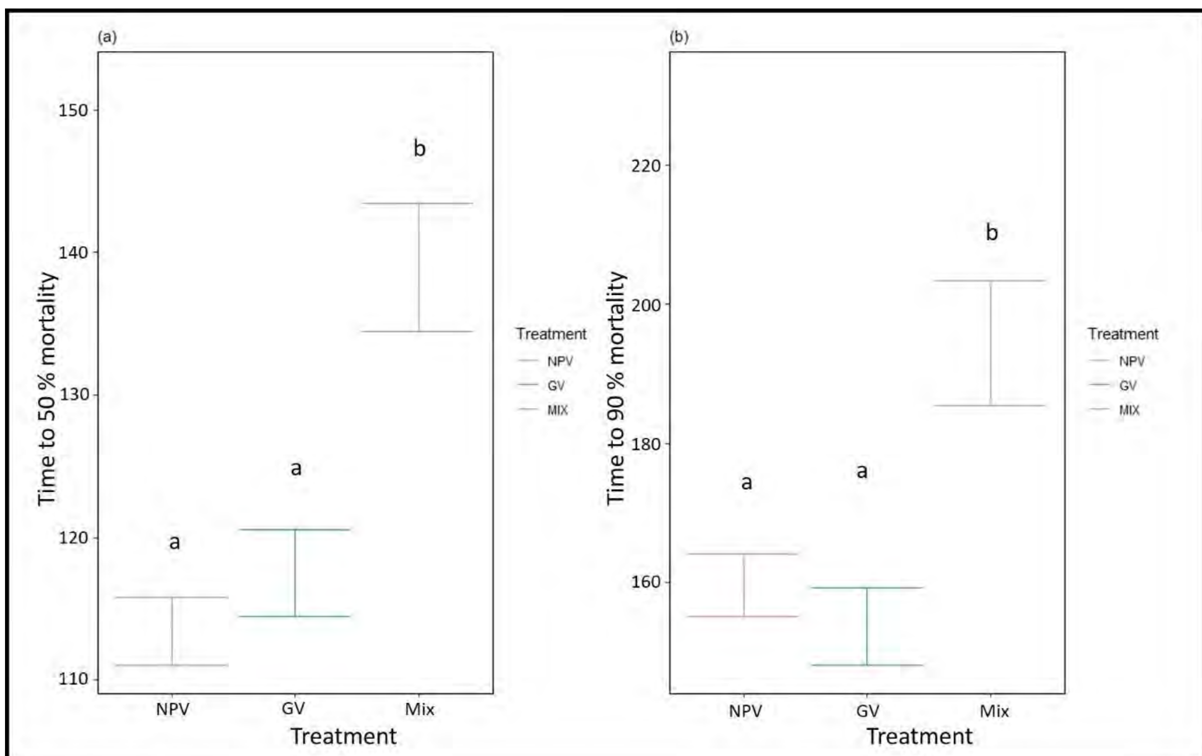

Figure 4.5. Confidence intervals (α = 0.05) for the LT_{50} (left) and LT_{90} (right) of CrleGV-SA (green), CrpeNPV (red) and a mixed infection of CrleGV-SA and CrpeNPV (blue) on *T. leucotreta* neonates. Different letters denote statistically significant differences.

4.4. Discussion

The purpose of this chapter was to establish and compare the speed at which CrleGV-SA, CrpeNPV and a 1:1 mixture of the two, kill *T. leucotreta* neonates. This was achieved by using a surface-dose time-response biological assay protocol adapted from Marsberg (2016). The data were then analysed and compared using a Wald's chi squared test with type two sum of squares and a logit regression model.

As with the dose-response biological assays, a surface-dose protocol was chosen to perform the time-response biological assays. For *T. leucotreta*, surface-treated diet is the most appropriate method of introducing the virus to the larva when performing biological assays. This is because *T. leucotreta* is a cryptic feeder (Daiber, 1979b) and so the only realistic way to introduce the virus into the larvae in the field is by spraying the surface of the fruit with a virus treatment before the larvae burrow through the rind (Moore et al., 2011).

The fastest killing viral treatment in this study was CrpeNPV, with a LT_{50} of 113.5 hours and a LT_{90} of 163.3 hours. While this treatment was the fastest-killing viral treatment, it was slower to kill than was reported by Marsberg (2016), as shown in Table 4.2. The LT_{50} reported here differs by 1 day and 9 hours, and the LT_{90} differs by 2 days and 15 hours to the values reported by Marsberg (2016) (Table 4.3). In contrast to this, the CrleGV-SA results reported in this study are comparable to those reported by Moore et al. (2011), with the LT_{50} being identical and the LT_{90} differing by less than 1 day (Table 4.3). This was the first study to use a single protocol and a single observer to perform time-response biological assays using CrleGV-SA and CrpeNPV against *T. leucotreta* neonates. Although the differences between the speed of kill of CrleGV-SA and CrpeNPV have been reported previously, it has only been done by meta-analysis (Marsberg, 2016). This study shows that, although CrpeNPV did appear to reach LT_{50} slightly quicker than CrleGV-SA, the difference was not as great as reported by Marsberg (2016) and the difference was not significant. It is important to note that Marsberg (2016) did not test whether the differences reported were significant. While Marsberg (2016) reported that CrpeNPV was substantially quicker than CrleGV-SA to reach 90 % mortality in *T. leucotreta*, this study found that CrleGV-SA was actually slightly quicker to cause 90 % mortality, although once again this difference reported here was not statistically significant. While the results reported here are very similar to the results reported by Moore et al. (2011), they differ substantially from the results reported by Marsberg (2016). This is potentially due to differences in the lethal concentration used or observer bias or slight differences in

methodology. While the total mortality reported by Moore (2002) for the time-response biological assays with CrleGV-SA are similar to those reported in this study, the mortalities reported for the time-response biological assays for CrpeNPV by Marsberg (2016) were substantially higher (Tables 4.1, 4.4). It is possible that the faster speed of kill reported by Marsberg (2016) could be attributed to a greater lethal dose being used than in this study. Nevertheless, the comparison of these results with Moore (2002) and Marsberg (2016) highlights the need for all time-response biological assays for any one study to be performed by a single observer.

Table 4.4. Total mortality of larvae in the time-response biological assays reported by Moore (2002) and Marsberg (2016).

Virus	Replicate	Number of larvae	Total mortality (%)	Mean mortality (%)
CrleGV-SA (Moore, 2002)	1	50	76	72
	2	50	62	
	3	50	78	
CrpeNPV (Marsberg, 2016)	1	50	94	95.3
	2	50	94	
	3	50	98	

Table 4.5. Comparison of time-mortality results reported in this study to time-mortality results reported by Marsberg (2016) and Moore et al. (2011).

Treatment		Time to Mortality (hours)		
		This study	Marsberg, 2016	Moore et al., 2011
CrpeNPV	LT_{50}	114	81	-
	LT_{90}	159	96	-
CrleGV-SA	LT_{50}	118	-	118
	LT_{90}	153	-	176
Mix	LT_{50}	139	-	-
	LT_{90}	193	-	-

This is the first study to report the time-mortality values of a mixture of CrleGV-SA and CrpeNPV on *T. leucotreta* neonates. While it was reported in chapter 3 that a mixture of CrleGV-SA and CrpeNPV was significantly more virulent than either virus by itself against *T. leucotreta* neonates, the mixture took significantly longer to kill than either virus by itself.

