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The isolation and genetic characterisation of a novel alphabaculovirus for the microbial control of *Cryptophlebia peltastica* and closely related tortricid pests

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ABSTRACT

Cryptophlebia peltastica (Meyrick) (Lepidoptera: Tortricidae) is an economically damaging pest of litchis and macadamias in South Africa. *Cryptophlebia peltastica* causes both pre- and post-harvest damage to litchis, reducing overall yields and thus classifying the pest as a phytosanitary risk. Various control methods have been implemented against *C. peltastica* in an integrated pest management programme. These control methods include chemical control, cultural control and biological control. However, these methods have not yet provided satisfactory control as of yet. As a result, an alternative control option needs to be identified and implemented into the IPM programme. An alternative method of control that has proved successful in other agricultural sectors and not yet implemented in the control of *C. peltastica* is that of microbial control, specifically the use of baculovirus biopesticides. This study aimed to isolate and characterise a novel baculovirus from a laboratory culture of *C. peltastica* that could be used as a commercially available baculovirus biopesticide.

In order to isolate a baculovirus a laboratory culture of *C. peltastica* was successfully established at Rhodes University, Grahamstown, South Africa. This is the first time a laboratory culture of *C. peltastica* has been established. This allowed for various biological aspects of the pest to be determined, which included: length of the life cycle, fecundity and time to oviposition, egg and larval development and percentage hatch. The results obtained from these studies found that the biology of *C. peltastica* was similar to that of *Thaumatotibia leucotreta* (Meyrick) (Lepidoptera: Tortricidae).

Once the laboratory culture had reached high densities, larvae showing symptoms of baculovirus infection were observed. Symptomatic larvae were collected and examined for the presence of a baculovirus. An alphabaculovirus (NPV) was successfully isolated and morphologically identified using purified OBs that were sectioned and observed by transmission electron microscopy. This was then confirmed by amplifying the polyhedrin gene region using degenerate primers. A BLAST analysis found a 93% similarity to a partial polyhedrin gene sequence to be that of *Epinotia granitalis* (Butler) (Lepidoptera: Tortricidae). The alphabaculovirus was then genetically characterised by generating restriction profiles and sequencing the whole genome. Due to the novelty of the virus, no comparison could be made.

The biological activity of the alphabaculovirus was then tested against *C. peltastica* and two closely related Tortricidae pests: *T. leucotreta* and *Cydia pomonella* (Linnaeous) (Lepidoptera: Tortricidae). The alphabaculovirus was highly virulent against all three species. The lethal concentrations (LC_{50} and LC_{90}) for the virus against *C. peltastica* was 8.19×10^3 and 3.33×10^5 OBs/ml. The LC_{50} and LC_{90} for *T. leucotreta* was 2.29×10^3 and 9.97×10^4 OBs/ml, respectively and *C. pomonella* had a LC_{50} of 1.43×10^3 OBs/ml and LC_{90} 1.26×10^4 OBs/ml. The virus was particularly virulent against *T. leucotreta* and *C. pomonella* as compared to *C. peltastica*. The biological activity of the alphabaculovirus was also tested against CpGV resistant European *C. pomonella*. From the results it was observed that the virus had the ability to overcome the resistance in *C. pomonella* and could potentially be used in the resistance management of *C. pomonella*.

With the successful biological activity results obtained from this study, preliminary investigation were made into the mass production of the alphabaculovirus using both the *in vivo* and *in vitro* production methods. For *in vivo* production both the homologous host (*C. peltastica*) and a heterologous host (*T. leucotreta*) were investigated. Preliminary studies focused on determining the biological activity in fifth instars of both hosts. Fifth instar LC_{50} and LC_{90} values for *C. peltastica* were 3.43×10^3 and 1.11×10^7 OBs/ml and for *T. leucotreta* the LC_{50} and LC_{90} values were 2.53×10^3 and 8.82×10^6 OBs/ml, respectively. The average yield of virus produced in each species was also determined. *Cryptophlebia peltastica* had the highest viral yield of 5.37×10^{10} OBs/larva and 2.93×10^{10} OBs/larva for *T. leucotreta*. The results obtained, from the preliminary investigation concluded that the virus could be produced *in vivo* in both *C. peltastica* and *T. leucotreta*, however further research is required into the mass production in both hosts. The *in vitro* production of the virus was also considered and the susceptibility of the virus was tested against the *C. pomonella* cell line, Cp14R. After infection of the Cp14R cells with budded virus collected from fifth instar *C. peltastica* larvae, OBs could be observed after three days. Thus, the alphabaculovirus is susceptible to the Cp14R cell line, thus has the potential to be produced *in vitro* and further characterised.

This study is the first to report of the identification and characterisation of a novel alphabaculovirus isolated from a laboratory reared culture of *C. peltastica* and the potential for it to be commercially developed into a bipoesticide and used against Tortricidae pests.