Since no other studies have investigated the time-mortality of a mixed infection of CrleGV-SA and CrpeNPV, the results reported here cannot be compared to any other results. However, time-mortality has been investigated with other baculoviruses on other lepidopteran pests. Biedma et al. (2015) reported that a mixed infection of EpapGV and AngeMNPV in 3rd instar *A. gemmatalis* larvae had a LT_{50} that was 2 days shorter than AngeMNPV by itself. In a different study it was reported that the addition of XecnGV to SpliNPV had an increased virulence against *S. litura* larvae over SpliNPV by itself, but that it had no significant effect on the speed of kill (Guo et al., 2007). It is important to note that in both of these cases, the granulovirus was not able to infect the larvae on which the mixture was being tested. The granulovirus OBs were used as a source of enhancin proteins that allowed the nucleopolyhedroviruses to move through the peritrophic membrane more easily (Biedma et al., 2015; Guo et al., 2007). This means that it would not be possible for the GVs and NPVs to compete with each other, since only one of the viruses was capable of replicating in the host cells. In contrast, *T. leucotreta* is a host to both CrleGV-SA and CrpeNPV, and thus there is the potential for competition between the viruses, potentially slowing down the time to larval mortality (Hefferon et al., 1999). The process of infection in a mixed infection needs to be investigated, since a mixed infection improves virulence in terms of the lethal concentration but causes the infection to take longer to kill. It is possible that one of the two viruses has a protein embedded in the OB that makes the peritrophic membrane more permeable to the other virus, increasing the potential for infection in the primary stage (Cheng and Lynn, 2009; Biedma et al., 2015). It is also possible that once the viruses have entered the cells and begin replicating, they compete in some way, slowing down the rate of replication in the systemic infection stage, thereby slowing down the speed of kill. These possibilities are speculation and need to be investigated.

This chapter reported and compared the biological activity of CrleGV-SA, CrpeNPV and a 1:1 mixture of CrleGV-SA and CrpeNPV OBs in terms of time-mortality. While chapter 3 reported a significant improvement in dose-response using a mixed infection, this chapter reported that the speed of mortality was slower for a mixed infection than for either virus by itself. This means that, although the control of *T. leucotreta* could potentially be improved in the long term by exploiting the increased dose-response of a mixed infection, a mixed infection could potentially result in increased fruit loss compared to single infection in the short term. Chapter 5 describes the use of multiplex PCR to determine which viruses were present in the cadavers from each treatment from the time-mortality biological assays.

Chapter 5

Molecular screening of baculoviruses recovered from *T. leucotreta* cadavers

5.1 Introduction

In Chapter 4 the speeds of kill of CrleGV-SA and CrpeNPV alone as well as a 1:1 mixture of the two were compared against *T. leucotreta* neonates. The 1:1 mixture had a significantly slower speed of kill than either virus alone, while the pure virus treatments were comparable to each other. It is therefore important from a pest-management perspective to know which viruses were present in the cadavers from each treatment as a first step in understanding how the viruses interact in mixed infections in *T. leucotreta*.

To be able to compare the different viral treatments, the cause of death of the larvae in each treatment must be known. However, covert infections of viruses not initially used to inoculate the larvae may be expressed due to stress caused by baculovirus infection (Burden et al., 2003), thereby potentially creating mixed infections when the larvae were only inoculated with a single virus species. Baculoviruses are transmitted either horizontally or vertically. Horizontal transmission requires there to be a high host density in the area to be successful, since it requires another individual to come into contact with the virus. Vertical transmission requires replication of the host to occur and is therefore directly affected by the fitness of the individual. Stresses that affect the potential fitness of an individual with a covert infection can trigger an overt infection, since a jump to a horizontal transmission strategy gives the virus a better chance of persisting and replicating when the reproduction potential of the host is compromised (Williams et al., 2017). One of the stresses known to trigger a covert infection to become overt is the overt infection by another baculovirus (Burden et al., 2003; Murillo et al., 2011; Jukes, 2018) It has been reported that when *T. leucotreta* larvae were inoculated with CrpeNPV, CrleGV-SA would often appear and even begin to outcompete CrpeNPV, and larvae inoculated with CrleGV-SA would occasionally also express CrpeNPV (Jukes, 2018). This phenomenon has the potential to skew the results. Screening for CrleGV-SA and CrpeNPV can be done by a multipex PCR reaction designed by Jukes (2018), described and discussed in Chapter 2.

Baculovirus infection of lepidopteran larvae often prevents or significantly reduces growth of the larvae due to reduced larval feeding (if an *egt* gene is absent) or due to the larvae succumbing to the infection before significant growth occurs (O'Reilly and Miller, 1991). This means that larvae inoculated with baculoviruses as neonates often fail to increase in size significantly from their original size. The result of this is that once the baculovirus infection has killed the larvae, the cadavers are of a similar size to the neonates, and therefore the baculovirus load is very small and traditional baculovirus DNA extraction techniques are not possible, since they usually involve the purification of OBs from later instar cadavers prior to the DNA extraction being performed (Jukes, 2018; Opoku-Debrah et al., 2013), and the quantity of tissue required exceeds the quantity available from a neonate-sized cadaver. It was therefore necessary to develop a DNA extraction protocol or modify an existing protocol to be suitable for the extraction of DNA from OBs inside neonate-sized cadavers.

This chapter describes the screening of cadavers from all of the time-response biological assays for CrleGV-SA and CrpeNPV and discusses the implications of covert infections and the simultaneous use of both viruses in the field. The first objective was to develop a suitable protocol for extracting DNA from OBs in neonate-sized cadavers. The second objective was to perform a multiplex PCR to screen the larvae from each viral treatment for CrleGV-SA and CrpeNPV, and the third objective was to perform the mPCR protocol using only *T. leucotreta* gDNA to eliminate that as a potential confounding factor in the reaction.

5.2 Materials and Methods

5.2.1 General Methodology: Optimisation of a DNA extraction method for baculoviruses in neonate-sized cadavers of *T. leucotreta*

Due to the small size of the cadavers, a DNA extraction method needed to be developed that minimised the loss of DNA. Five different extraction methods were attempted, all based on the CTAB extraction described by Opoku-Debrah et al. (2013). The first method (Method 1, Figure 5.1) was a crude extraction without cleaning steps. This involved the homogenisation of the cadaver in 5 µL ddH_2O, followed by the addition of 2.5 µL of 1M Na_2CO_3, after which the suspension was incubated at 37 °C for 30 minutes. Following the incubation step, 3 µL Tris-HCl (pH 6.8), 1.25 µL 10 % SDS (v/v) and 0.5 µL proteinase K (25 mg/mL) were added and the suspension was incubated at 37 °C for 30 minutes. This was followed by the addition of 0.5 µL of RNase A (10mg/mL) and the suspension was once again incubated at 37 °C for 30 minutes. An attempt to deactivate the proteinase K was made by heating the suspension to 95

°C for 10 minutes. This was done to prevent the proteinase K from denaturing the *Taq* polymerase during mPCR.

The second method (Method 2, Figure 5.1) involved performing the same process as the first method without the final 95 °C step. This was followed by the addition of 50 µL of ice-cold isopropanol and the suspension was then left at -20 °C overnight. The suspension was then centrifuged at 12100 × *g* for 20 minutes, after which the supernatant was discarded and 50 µL of ice cold 70 % (v/v) EtOH was added. This was followed by a centrifugation step at 12100 × *g* for 5 minutes, after which the supernatant was discarded. The tubes were left to dry, and the DNA was resuspended in 10 µL ddH_2O.

The third method (Method 3, Figure 5.1) involved the same steps as method 1 without the final 95 °C proteinase K deactivation step. This was followed by a centrifugation step at 12100 × *g* for 2 minutes. The supernatant was collected and placed into new tubes containing 10 µL CTAB buffer at 70 °C. An incubation step of 45 minutes at 70 °C was then performed. Following the incubation, 50 µL of chloroform at 4 °C was added to each tube and the tubes were inverted a few times. They were then centrifuged at 6700 × *g* for 10 minutes and the upper aqueous layers were added to tubes containing 50 µL of ice-cold isopropanol for precipitation. The suspensions were left overnight at -20 °C, after which the tubes were centrifuged at 12100 × *g* for 20 minutes. The supernatant was discarded and 100 µL of ice-cold 70 % (v/v) EtOH was added, after which the tubes were centrifuged at 12100 × *g* for 5 minutes. The supernatant was discarded, the tubes were left to dry and finally the DNA was resuspended in 10 µL of ddH_2O.

The fourth method (Method 4, Figure 5.1) involved using purified CrleGV-SA OBs in place of the template gDNA in the mPCR protocol, and the fifth method (Method 5, Figure 5.1) involved boiling purified CrleGV-SA OBs for 10 minutes and using them in place of gDNA in the mPCR protocol.

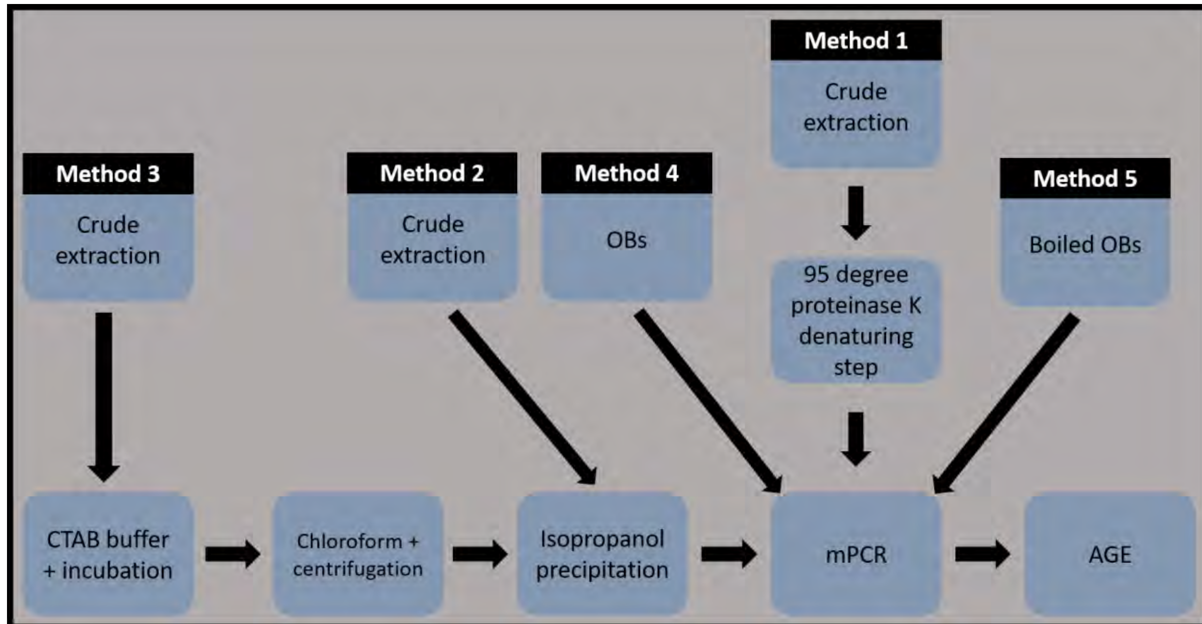

Figure 5.1. Flow diagram illustrating the methods used to develop an effective protocol for the extraction of baculovirus gDNA from neonate-sized *T. leucotreta* cadavers, as well as to assess different reagents for interference with the mPCR.

Since screening was to be done by mPCR, the success of the extraction methods was tested using the mPCR protocol. An initial PCR test was performed on just the first method, which was unsuccessful (see section 5.3.1). A second round of tests were then performed on all three extractions as well as tests using confirmed CrleGV-SA gDNA in combination with one of each chemical in the crude extraction to test whether the chemicals were to blame for the initial failure.

The PCR tests included 12.5 µL of *Taq* MasterMix, 1 µL of each primer (CpNPV-Pif1F, CpNPV-Pif1R, ClGV-L4F, ClGV-L4R) at 10 µM, 4 µL of DNA extraction product, and ddH_2O to increase the total volume to 25 µL. As well as this, a positive control containing 1 µL *Pif-1* and *Lef-4* regions, and a negative control containing no template was used. Additional reactions were set up in the same way, but each extraction was tested in a reaction, as well as CrleGV-SA and CrpeNPV gDNA in combination with a single chemical used in the crude extraction method (Na_2CO_3, SDS, proteinase K and Tris-HCl) in the same concentrations as the first attempt (Figure 5.2). The heat cycling was performed according to the methods described in chapter 2.

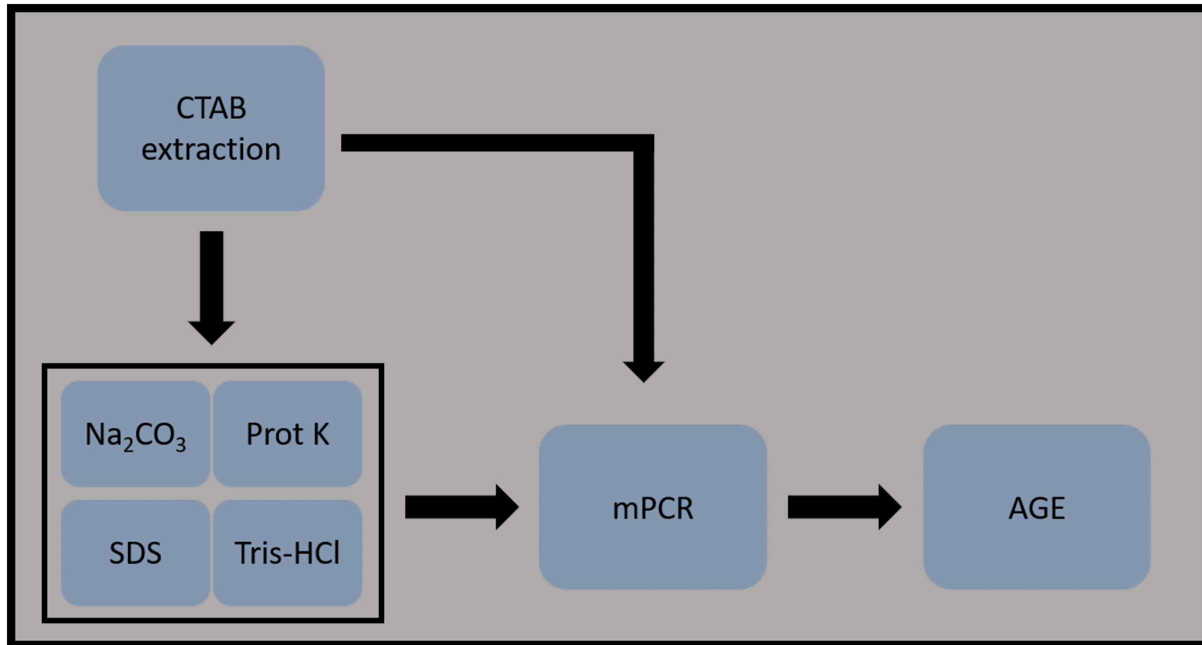

Figure 5.2. Flow diagram showing methods used to test the interference of reagents in the PCR process.

5.2.2 DNA extraction

DNA was extracted from OBs from cadavers from the time-response biological assays using a modified CTAB extraction method (Method 3) based on the method described by Opoku-Debrah et al. (2013). Three *T. leucotreta* cadavers from each time-response replicate (9 per treatment) were selected, placed in separate 1.5 mL tubes. They were then homogenised in 5 µL of ddH_2O using a sterile pipette tip. Once the cadavers had been homogenised, 2.5 µL of Na_2CO_3 (1 M) was added to each tube, after which they underwent an incubation period of 30 minutes at 37 °C. After the initial incubation period, 3 µL Tris-HCl (pH6.8), 1.25 µL SDS (10 % v/v) and 0.5 µL of proteinase K (25 mg/mL) were added to each tube and the incubation was repeated as before. Following the second incubation period, 0.5 µL RNase A (10mg/mL) was added to each tube and incubation was repeated as before. The tubes were then centrifuged at 2100 g for 2 minutes and the supernatants were collected and placed in new tubes containing 10 µL CTAB buffer at 70 °C. An incubation period of 45 minutes at 70 °C was then performed. Following the incubation, 50 µL of chloroform at 4 °C was added to each tube and the tubes were inverted a few times. The tubes were then centrifuged at 6700 × *g* for 10 minutes, after which the upper aqueous layer was collected from each tube and placed into new tubes containing 50 µL of ice-cold isopropanol. The tubes were left in a freezer overnight to promote precipitation of the DNA. They were then centrifuged for 20 minutes at 2100 × *g* and the

supernatant was discarded. One hundred µL of ice-cold 70 % (v/v) ethanol was added to each tube and the tubes were centrifuged for 5 minutes at 2100 × *g*. The supernatant was discarded and the tubes were left with the lids open in a heating block at 55 °C until the pellets were dry. The DNA was resuspended in 10 µL ddH_2O. The concentration of DNA was then measured using a NanoDrop™ 1000 spectrophotometer (Thermo Fisher Scientific, USA).