TABLE OF CONTENTS

LIST OF TABLES

CHAPTER 4:

CHAPTER 5:

CHAPTER 6:

CHAPTER 7:

LIST OF FIGURES

CHAPTER 1:

CHAPTER 2:

CHAPTER 3:

CHAPTER 4:

CHAPTER 5:

CHAPTER 6:

CHAPTER 7:

ABBREVIATIONS

%	percentage
&	and
±	approximately
©	copyright
®	registered
°	degrees
°C	degrees Celsius
AcNPV	*Autographa californica* nucleopolyhedrovirus
AGE	agarose gel electrophoresis
AgNPV	*Anticarsia gemmatalis* nucleopolyhedrovirus
ApE	A plasmid editor
BLAST	Basic Local Alignment Tool
bp	base pair
CE	controlled environment
CGA	citrus growers association
cm	centimetre
CM	codling moth
CpGV	*Cydia pomonella* granulovirus
CrleGV	*Cryptophlebia peltastica* granulovirus
CrpeNPV	*Cryptophlebia peltastica* nucleopolyhedrovirus

CTAB	Cetrimonium bromide
DBM	diamondback moth
ddH_2O	double distilled water
dH_2O	distilled water
DNA	deoxyribonucleic acid
E	east
e.g.	example
egt	ecdysteroid UDP-glucoslytransferase
et al.	*et alia* (and others)
FCM	false codling moth
FCS	foetal calf serum
g	gram (s)
g	G-force
GmNPV	*Galleria mellonella* nucleopolyhedrovirus
GV	granulovirus
HearNPV	*Helicoverpa armigera* nucleopolyhedrovirus
i.e.	id est (that is)
ie	immediately early
IPM	integrated pest management
Kbp	kilobase pair
Kv	kilovolts
LC_{50}	median lethal concentration for 50% mortality
LC_{90}	median lethal concentration for 90% mortality

lef	late expression
LT_{50}	median lethal time for 50% mortality
LT_{90}	median lethal time for 90% mortality
M	molar
ml	millilitre
mm	millimetre
MOI	multiplicity of infection
n	number of replicates
Na_2CO_3	sodium carbonate
NCBI	National Center for Biotechnology Information
ng	nanogram
NGS	next-generation sequencing
nm	nanometer
NPV	nucleopolyhedrovirus
OB	occlusion body
ODV	occlusion derived viruses
ORF	open reading frame
p.i.	post infection
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PE	production efficiency
pfu	plaque-forming unit
PlxyGV	*Plutella xylostella* granulovirus

pM	picoMole
PO_4	phosphate
polH	polyhedrin
PR	productivity ratio
qPCR	quantitative PCR
R	rand (s)
REN	restriction endonuclease
RNA	ribonucleic acid
rpm	revolutions per minute
RT-qPCR	real time quantitative PCR
S	south
SDS	Sodium Dodecyl Sulphate
SE	standard error
SfNPV	*Spodoptera frugiperda* nucleopolyhedrovirus
SNPs	single nucleotide polymorphisms
TAE	tris-acetate EDTA
$TCID_{50}$	tissue culture infectious dosage
TEM	transmission electron microscope
Tris-HCL	tris-hydrochloric acid
UV	ultra violet
VHT	vapour heat treatment
µl	micro liter
µm	micro meter

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Chapter 1

LITERATURE REVIEW

1.1 INTRODUCTION

The advancement and growth in the human population has amplified the need for world-wide trade of agricultural products, and with this comes an increase in transport. The increase in population and travel will ultimately lead to the exponential growth in the number of pests establishing in new areas. These pests may have been naturally, accidently or intentionally introduced into these areas causing ecological and economic concerns on a global scale (Hajek *et al*. 2007). The major pest species causing economic damage in the agricultural sector are moths in the family Tortricidae. There are several tortricid species impacting the fruit industry, however the major species are; false codling moth, *Thaumatotibia leucotreta* (Meyrick); macadamia nut borer, *Thaumatotibia batrachopa* (Meyrick) (1908) (Lepidoptera: Tortricidae); litchi moth, *Cryptophlebia peltastica* (Meyrick) (1921) (Lepidoptera: Tortricidae); carnation worm, *Epichoristodes acerbella* (Walker) (1864) (Lepidoptera: Tortricidae); the oriental fruit moth, *Gapholita molesta* (Busck) (1916) (Lepidoptera: Tortricidae) and codling moth, *Cydia pomonella* (Linnaeous) (1758) (Lepidoptera: Tortricidae) (Timm *et al.* 2006). Three of these species are of major economic importance with overlapping host crops, macadamias and litchi: *T. leucotreta* (false codling moth), *T. batrachopa* (macadamia nut borer) and *C. peltastica* (litchi moth). Although *T. leucotreta* and *C. peltastica* are pests of litchis, co-existing in the same environment, *C. peltastica* causes 15% more damage than *T. leucotreta* (Timm *et al.* 2006; Timm *et al*. 2010; van den Berg *et al.* 2010).

1.1.1 The host plant

Litchi chinensis (Sonnerat) (Sapindales: Sapindaceae), also known as litchi or lychee, is a medium to large, tropical, evergreen tree that bears fruit on the terminal shoots, producing new

leaves and stems in successions of six to eight, with dormant stages (Storey 1973; Hieke *et al.* 2002). The pedicillate flowers are unisexual, with a panicle inflorescence (Banerji & Chaudhuri 1944). According to Jiang *et al.* (2003) the fruit is "small, conical, heart-shaped or spherical in shape and bright red in colour". *Litchi chinensis* is popular for its fleshy, juicy and sweet white translucent pulp which surrounds the large brown, glossy seed (Jiang *et al.* 2003). Before the fruit can be harvested, the tree undergoes several reproductive developmental stages including, panicle differentiation, panicle emergence and fruit maturation (Table 1.1) (Menzel 1984).

Table 1.1: Reproductive developmental stages of *Litchi chinensis* (Menzel 1984).

Stage	Duration (weeks)	Time
Panicle differentiation (initiation – emergence)	2-4	May - June
Panicle growth	5-8	July – Aug.
Flowering (anthesis, anther dehiscence and pollination)	3-6	Aug – Sept.
Fruiting (Fruit set – fruit maturity)		
• Mainly skin, embryo and tests growth	7-8	Oct. – Dec.
• Cotyledons and beginning of aril growth	2-3	
• Mainly aril growth	5-6	

The litchi tree originated from the southern subtropics of China and is a commercial crop in subtropical countries around the world including: Asia, Australia, India, Thailand, Taiwan, Reunion Island, Vietnam and South Africa, as well as the island of Hawaii (Jacobi *et al.* 1993; Batten *et al.* 1994; Jiang & Chen 1995). *Litchi chinensis* is a traditional Chinese delicacy that has been cultivated in South-East Asia for approximately 3500 years (Menzel 1983; Menzel 1985; Piper 1988). Some records show that the Chinese cultivated litchis as early as the second century BC or 1766 BC depending on official or unofficial records (Storey 1973; Waite & Hwang 2002).

Litchi chinensis was first introduced into Europe and America in the early 16th century when voyager's visited China. It was then further introduced into Burma and India, followed by introductions to tropical and subtropical countries around the world, during Magellan's circumnavigation in the 16th and 17th century (Storey 1972-73; Menzel 1983). The first official

record of litchis being cultivated in South Africa was in 1876, when litchi trees were imported from Mauritius. However, there are unofficial records that have found earlier imports of litchis, which were observed in Kwa-Zulu Natal in 1875. During this time a variety of cultivars were established in Kwa-Zulu Natal, which later spread to the lowveld of Mpumalanga and various other frost-free areas of South Africa (Figure 1.1) (Department of Agriculture, Forestry and Fisheries 2011 & 2013).

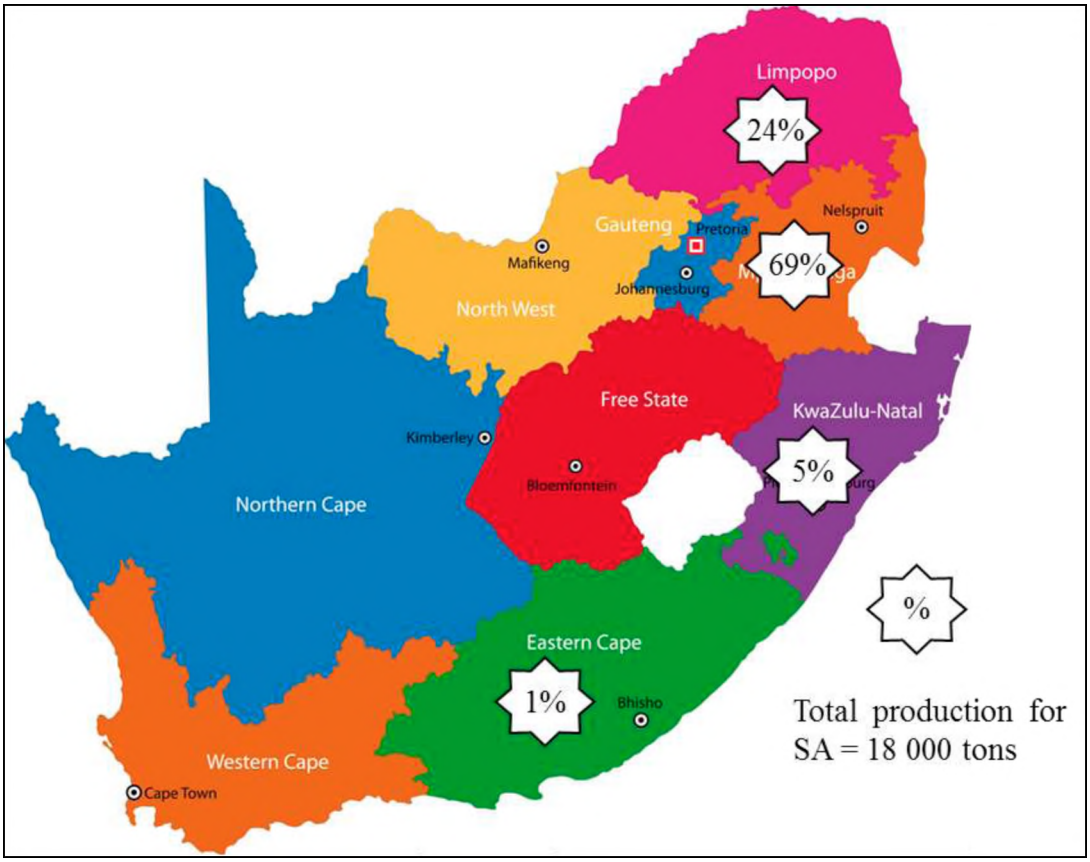


Figure 1.1: Main litchi producing provinces in South Africa (Department of Agriculture, Forestry and Fisheries 2011 & 2013).

According to a report from the Department of Agriculture, Forestry and Fisheries there are three different main cultivar groups of litchis that are grown in South Africa: Mauritius group, Chinese group and Madras group. The Mauritius group contain good quality fruit that produces higher yields than the other groups. They are therefore the preferred cultivar, both nationally and internationally. Some of the cultivars that are part of this group are: HLH Mauritius, Late Large Red, Rose-Scented and Saharan. The Chinese cultivar group contains fruit of exceptional

quality but produces lower yields than that of the Mauritius group (Figure 1.2). Cultivars that are found in this group are: Haak Yip, Kontand, Glutinous Rice, Shang Shou Haui and Three Months Red. The Madras group produces poor quality fruit (soft and watery flesh), as the majority of the energy is used to produce a larger seed and a richer red colour fruit. Fruit yields are unreliable, as they vary from year to year. Cultivars that belong in this group are: Bedana, Brewster, Durbhanga, Emmerson, Hazipur, Johnstone's Favorite, Kafri, McLean's Red, Maries and Mooragusha. The majority of cultivars grown in South Arica are the HLH Mauritus (75%) and McLean's Red (23%) (Figure 1.1) (Department of Agriculture, Forestry and Fisheries 2011 & 2013).