To extract DNA from larger *T. leucotreta* larvae that were not displaying signs of viral infection, the same protocol was used, but with volumes being: 200 µL ddH_2O for homogenisation, 90 µL Na_2CO_3, 120 µL Tris-HCl (pH6.8), 50 µL SDS, 20 µL proteinase K, 10 µL RNase A, 400 µL CTAB, 400 µL chloroform, 400 µL isopropanol, 1000 µL ethanol. The concentrations of each of these chemicals remained the same as above.

5.2.3 Multiplex polymerase chain reaction

The DNA extracted from each cadaver (3 per replicate, 9 per treatment) was screened for the presence of both CrleGV-SA and CrpeNPV using the multiplex PCR primers and protocol developed by Jukes (2018). Each PCR reaction received 12.5 µL Ampliqon 2 × MasterMix (Ampliqon, Denmark), 1 µL CpNPV-Pif1F (10 µM), 1 µL CpNPV-Pif1R (10 µM), 1 µL ClGV-L4F (10 µM), 1 µL ClGV-L4R (10 µM) oligonucleotides (Jukes, 2018), 5 µL of one of the gDNA extracted from one of the cadavers and the total volume was increased to 25 µL by adding the difference of ddH_2O. A positive control containing 2 µL of CrleGV-SA gDNA suspension and 2 µL of CrpeNPV gDNA suspension was also made, as well as a negative control containing ddH_2O in the place of gDNA. The thermal cycling protocol included an initial denaturation step of three minutes at 95 °C. After this, a denaturation step of 30 seconds at 95 °C was followed by an annealing step of 30s at 55 °C and an elongation step of 30 seconds at 72 °C. These three steps were repeated in order 30 times, after which a final elongation step of one minute at 72 °C took place. Samples were kept at 4 °C until they were analysed using AGE.

5.2.4 Agarose Gel Electrophoresis

Following the PCR, AGE was performed to determine whether the reaction was successful and to estimate the lengths of the amplicons produced in each reaction. A gel was also run to determine whether the CTAB extraction process was successful at extracting gDNA from *T. leucotreta* cells. For the PCR products, a 2% gel was used with ethidium bromide staining. The gel was run at 70 V for 45 minutes, while the *T. leucotreta* gDNA was run through a 1 % gel with ethidium bromide staining at 100 V for 45 minutes.

5.3 Results

5.3.1 **General Methodology: Optimisation of a DNA extraction method for baculoviruses in neonate-sized cadavers of *T. leucotreta***

The mPCR and AGE of the different extraction attempts showed that both the second method (crude extraction with a precipitation step) and the third method (full CTAB extraction with smaller volumes) were successful (Figure 5.3). The Na_2CO_3, SDS and proteinase K all interfered with the PCR reaction, while the Tris-HCl appeared to have no effect (Figure 5.3). The CTAB with reduced volumes was therefore chosen as the extraction method for the future extractions. Lanes 1A, 2A and 3A all produced bands of between 350-400 bp (*lef-4*), with lane 1A being the PCR product when using the standard CTAB reaction performed exactly as described by Jukes (2018) with purified CrleGV-SA OBs, lane 2A being the PCR product of the reduced-volume CTAB extraction (Method 3) and lane 3A showing the PCR product of the crude extraction with an isopropanol-precipitation step (Method 2) (Figure 5.3). Lane 4A depicts the PCR product when using the crude extraction method (Method 1) and it produced no bands showing that this technique was not suitable. Lanes 5A and 6A depict the PCR products when using purified CrleGV-SA OBs (Method 4) and boiled CrleGV-SA OBs (Method 5), respectively, in the place of gDNA. Both showed faint *lef-4* bands. Lane 7A was the positive control which produced two bands, one being *lef-4* and the other being *pif-1* (between 150-200 bp). Lane 1B (no-template control) produced no bands, and lanes 2B, 3B and 4B produced no bands. These lanes showed the result of a PCR that used a mixture of CrleGV-SA and CrpeNPV gDNA as the template, but also had an additional reagent added to test the potential of the reagents to interfere with the reaction. The lanes show the results for SDS, proteinase K and Na_2CO_3 respectively. Finally, lane 5B shows the products of the PCR using both CrleGV-SA and CrpeNPV gDNA as a template, but with the addition of Tris-HCl at pH 6.8. This produced bands of both *lef-4* and *pif*-1 (Figure 5.3).

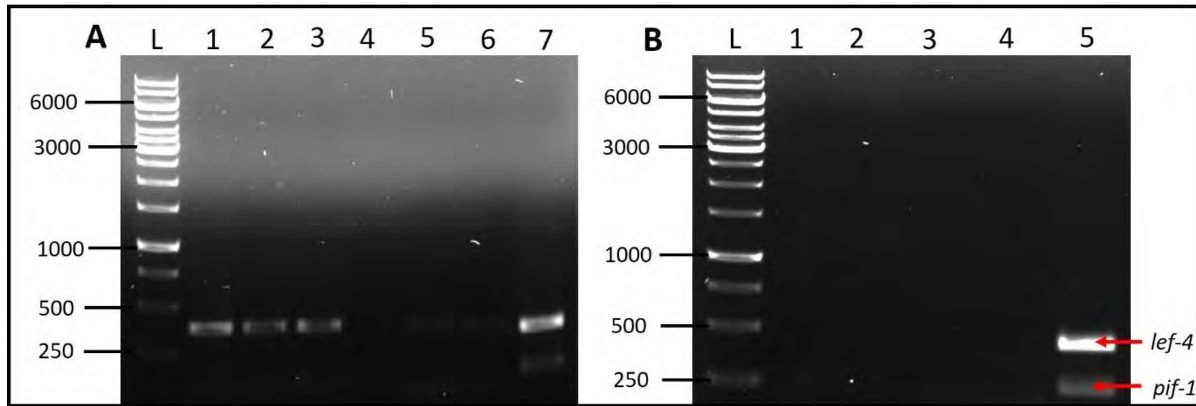

Figure 5.3. AGE with ethidium bromide staining of the mPCR products of different DNA extraction methods. A) Lane L: GeneRuler 1kb ladder, lane 1: CTAB extraction of purified CrleGV-SA OBs, lane 2: small-volume CTAB, lane 3: crude extraction with isopropanol precipitation step, lane 4: crude extraction, lane 5: direct CrleGV-SA OBs, lane 6: boiled CrleGV-SA OBs, lane 7: positive control, B) lane L: GeneRuler 1kb ladder, lane 1: no-template control, lane 2: SDS with CrleGV-SA and CrpeNPV gDNA, lane 3: proteinase K with CrleGV-SA and CrpeNPV gDNA, lane 4: Na_2CO_3 with CrleGV-SA and CrpeNPV gDNA, lane 5: Tris-HCl with CrleGV-SA and CrpeNPV gDNA.

5.3.2 Multiplex polymerase chain reaction

A multiplex PCR reaction described by Jukes (2018) was performed to determine which viruses were present in the cadavers of each reaction following a DNA extraction using the modified CTAB extraction technique (Method 3) developed in section 5.2.1. A 187 bp region of the *pif-1* gene of CrpeNPV and a 378 bp region of the *lef-4* gene of CrleGV-SA were screened to determine which viruses were present in each case.

Following PCR of the DNA extractions of the larvae treated with CrleGV-SA, 7 out of 9 showed *lef-4* bands (Figure 5.4). In 4 out of 9 cases, faint bands consistent with the expected size of the *pif-1* amplicon were present, suggesting the presence of CrpeNPV in the cadavers (Figure 5.4). Lanes 1-5, 7 and 8 showed amplicons consistent with *lef-4*, while lanes 9 and 10 showed no bands. Lane 6 showed a faint unidentified band of between 250-300 bp. In addition to the *lef-4* bands, lanes 1-4 also showed faint bands consistent with *pif-1* and lanes 2, 3, 5, 7 and 8 showed faint unidentified bands of between 250-300 bp (Figure 5.4). Lane 11 (positive control) showed bands of both *lef-4* and *pif-1*.