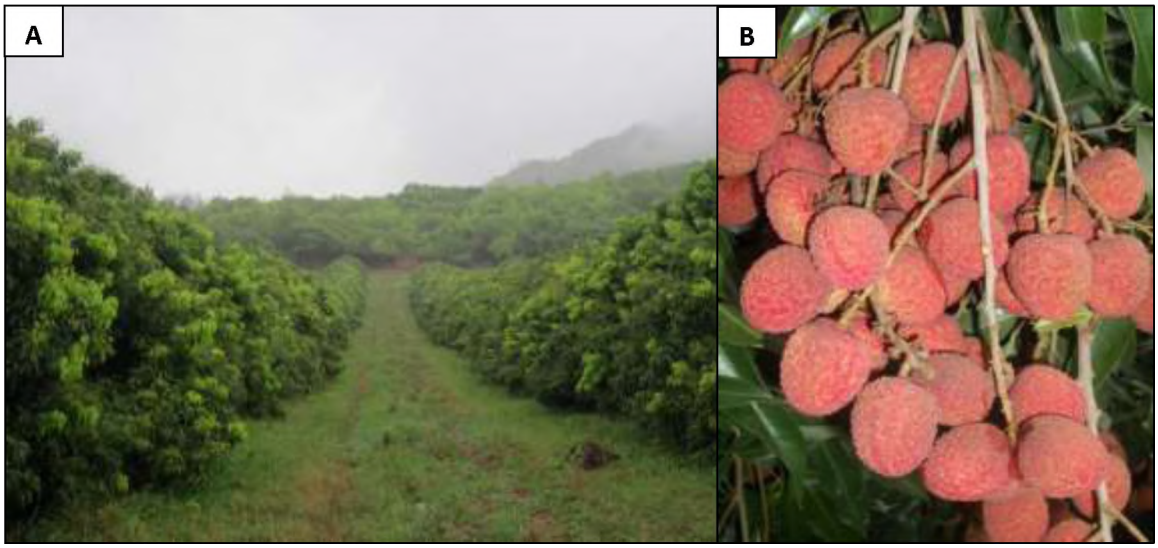


Figure 1.2: (A) Litchi orchard in Malelane, Mpumalanga, (B) The fruit, Mauritius cultivar (Wilna Stones).

The majority of the cultivar groups are irregular bearers, producing variable yields from year to year. Yields are affected either by variable fruit set, fruit retention or fruit development. Most countries, including South Africa cultivate these irregular bearing cultivars (Menzel & Simpson 1987). Due to the irregular bearing of litchis, the crop has not yet reached the status of a major horticultural crop around the world. The irregular bearing is due to several factors such as early flower and fruit drop due to adverse conditions, failure to flower or lack of flowering, poor fruit development, high flower yields but few fruit and low female to male sex ratio, as well as poor pollination (Menzel 1983; Menzel 1984). Irregular bearing may also be

related to climatic conditions and soil moisture. Although litchis have the ability to be cultivated in a wide range of soil types such as, alluvial sands, loams, heavy clay, organic soils, rock piles and calcareous soils, soil moisture is imperative for the development of quality and adequate yields of fruit. Soil moisture is important for the initial period of fruit development, as with low levels of moisture the skin develops into a hard, inelastic outer covering which leads to sun-burning and skin-cracking (Menzel 1984; Menzel & Simpson 1987). *Litchi chinensis* requires a warm tropical or subtropical climate, with short winters that have minimal frost and long hot, humid summer periods (Chen 1949). Another reason for irregular bearing is that of sun-burning and skin-cracking, which occurs at elevated temperatures, low humidity and low soil moisture. Another factor that affects the yields of litchis is browning. Browning is an enzymatic reaction, mainly the oxidation of phenolics causing bruising in fruits. Browning is mainly a post-harvest concern, however it is caused by several factors during development such as; heat stress, chilling injury, pests or pathogens (Cheng & Crisosto 1995; Holcroft & Mitcham 1996).

Litchi chinensis has a low overall gross value as compared to other horticultural crops. An example of this is records from 2009/10 where the litchi industry contributed a total gross value of R 78 million, South Africa producing R 2.1 million of this value. This is compared to a major horticultural crop: citrus (e.g. oranges) where the total gross value in 2011 was R 4 billion in South Africa. However, the litchi industry is currently focusing on expanding planting and production facilities in order to increase yields, which is approximately 12.7 tons per hectare (Cronje & Mostert 2008; Department of Agriculture, Forestry and Fisheries 2011; CGA 2012). Over the past few years the total world production of litchis has been approximately two million tons per annum. South Africa produces approximately 18 000 tons; only 1000 tons are exported to countries such as European Union, Hong Kong and Japan. The majority of exports is fresh fruit and the remaining is preserved litchis (Evans & Degner 2005; Department of Agriculture, Fisheries & Forestry 2013). These annual yields could be greater if the range of insect pests (Table 1.2) and diseases that affect litchi production were reduced. In South Africa in particular, there is a major production loss of litchis due to *C. peltastica* and thus reducing pest levels to below economic thresholds could help increase the number of tons of litchi production and exports.

Table 1.2: The major pests of *Litchi chinensis* and countries where they commonly occur (Jiang *et al.* 2003; Waite 2005)

Common name	Insect	Region
Litchi moth	*Cryptophlebia peltastica* (Meyrick) (1921) (Lepidoptera: Tortricidae)	South Africa, Mauritius, Seychelles, Réunion, Madagascar
Litchi stink bug	*Tessartoma papillosa* (Lepeletier & Serville) (1825) (Hemiptera: Tessaratomidae)	China, Vietnam, Thailand, Mynmar, Philippines, India
Macadamia nut borer	*Cryptophlebia ombrodelta* (Lower) (1898) (Lepidoptera: Tortricidae)	Australia, Thailand, China, Taiwan, Japan, Hawaii
Mediterranean fruit fly	*Ceratitis capitata* (Wiedemann) (1824) (Diptera: Tephritidae)	South Africa, Réunion, Hawaii
Natal fly	*Ceratitis rosa* (Karsch) (1887) (Diptera: Tephritidae)	South Africa, Réunion
Oriental fruit fly	*Bactrocera dorsalis* (Hendel) (1912) (Diptera: Tephritidae)	Hawaii
Shot hole borer	*Acrocerocops cromerella* (Meyrick) (1918) (Lepidoptera: Gracillarinae)	China
False codling moth (FCM)	*Thaumatotibia leucotreta* (Meyrick) (1913) (Lepidoptera: Tortricidae)	South Africa

1.1.2 The pest: Cryptophlebia peltastica

Cryptophlebia peltastica (Meyrick) (1921) (Lepidoptera: Tortricidae), is a major ecological and economic pest of litchis and macadamia in South Africa. It is also a pest of *Delonix regia* (Hook), *Tamarindus indica* (Linnaeus), *Bauhinia* spp. (Linnaeus), *Caesalpinia pulcherina* (Linnaeus), *Acacia* spp. (Linnaeus) and *Cassia* spp. (Linnaeus) (Follet & Lower 2000; Waite & Hwang 2002; Manrakhan *et al.* 2008). In addition, *C. peltastica*, along with a few other Lepidoptera, are involved in the galling of *Acacia karoo* (Hayne) and *Acacia saligna* (Labillardiere) (Myburgh 1987; Timm *et al.* 2006). *Cryptophlebia peltastica* does not only infest litchis in South Africa but is also a pest in Mauritius, Madagascar, Seychelles and Réunion Island (Mankrakhan *et al.* 2008).

1.1.2.1 Pest Description

Cryptophlebia peltastica eggs are approximately 1 mm in diameter and are oviposited on the fruit. The eggs are oblate in shape and appear creamy-white in colour. The larvae have five instars, the first instar which is creamy-white in colour, burrows into the fruit towards the seed, where it starts to feed. The final instar is similar to that of *T. leucotreta*, as it has the same reddish-pink colouring and is approximately 15 mm long. *Cryptophlebia peltastica* pupates in its outer cuticle within the fruit or the soil, forming a brown pupae. (Myburgh 1987; Booysen *et al.* 2006). The adult moths have a similar appearance to that of *T. leucotreta*; the only difference between the two adult moths is that *C. peltastica* is a more robust, larger moth with light grey colouring. The main feature to identify each moth correctly is through the wing characteristics. *Cryptophlebia peltastica* has a more noticeable wing characteristics such as the subterminal band on the forewing and the tornal macula (Myburgh 1987; Krüger 1998; Booysen *et al.* 2006).

1.1.2.2 Life Cycle

The adult females will usually oviposit a single egg onto the fruit, either during the initial development phase of the fruit or once the fruit has begun to ripen. Once the larvae hatch, the first instar burrows into the fruit and make its way to the seed. Larvae will feed on the seed and move onto the flesh of the fruit. The larvae remain inside the fruit, until they have developed into fifth instars, unless the fruit is too small. In that case the larva will move onto the next fruit. Once larvae reach fifth instar they will begin to pupate within the fruit or drop to the soil for pupation. After a certain period the adult moths will eclose and the life cycle will begin again (Figure 1.3). There is relatively little information on the life cycle of *C. peltastica*, especially with regards to the time it takes to complete the life cycle. This is largely a consequence of difficulties faced during the rearing of *C. peltastica* in the laboratory (Grové 2000; Timm *et al.* 2006; van den Berg *et al.* 2010).