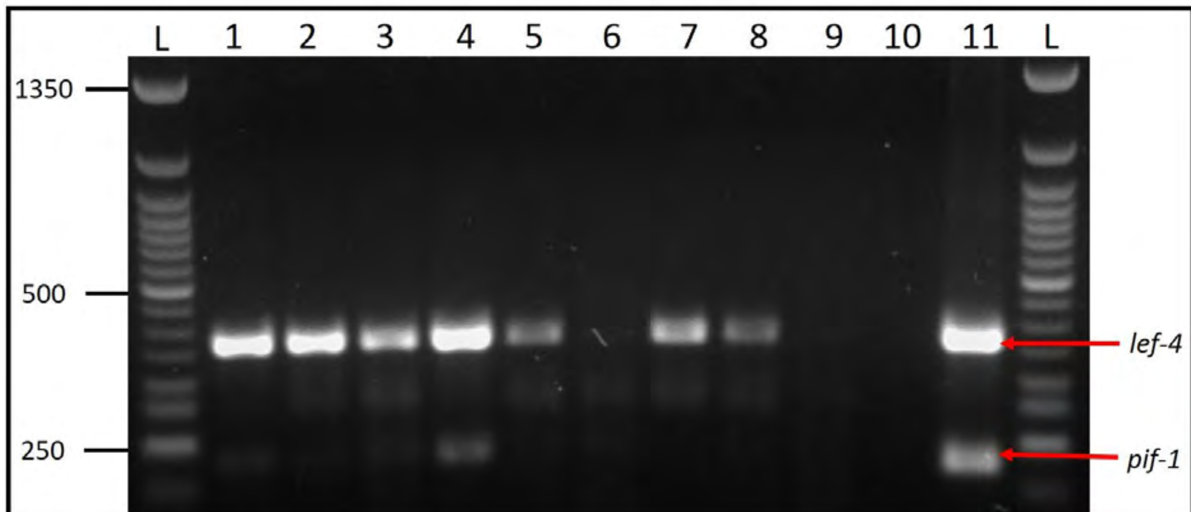

Figure 5.4. AGE with ethidium bromide staining of the mPCR amplicons resulting from gDNA extracted from larval cadavers inoculated with CrleGV-SA. Lanes 1 to 9: amplicons from DNA extracted from cadavers from the CrleGV-SA time-response biological assays, lane 10: no-template control, lane 11: positive control, lanes L: NEB 50bp ladder.

The AGE of the PCR products of larvae treated with CrpeNPV showed that *pif-1* amplicons were present in all 9 cases (Figure 5.5). Faint *lef-4* bands appeared in 5 out of 9 cases, and bright bands of the same size appeared in 3 out of 9 cases, suggesting the presence of CrleGV-SA in 7 out of 9 cases. Lanes 1-9 showed *pif-1* band. Additionally, lanes 1, 2, 5, 6 and 9 showed *lef-4* bands, suggesting the expression of covert infections of CrleGV-SA. Lanes 1, 2, 3, 6 and 9 also showed faint unidentified bands of between 250-300 bp. Lane 10 (no-template control) showed no bands, while lane 11 (positive control) showed both *pif-1* and *lef-4* (Figure 5.5).

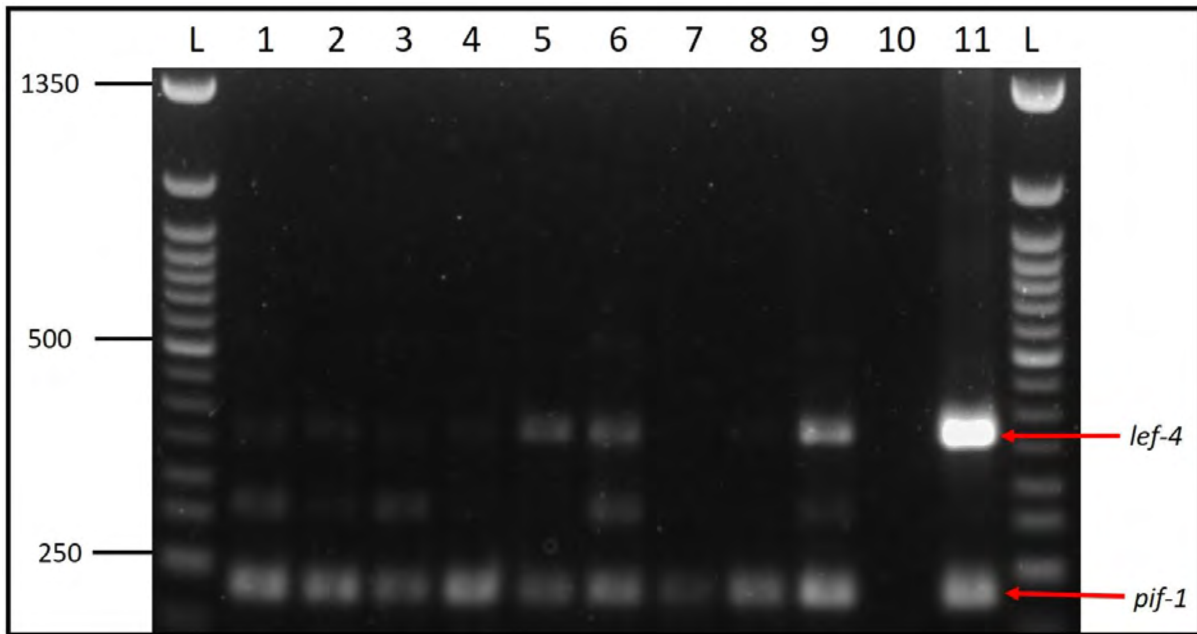

Figure 5.5. AGE with ethidium bromide staining of the mPCR amplicons resulting from gDNA extracted from larval cadavers inoculated with CrpeNPV. Lanes 1 to 9: amplicons from DNA extracted from cadavers from the CrpeNPV time-response biological assays, lane 10: no-template control, lane 11: positive control, lanes L: NEB 50bp ladder.

The AGE of the PCR products from the larvae treated with a 1:1 mixture of CrleGV-SA and CrpeNPV OBs produced *lef-4* bands in all 9 cases, and fainter *pif-1* bands in 8 out of 9 cases, suggesting the presence of both CrleGV-SA and CrpeNPV in 8 out of 9 cases and only CrleGV-SA in 1 out of 9 cases (Figure 5.6). In all cases the *lef-4* band was brighter than the *pif-1* band, suggesting that either CrleGV-SA becomes dominant over CrpeNPV in mixed infections or the DNA extraction methods or mPCR protocol favours one of the two viruses. Lanes 1-3 and 5-8 produced bands of both *lef-4* and *pif-1*, suggesting both CrleGV-SA and CrpeNPV were present in these cases. Lanes 4 and 9 showed only *lef-4*, suggesting the presence of only CrleGV-SA. Lanes 5 and 6 showed additional faint unidentified bands of between 250-300 bp. Lane 10 (NTC) showed no bands, and lane 11 (positive control) showed both *lef-4* and *pif-1* (Figure 5.6).