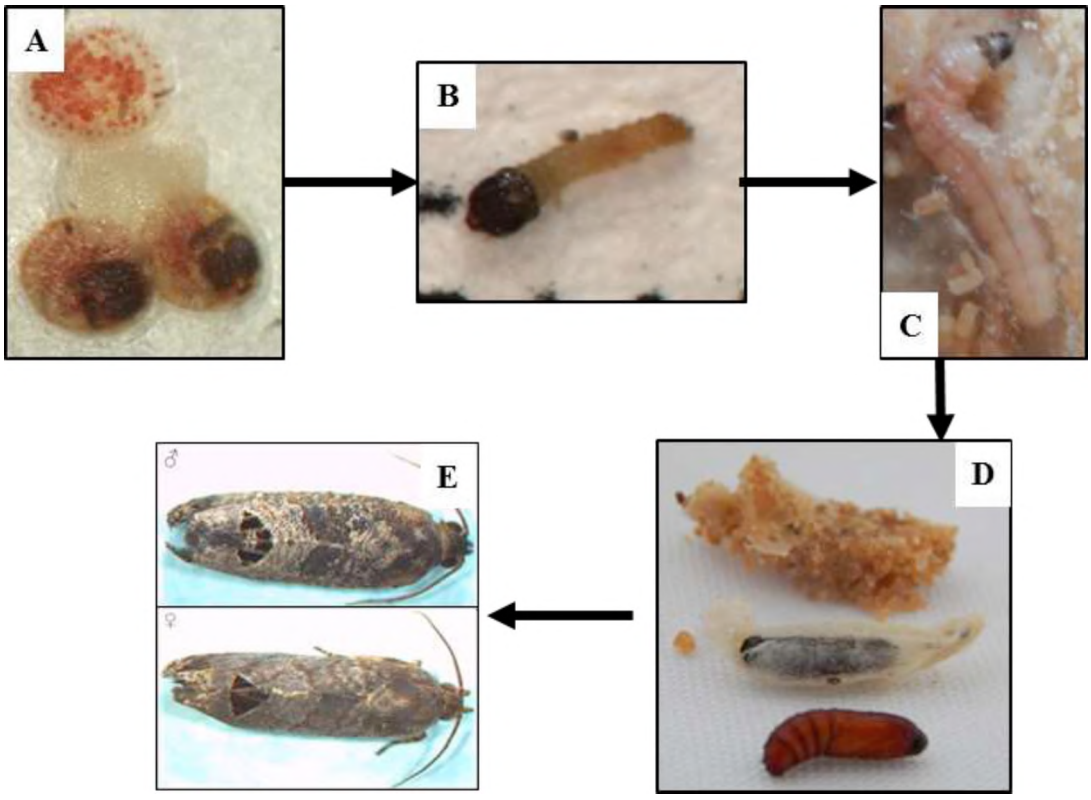


Figure 1.3: The life cycle of *Cryptophlebia peltastica*. (A) Developing egg, (B) first instar larva, (C) fifth instar larva, (C) pupa and pupal casing and (E) adult female and male moths (Marsberg, personal photos).

1.1.2.3 Damage and control techniques of Cryptophlebia peltastica

Cryptophlebia peltastica is just one of many pests found on litchis (see Table 1.2). It is however, considered to be the major pest of litchis and is considered a phytosanitary risk, reducing the yield of export, which subsequently reduces the overall profits (Figure 1.4) (de Villiers 1992). Various control methods have been implemented to help reduce the level of damage and increase production yields.

Figure 1.4: *Cryptophlebia peltastica* damage to litchi fruits (Wilna Stones).

There are a number of pre-harvest control techniques that are used against *C. peltastica*. A favourable control technique is the use of chemical pesticides. However the use of chemical pesticides is slowly being reduced due to the harmful side effects these chemical pesticides have on the environment, humans, animals, beneficial insects and the more stringent residue restrictions imposed by export markets (de Villiers 1992). The two main insecticides registered for the control of *C. peltastica* are triflumuron (Alsystin 480 SC) and teflubenzuron (Nomolt 150 SC). Both insecticides function as chitin synthesis inhibitors and are applied as full cover sprays (de Villiers 1992; Grové 2000).

The use of control techniques as mentioned above are determined by an economic threshold, also known as integrated pest management (IPM) programme. When these control techniques are implemented pest numbers should be reduced below the economic threshold (Hunter-Fujita *et al.* 1998). One of the most important techniques is that of cultural control. Cultural control should be practised throughout the year and in combination with other control methods. Cultural control involves: practising orchard sanitation and fruit bagging. Orchard sanitation encompasses the collection and removal of dropped fruit, pruning, weeding and grass cutting. Fruit that is removed from the orchard is burnt and can be buried in trenches to prevent further infestation. Fruit bagging involves tying a brown paper bag around developing fruit after the first drop and continues until harvest. The paper bags prevent pests from accessing the fruit (van den Berg *et al.* 2010). These bags are also known as Birdspun (Plastrip cc, South Africa),

which are effective control methods but highly impractical and labour intensive (Grové & de Beer 2005).

Pheromone control methods used are mainly mating disruption, in this case "Isomate® - CM FLEX" (Pacific Biocontrol corporation, Vancouver) mating disruption. Pheromone traps do not eradicate the pest but help to reduce their numbers (Booysen *et al.* 2006; Schoeman *et al.* 2009). Another control technique used against *C. peltastica* is augmentative biological control. This involves the release of an egg parasitoid, *Trichogrammatoidea* species (van den Berg *et al.* 2010). Microbial control, which has a wide range of success stories but has not been implemented in litchi as of yet.

The litchi fruit does not only need to be protected before harvest, but it also needs postharvest techniques to prevent further infestation, fungal contamination and disease before export. These methods include; vapour heat treatment (VHT), cold storage and gamma irradiation. VHT involves heating the fruit to approximately 45°C for thirty minutes under high humidity. Cold storage is where the fruit is kept at 1°C for approximately two weeks and then dipped in hot water (49°C) for 20 minutes in order to disinfect the fruit and preserve its quality. Another disinfection method is the use of gamma irradiation, which is used in dosages 75 to 300 gamma rays. Gamma irradiation helps disrupt the insect hosts life cycle without affecting the fruit (Jacobi *et al.* 1993; Jiang & Chen 1995; Holcroft & Mitcham 1996).

1.1.3 Microbial control

The traditional method of control is the use of chemical insecticides, however there is a wide range of complications that accompany their use such as resistance, safety risks for humans, animals, beneficial insects and the environment. Due to the wide area of concern, an alternative method to chemical insecticides needed to be developed. An alternative control option is the use of microbial control agents as they function in a similar fashion to that of chemical insecticides and can be used in conjunction with chemical control options (Hunter-Fujita *et al.*1998; Dolinski & Lacey 2007; Knox *et al*, 2015; Dutta 2015). Further favourable characteristic of microbial control agents which are similar to that of chemical control is the ability to be applied with conventional equipment and can be stored for prolonged periods, under the correct conditions (Lacey *et al.* 2001; Lacey *et al.* 2015). Microbial control agents have the potential as successful alternative control options with advantages such as the

conservation of biodiversity as they are less toxic, counteract resistance to insecticides, environmentally friendly, and species specific with low to no impact on natural enemies. Microbial control agents have the ability to be used in IPM programmes as well as with conventional techniques (Lacey & Shapiro-Ilan 2003; Lacey & Shapiro-Ilan 2008; Vasantharaj 2008, Knox *et al*. 2015; Lacey *et al*. 2015).

Microbial control agents are also known as entomopathogens (Table 1.3). This includes bacteria, fungi, nematodes, protozoa and viruses (Ravensberg 2011; Dutta 2015; Sherwani & Khan 2015). These entomopathogens are obligate and facultative pathogens that occur naturally within the insects or in the insects' environment. Microbial control agents kill or impair their insect hosts by causing toxemia infection or certain diseases. In order to use these microbial agents, the entomopathogens need to be isolated and characterised from unhealthy host insect cadavers, which involves several steps (Jaques 1983; Harper 1987; Shermani & Khan 2015).

Table 1.3: Examples of entomopathogens that have been used as microbial control agents (Jaques 1983; Gwynn & Maniania 2010).

Viruses	Bacteria	Fungi	Protozoa
Choristoneura fumiferana NPV*	*Bacillus thuringiensis*	*Beauveria bassiana*	*Nosema fumiferanae*
Mamestra brassicae NPV*	*Bacillus lentimorbus*	*Metarrhizium anisopliae*	*Nosema locustae*
Agrotis segetum NPV*	*Bacillus popilliae*	*Verticillium lecanii*	*Nosema pyraustae*
Cydia pomonella GV#	*Bacillus sphaericus*	*Conidiobolus osmodes*	*Vairimorpha necatrix*
Pieris brassicae GV#		*Nomuraea rileyi*	
Dendrolimus spectabilis CPV%			
Melolontha melolontha EPV^			

*NPV (nucleopolyhedrovirus), #GV (granulovirus), %CPV (cytoplasmic polyhedrosis virus), ^EPV (Entomopox viruses)

The most commercialised microbial control agent is bacteria, but only one genus of bacteria has been commercialised: *Bacillus*, mainly *Bacillus thuringensis* (Berliner) (1915) (Bacillales:

Bacillaceae) and *Bacillus popilliae* (Dutky) (1941) (Bacillales: Pacnibacillaceae). Insects that are susceptible to this genus of bacteria are required to have an alkaline midgut, suitable proteolytic enzymes and tissue receptors. The microbial activity of *Bacillus* relies on its crystal protein toxins to kill the host. These toxins enter the midgut of the insect host. This causes the insects to stop feeding and paralyses the gut, consequently killing the insect (Flexner *et al.* 1986; Lacey & Shapiro-Ilan 2008; Dutta 2015). However, some insect hosts have recently developed the ability to become resistant to *B. thuringensis*, making it a less favourable microbial control agent.

Entomopathogenic nematodes, belonging to the families Steinernematidae and Heterorhabditidae are one of the more recent methods used in microbial control practices. The nematodes themselves do not kill the insect host, but two mutualistic bacterial genera, *Xenorhabdus* and *Photorhabdus,* that are associated with the nematodes, causes the insect host to die of bacterial infection (Cross *et al.* 1999). Infection occurs when the infective juvenile nematode finds an insect host in the soil. The nematode will enter into the insect host through any hole in the body such as the mouth, anus or spiracles. They will then move into the body cavity of the insect where they release the mutualistic bacterium that occurs in their intestines. The bacterial infection will kill the insect within 48 hours. The bacterium will then release antibiotics which prevents further infection of the insect cadaver. The nematodes will only leave the cadaver once all the nutrients are depleted, this can take several generations. The nematodes will return to the soil for reinfection of another host (Dolinski & Lacey 2007).

The entomopathogenic fungi are a favourable group of control agents as they are widely distributed and are common in all soil environments. The two major groups of fungi used as control agents are, Hypocreales and Entomopthoromycota. The Hypocreales are the species most widely used in control of insect pests, including, *Beauveria bassiana*, *Metarhizium anisopliae*, *Paecilomyces farinosus* and *Verticillium lecanii* (Flexner *et al.* 1986; Cross *et al.* 1999; Lacey & Shapiro-Ilan 2008; Ravensberg 2011). Infection occurs when the insect host comes into contact with fungal spores. The fungus will then germinate, penetrating the cuticle of the insect. Fungal spores can also enter through the spiracles. Once inside the insect host, the mycelium of the fungus attacks the host tissue through enzymatic and mechanical processes, which then multiply in the haemocoel. The fungus will cause death of the host in two ways: either through the release of toxins or through tissue damage. Hyphae will grow out of the cadaver, eventually erupting and releasing fungal spores for reinfection (Flexner *et al.* 1986). The only major disadvantage of entomopathogenic fungi is that they require high

humidity to develop. Without optimal humidity and depending on the fungal isolate, they may not develop (Cross *et al.* 1999).

The last group of microbial control agents are the viruses. Several groups of viruses have been commercialised, such as, *Baculoviridae*, *Entomopoxviridae* and *Beoviridae*. Virus microbial control agents are well known for their host-specificity, as many viruses have narrow host ranges, sometimes only affecting a single species (Dolinski & Lacey 2007 Szewczyk *et al.* 2009). The baculoviruses are the most favoured and successfully commercialised viral control product on the market (Lacey & Shapiro-Ilan 2008). Baculoviruses were the focus for this study.