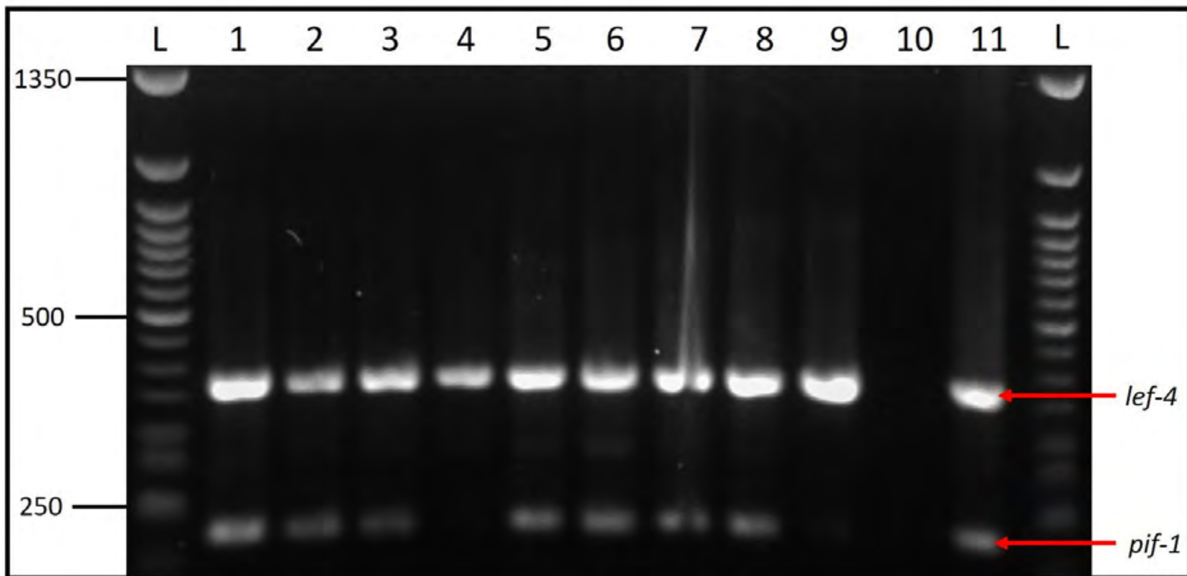

Figure 5.6. AGE with ethidium bromide staining of the mPCR amplicons resulting from gDNA extracted from larval cadavers inoculated with a 1:1 mixture of CrleGV-SA and CrpeNPV. Lanes 1 to 9: amplicons from DNA extracted from cadavers from the mixed infection time-response biological assays, lane 10: no-template control, lane 11: positive control, lanes L: NEB 50bp ladder.

A faint unidentified band of between 250 and 300 bp appeared on all three gels (Figures 5.4, 5.5, 5.6). This band was present in 6 out of 9 cases on the CrleGV-SA gel, including in one lane where no other bands appeared (Figure 5.4). On the CrpeNPV gel, this band appeared in 5 out of 9 cases (Figure 5.5), and on the mixed infection gel this band appeared in 3 out of 9 instances (Figure 5.6). It was never present in the positive control and it was never present in the no-template control. Multiplex PCR using *T. leucotreta* gDNA as the template produced no bands, discounting the possibility that the primers were binding to genomic DNA from *T. leucotreta* or gut bacteria from *T. leucotreta* larvae (Figure 5.7). Lanes 1-3 (*T. leucotreta* gDNA) and lane 4 (NTC) produced no bands and lane 5 (positive control) showed both *lef-4* and *pif-1* (Figure 5.7).

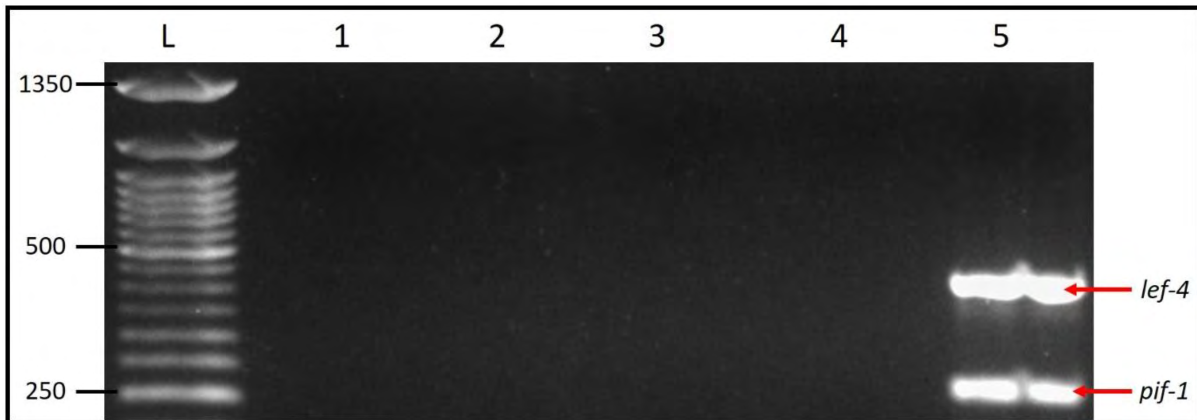

Figure 5.7. Products from the mPCR using *T. leucotreta* gDNA as a template. Lanes 1 to 3: products of the PCR reaction containing *T. leucotreta* gDNA, lane 4: no-template control, lane 5: positive control, Lane L: NEB 50 bp ladder.

5.4 Discussion

The purpose of this chapter was to investigate the virus or viruses responsible for the deaths of the larvae for each time-response biological assay treatment (described in chapter 4) and determine whether covert infections are expressed as a result of stress caused by viral infection. This was achieved by developing and using a modified CTAB DNA extraction method suitable for small quantities of viruses followed by a multiplex PCR and AGE for detection of viruses.

Covert infections were present in all treatments, although not in all larvae. In the CrleGV-SA treatments, of the 7 larvae that produced bands, only 4 produced bands of the correct length for CrleGV-SA and 3 produced two bands consistent with the lengths expected from both viruses. Where both bands were present, the band consistent with the length expected from CrpeNPV was faint. This suggests that covert infections of CrpeNPV are possible (Burden et al., 2003). This phenomenon has been reported once before by Jukes (2018). A similar phenomenon was observed in the CrpeNPV-treated larvae. All 9 samples produced bands consistent with the lengths expected from CrpeNPV, but 7 of the 9 samples also produced faint bands of CrleGV-SA. This suggests that CrpeNPV was dominant in the infection, but covert infections of CrleGV-SA began to be expressed. Finally, in the mixed infection, all 9 samples produced bands and 8 out of the 9 produced two bands consistent with the lengths expected for CrleGV-SA and CrpeNPV. In all of the cases where both bands were present, the band consistent with CrleGV-SA was brighter than that of CrpeNPV, despite the fact that the proportion of OBs used in the treatment was the same for each virus. It is important to note that the number of virions is higher per OB of CrpeNPV than CrleGV-SA, meaning that a 1:1 mixture of the two

is technically CrpeNPV-dominant (Marsberg, 2016; Jukes, 2018). In the mPCR test used, this could potentially be compounded by the fact that the test is more sensitive to CrpeNPV than to CrleGV-SA (Jackson, 2018). The dominance of the CrleGV-SA bands in the mixed infection suggests that CrleGV-SA began to outcompete CrpeNPV, a phenomenon also reported by Jukes (2018). This phenomenon has also been reported in other mixed baculovirus infections where one baculovirus begins to outcompete another for resources (Wennman et al., 2015).

The presence of unexpected bands with a length of between 250 and 300 bp remains unexplained. It was shown that this could not be explained by the oligonucleotides binding to regions of the *T. leucotreta* genome, or to regions of the genome of one of the gut bacteria of *T. leucotreta* larvae, since the protocol for DNA extraction and multiplex PCR produced no bands when performed on larvae showing no signs of baculovirus infection (Figure 5.5). The positive controls also remained free of this band. Due to the time restrictions placed on this study in conjunction with the lock-downs put in place by the South African government to deal with the SARS-Cov-2 pandemic, there was not sufficient time to isolate this band from the gels to be sequenced.

This is the second study to report covert infections of CrpeNPV in *T. leucotreta* larvae (Jukes, 2018). In both cases, the same captive *T. leucotreta* colony was used to perform the study. This is also the same colony against which CrpeNPV was originally tested (Marsberg, 2016) and it is therefore possible that, through exposure to the virus, the laboratory colony used for this study has acquired a covert infection of CrpeNPV that would not usually be present in wild populations. If this is the case, the use of CrpeNPV in the field as a tool for managing *T. leucotreta* could result in covert infections of CrpeNPV occurring in wild populations. The potential implications for this are that mortality could be increased when spraying CrleGV-SA in the field where covert infections of CrpeNPV occur as shown in the lethal concentration biological assays in this study as well as by Jukes (2018), although this may simultaneously reduce the speed of kill. Field studies to determine whether or not this is the case must be conducted as the information has implications for the management of *T. leucotreta.* Furthermore, the Rhodes University laboratory colony should be screened for covert infections of both CrleGV-SA and CrpeNPV, as this information may affect future studies with this colony.

Chapter 6

General Discussion

6.1 Thesis overview

The discovery of the synergistic interaction between CrleGV-SA and CrpeNPV in *T. leucotreta* (Jukes, 2018) opened the opportunity to study this interaction in greater depth. The first objective of this study was to acquire and enumerate pure stocks of CrleGV-SA and CrpeNPV OBs. The acquisition, purification, enumeration, and screening of these stocks was covered in Chapter 2. Pure stocks of CrpeNPV and CrleGV-SA were successfully obtained, allowing for a dose-mortality study to be conducted on *T. leucotreta* neonates, comparing pure virus inoculations and a 1:1 mixed inoculation of CrleGV-SA and CrpeNPV, described in chapter 3. The confirmation of improved lethal concentration in the mixed infection over single infections raised the question of the speed of kill of the single infections versus the mixed infection (Chapter 4). It was found that the mixed infection was significantly slower to kill than the single infections, which allowed for the study of the viral species composition in the cadavers of larvae from the time-response study. This was done using a multiplex PCR protocol and is described in chapter 5. Finally, this chapter discusses the findings of this study in relation to other literature and highlights some questions raised by this study for future research to answer.

6.2 Improved lethal concentration of a CrleGV-SA and CrpeNPV mixed infection

This study found that a 1:1 mixture of CrleGV-SA and CrpeNPV had a significantly reduced lethal concentration when compared to either virus alone when used against *T. leucotreta* neonates. In addition, it was shown that CrpeNPV alone was as effective as CrleGV-SA in terms of lethal concentration. While this was a study conducted using a captive colony of *T. leucotreta* in controlled conditions, it is a promising first step in improving the efficacy of the current baculovirus-based biopesticides that are used for the management of *T. leucotreta* in citrus orchards in South Africa. This was not the first instance of synergism reported between CrleGV-SA, with Jukes (2018) reporting that a 3:1 mixture of CrpeNPV:CrleGV-SA had an improved lethal concentration over either virus alone.

The improved virulence reported in this study may be a tool for future management of *T. leucotreta*. CrleGV-SA has been successfully used as a biological control agent for the management of *T. leucotreta* for 16 years (Moore et al., 2015b), and the longer an agent is used, the greater the chance of the development of resistance by the pest to that agent. The precedent for the development of resistance in the field to a baculovirus was set by *C. pomonella* in Europe, where field populations in France and Germany became resistant to CpGV-M (Asser-Kaiser et al., 2007; Eberle and Jehle, 2006). However, resistance to baculoviruses has been selected for in other controlled studies in many different pests (Abot et al., 1996; Reeson et al., 1998; Milks and Myers, 2000; Nakai et al., 2017). It is therefore important to identify novel baculovirus-based methods for the management of *T. leucotreta* to prevent resistance from developing, or as alternative tools for the management of *T. leucotreta* if resistance does develop.

This study has also shown that CrpeNPV is potentially a viable alternative to CrleGV-SA, should the latter become ineffective due to the development of resistance. This adds to the work reported by Marsberg (2016) and Jukes (2018) who also found that CrpeNPV was at least as effective in terms of dose-response as CrleGV-SA. In addition, the improved virulence of the mixed infection could be used in future baculovirus-based pesticide formulations to either decrease the quantity that is sprayed per hectare or increase the time between treatments, thereby reducing the cost of pest management and increasing the profit margins of farmers.

An added benefit of mixed infections is that they may be resistance-breaking in baculovirus-resistant pests. For example, it was shown that the resistance in *Cydia pomonella* to CpGV-M could be overcome by the addition of a second virus strain (CpGV-R5), even in low quantities, to a CpGV-M formulation (Graillot et al., 2016). While it would require further investigation to establish whether this would be the case for CrleGV-SA and CrpeNPV, it is possible that a mixed infection could overcome potential future resistance to a baculovirus in *T. leucotreta.*

6.3 Slower speed of kill of a mixed infection of CrleGV-SA and CrpeNPV

While this study found that a mixed infection had an improved lethal concentration, the cost of this appeared to be a reduced speed of kill. The reason for this phenomenon is unknown, although it is likely that the answer could be found in the typical progression of a baculovirus infection. This progression has two main parts: the primary infection and the secondary/systemic infection (described in section 1.3.3). It is possible that embedded in the crystalline protein matrix of one of the viruses are proteins that damage the peritrophic

membrane, allowing for a greater likelihood of primary infection of both viruses to occur, thereby reducing the concentration required for an infection to take hold. This phenomenon has been reported previously by Biedma et al. (2015), where the lethal concentration of AngeMNPV when used to infect *A. gemmatalis* larvae was improved significantly by the addition of the granulovirus of *Crocidosema* (=*Epinotia*) *aporema* (Walsingham) (Lepidoptera: Tortricidae), Epinotia aporema granulovirus (EpapGV), despite the fact that EpapGV can-not infect *A. gemmatalis*. Biedma et al. (2015) then investigated the reason for the improvement by inspecting the midguts of the larvae infected with AngeMNPV and comparing them to the midguts of larvae inoculated with a mixture of AngeMNPV and EpapGV. They found that there were factors in the protein matrix of the EpapGV that damaged the peritrophic membrane, increasing the size of the pores, thereby making it more permeable to AngeMNPV and increasing the probability of the viruses coming into contact with the midgut epithelial cells. This increased the efficiency of the primary infection, resulting in a lowering of the lethal concentration of AngeMNPV against *A. gemmatalis*. The protein that was likely responsible for this is encoded by the *Gp37* region of the EpapGV genome (Liu et al., 2011).

Similarly, the synergistic interaction between SpliGV and SpliNPV described by Guo et al. (2007) found that factors associated with the protein matrix of SpliGV, most notably enhancin, damaged the peritrophic membrane, aiding the passage of the viruses across the membrane. In a similar study an increase in the virulence of MabrNPV against *Autographa nigrisigna* (Walker) (Lepidoptera: Noctuidae) second instar larvae was achieved by the addition of granulovirus capsule proteins derived from OBs of XecnGV (Mukawa and Goto, 2011). Once again, this improved virulence was putatively ascribed to the disruption of the peritrophic membrane caused by the proteins.

What is common to these three studies is that the proteins responsible for the peritrophic membrane disruption are all derived from granuloviruses. It is therefore likely that the improved lethal concentration of the mixed CrleGV-SA and CrpeNPV infection is due to interference by proteins embedded in the CrleGV-SA OBs with the peritrophic membrane, allowing for the probability of an initial infection by CrpeNPV to increase. This scenario could also explain the longer speed of kill of the mixed infection over the single infection. The OB-associated proteins would only increase the efficiency during the primary stage of infection, allowing an infection to take hold and ensuring that the larva will die. However, in the secondary systemic stage of infection, there is the potential for the viruses to compete for

resources. While this would not eliminate the infection and would not prevent the ultimate death of the larva, it could potentially reduce the rate of viral replication and reduce the viral load, thereby slowing down the infection progression. This hypothetical phenomenon is not without precedent, as a study by Wennmann et al. (2015) showed that production of OBs was lower in mixed infections of Agrotis segetum nucleopolyhedrovirus (AgseNPV) and Agrotis segetum granulovirus (AgseGV) in *Agrotis segetum* (Denis and Schiffermuller) (Lepidoptera: Noctuidae) larvae than in single infections of either virus, suggesting the competition for resources between the two viruses.

The outcomes of this study have potential implications for the application of these viruses in the field. While overall larval mortality per unit of pesticide is very important in the field in the long term, the knockdown of pests is extremely important in the short-term, since it limits damage to fruit. Given that *T. leucotreta* larvae typically take around 4 days (96 hours) to penetrate citrus fruits (Moore et al., 2015b), a LT_{90} of 216 hours could potentially mean that fruit is damaged by the larva before the mixed infection kills the larva. While both CrleGV-SA and CrpeNPV fall short of the four-day threshold, they are close enough that the behaviour change caused by baculovirus infection could potentially prevent feeding before the larva has penetrated the fruit. It was observed, but not recorded, in the time-response assays, that larvae tended to stop feeding and reverse out of the diet a few days before dying. While this typically occurred within the 96-hour threshold in the CrleGV-SA and CrpeNPV biological assays, the proportion of larvae displaying a change in behaviour before 96 hours was far lower in the mixed infection biological assay. A difference in the size of the larvae at mortality among the different treatments could potentially be used in the future to indicate the difference in feeding of the larvae while they were still alive, but this was not measured in this study. Using a mixture of CrleGV-SA and CrpeNPV or spraying both on the same orchard without leaving a sufficient time-interval could potentially allow for fruit loss to occur before the *T. leucotreta* larvae are knocked down, although the improved lethal concentration should decrease the chances of live larvae being exported.

6.4 Molecular analysis

Molecular screening by mPCR of *T. leucotreta* cadavers was carried out to determine which virus(es) caused the deaths of the larvae in each treatment. This was done to show that the treatment being applied was also what killed the larvae, and it was also to test whether covert infections of either virus became overt during the progress of the disease. Both viruses were

present in the cadavers of larvae inoculated with the mixed suspension, but CrleGV-SA was also present in some of the larvae treated with CrpeNPV only and vice versa.

The appearance of a CrpeNPV infection in the cadavers of larvae inoculated with only CrleGV-SA could be explained by one of two scenarios. The first is that it is possible that there was slight contamination of the CrleGV-SA virus stock. While the stock was screened by mPCR, the technique has limited sensitivity. Jackson (2018) tested the sensitivity of the mPCR protocol by using serial dilutions of viral gDNA from both CrleGV-SA and CrpeNPV as the template portion of the reaction. This data was used to determine the minimum concentration of DNA of each virus required to obtain a positive result, with it being sensitive to CrleGV down to 3 pg/µL of gDNA for CrpeNPV and between 50-500 pg/µL gDNA for CrleGV-SA. This means that a negative result for a virus in a stock solution does not necessarily mean that the virus is not present, simply that it is not present in high quantities. It is therefore possible that slight contamination of the stock due to covert infection of the cadavers used for the creation of the stock resulted in unexpected infections occurring in the larvae in the time-response bioassays.

An alternative explanation is that the *T. leucotreta* colony used for this study carries a covert infection of CrpeNPV. While *T. leucotreta* is not currently considered a homologous host for CrpeNPV, NGS data detected the presence of an alphabaculovirus present in samples of OBs purified from diseased *T. leucotreta* larvae from as early as 2001 (unpublished data, Jukes, 2018). This was once again detected by mPCR in samples from 2013 onward by Jukes (2018), with the disparity in the earliest date of detection being put down to the differing sensitivities of the detection methods used. This NPV was initially named Thaumatotibia leucotreta nucleopolyhedrovirus (ThleNPV), but was found to be identical to CrpeNPV when the genomes were compared (Jukes, 2018). It is therefore probable that CrpeNPV is a virus that occurs naturally in a covert state in *T. leucotreta* populations and therefore in the captive colony. This is not surprising, since NPVs often have broader host ranges than GVs, with CrpeNPV so far being known to infect *C. peltastica, T. leucotreta* and *C. pomonella*, while CrleGV-SA is only known to infect *T. leucotreta* (Gröner, 1986; Moore and Jukes, 2019). CrpeNPV has not yet been tested against all of the pests closely related to *T. leucotreta*, but it is likely to infect *G. molesta* and *Thaumatotibia batrachopa* (Meyrick) (Lepidoptera: Tortricidae) as well (Moore and Jukes, 2019). It is therefore possible that the appearance of CrpeNPV was due to a covert infection of CrpeNPV becoming overt due to stress caused by CrleGV-SA infection (Burden et al., 2003; Williams et al., 2017). There are potential

implications for the production of CrleGV-SA, since CrleGV-SA production can only take place in *T. leucotreta*. If a covert CrpeNPV infection switches to an overt infection in the CrleGV-SA production process, the purity of the final product will be compromised, and the complications associated with the mixed infections discussed above may alter the efficacy of the pesticide. While CrleGV-SA appeared in cadavers inoculated with CrpeNPV, this is not as problematic for CrpeNPV production, since it can be produced in both *C. peltastica* and *C. pomonella*, both of which are non-permissive of CrleGV-SA infection.

6.5 Potential future research

This study reported improved virulence in terms of lethal concentration of a 1:1 mixture of CrleGV-SA and CrpeNPV. However, only one ratio of CrleGV-SA:CrpeNPV was compared. Jukes (2018) found that a CrpeNPV-dominant infection was more effective than a CrleGV-SA-dominant infection. Future research should optimise the ratio of CrpeNPV:CrleGV-SA for the highest virulence in terms of lethal concentration as a starting point to improve the current baculovirus-based biopesticides for management of *T. leucotreta*.

While this study reported that a mixed infection of CrleGV-SA and CrpeNPV in *T. leucotreta* larvae took significantly longer to kill the larvae than either virus alone, it may be possible to exploit the improved lethal concentration of the mixed infection while ensuring that competition between the viruses does not result in a decrease in the speed of kill. Firstly, the mechanism by which the mixed infection results in an improved lethal concentration must be established. This would involve the inspection of the peritrophic membranes of insects infected with both viruses as well as with each virus in isolation, and potentially larvae that have been fed with inactivated OBs of each virus. Similar studies have been done with other viruses and hosts. By using a combination of SDS-PAGE and scanning electron microscopy, Biedma et al. (2015) were able to determine that proteins embedded in the OBs of EpapGV were responsible for disrupting the peritrophic membrane of *A. gemmatalis*, causing an improvement in the lethal concentration of AngeMNPV. This would establish whether the improved lethal concentration is due to proteins embedded in the OBs disrupting the peritrophic membrane. If this is the case it would suggest that one of the viruses has a homologue of enhancin or gp37, or a novel protein with a similar function, embedded in the OB. A repeat of this study could be conducted using a mixed infection where one of the viruses is inactivated, thereby potentially supplying proteins to disrupt the peritrophic membrane without allowing competition between the viruses in the later stages of infection.

Similarly, a study should be conducted where a GV that produces enhancin or gp37 for which *T. leucotreta* is not a host is added to a formulation of either CrleGV-SA or CrpeNPV to provide peritrophic membrane-disrupting proteins without there being a possibility of viral competition. A similar study has been conducted on *A. gemmatalis* where the addition of EpapGV improved the efficacy of AngeMNPV without interfering with the reproduction of the virus in the later stages of infection (Biedma et al., 2015).

A study should also be conducted to analyse any potential impacts of the *per os* infectivity factors (PIFs) between the two viruses. These proteins control the entry of the virion into the insect cells. (Boogaard 2020). Should the PIF proteins of the two viruses inhibit the entrance of the other virus into a cell, this could be used as a potential explanation for the slower speed of kill of the mixture over either virus by itself. If the PIFs of each virus do inhibit each other, it is possible that this effect could be mitigated by spraying the different viruses asynchronously to give one virus the time to begin infecting before another is introduced.

Once more is known about the interactions between CrleGV-SA and CrpeNPV in mixed infections and ways to mitigate competition between the two viruses have been investigated, field trials must be conducted to test whether the results obtained in this study are applicable in the field. Initial field trials (unpublished data) seem to indicate that a mixed infection and CrpeNPV alone are less effective than the current commercial CrleGV-SA-based products.

6.6 Conclusion

The primary aim of this study was to test for synergistic interactions between CrleGV-SA and CrpeNPV. Both dose-response and time-response surface-dose biological assays were conducted and showed that a mixed infection of the two viruses provided an improvement in the lethal concentration against *T. leucotreta* neonates, but resulted in a deterioration in the speed of kill compared to either virus by itself. This provided further insight into the relationship and interactions between the two viruses in a mixed infection. These results may enable future investigation into the interaction between CrleGV-SA and CrpeNPV that could allow for the improvement of baculovirus-based biopesticides for the management of *T. leucotreta* in citrus orchards.

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