1.1.4 Baculoviruses

Baculoviruses were first discovered in the 19th century, with the 'wilting' disease of the silkworm, *Bombyx mori* (Lepidoptera: Bombycidae) (Linnaeus) (1758). When the 'wilting' disease was studied crystalline polyhedral bodies were observed. It was thus thought that these polyhedral bodies were responsible for the disease in silkworms. In the 1900's the polyhedra were then associated with a virus. Further characterisation in 1940 by Bergold found that the polyhedron contain the characteristic rod shaped virus (Knipe *et al.* 2007). Initially, the use of viruses as an alternative control option to chemical insecticides was not favourable. It wasn't until many years later, when approximately two million hectares of soyabean in Brazil, were treated with a baculovirus to control velvet bean caterpillar, *Anticarsia gemmatalis* (Hübner 1818) (Lepidoptera: Noctuidae) and successfully suppressed the caterpillar population (Moscardi 1999; Szewczyk *et al.* 2009).

Baculoviruses are a DNA group that infect arthropods, specifically insects in the order Diptera, Hymenoptera and Lepidoptera. The Baculoviridae family is divided into four genera: alphabaculoviruses, known as nucleopolyhedrovirus (NPV) which are associated with Lepidoptera; betabaculoviruses are also lepidopteran specific virus but are granuloviruses; gammabaculoviuses are Hymenoptera NPV specific viruses and deltabaculoviruses are specifically dipteran NPVs. The viruses get their name from their characteristic rod shape nucleocapsids and large size (O'Reilly *et al.* 1992; Cory & Myers 2003; Moscardi *et al.* 2011; Rohrmann 2014). There are three subgroups of the family Baculoviridae, A; nucleopolyhedrovirus (NPV), B; granuloviruses (GV) (Figure 1.5) and C; non-occluded

viruses (Mielie *et al.* 2011). NPVs have a polyhedral coat which forms multiple virions packaged in occlusions (protein matrix), whereas GVs are composed of single virions forming granules. NPVs (0.15-15 µm) are larger than GVs (0.3-0.5 µm) (Cory & Myers 2003; Possee *et al.* 2010).

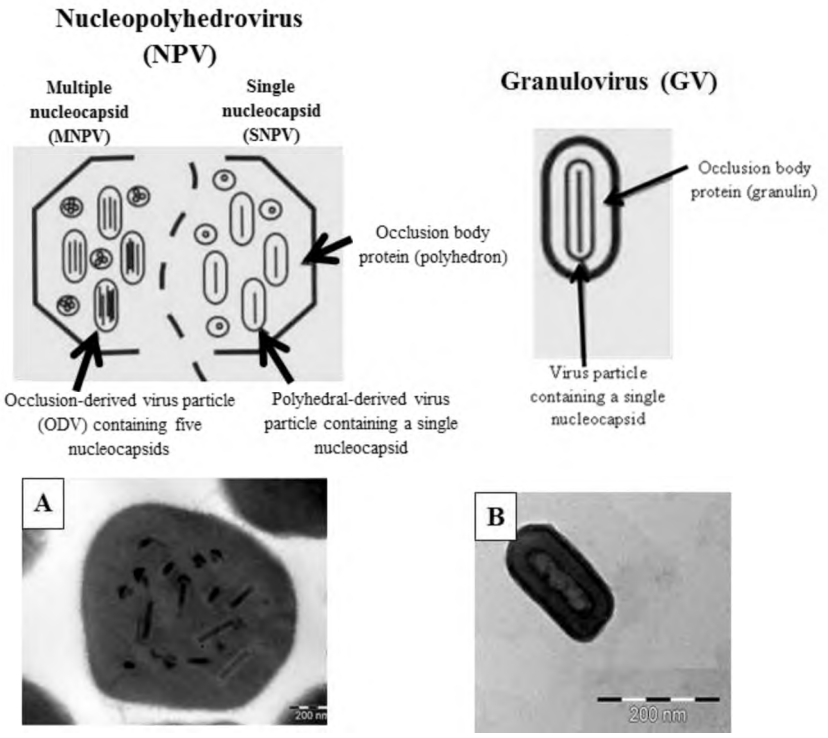


Figure 1.5: Diagrams representing the physical appearance of a nucleopolyhedrovirus (NPV) and granulovirus (GV) (Image A: TEM image of a NPV and B: TEM image of a GV) (Hunter-Fujita *et al*, 1998; Chang *et al.* 2003; Marsberg, personal photo).

The infection cycle of baculoviruses is divided into a primary and secondary phase. The primary phase begins when insects ingest (either GV or NPV) contaminated food (Figure 1.5). The virus will then enter into the midgut of the insect, where it will be exposed to an alkaline environment, causing the protective protein body (occlusion body (OB)), surrounding the virus to dissolve. Once the virus is released, protease activity causes the virions to move through the peritrophic membrane and attach to epithelial cells, where replication occurs. The virions are then transported to the basal membrane of the midgut where budding is promoted. From here they then move into the haemocoel, where the secondary phase is initiated (Figure 1.6). The virus moves around the insect's body by attaching to haemocytes, where further replication

occurs to multiply the number of viruses (Hunter-Fujita *et al.* 1998; Knipe *et al.* 2007; Muthamia *et al.* 2011). The virus will have invaded all internal tissue controlling the insect's cytoskeletal and nuclear structure. They also alter the insect's behaviour, causing the insect to climb to the highest point on the vegetation. This technique is used to spread OBs, as once the larvae has made its way to the highest point, the cuticle will rupture releasing OBs to the vegetation below, thus the infection process can begin again (Figure 1.6) (Hunter-Fujita *et al.* 1998; Knipe *et al.* 2007; Muthamia *et al.* 2011).

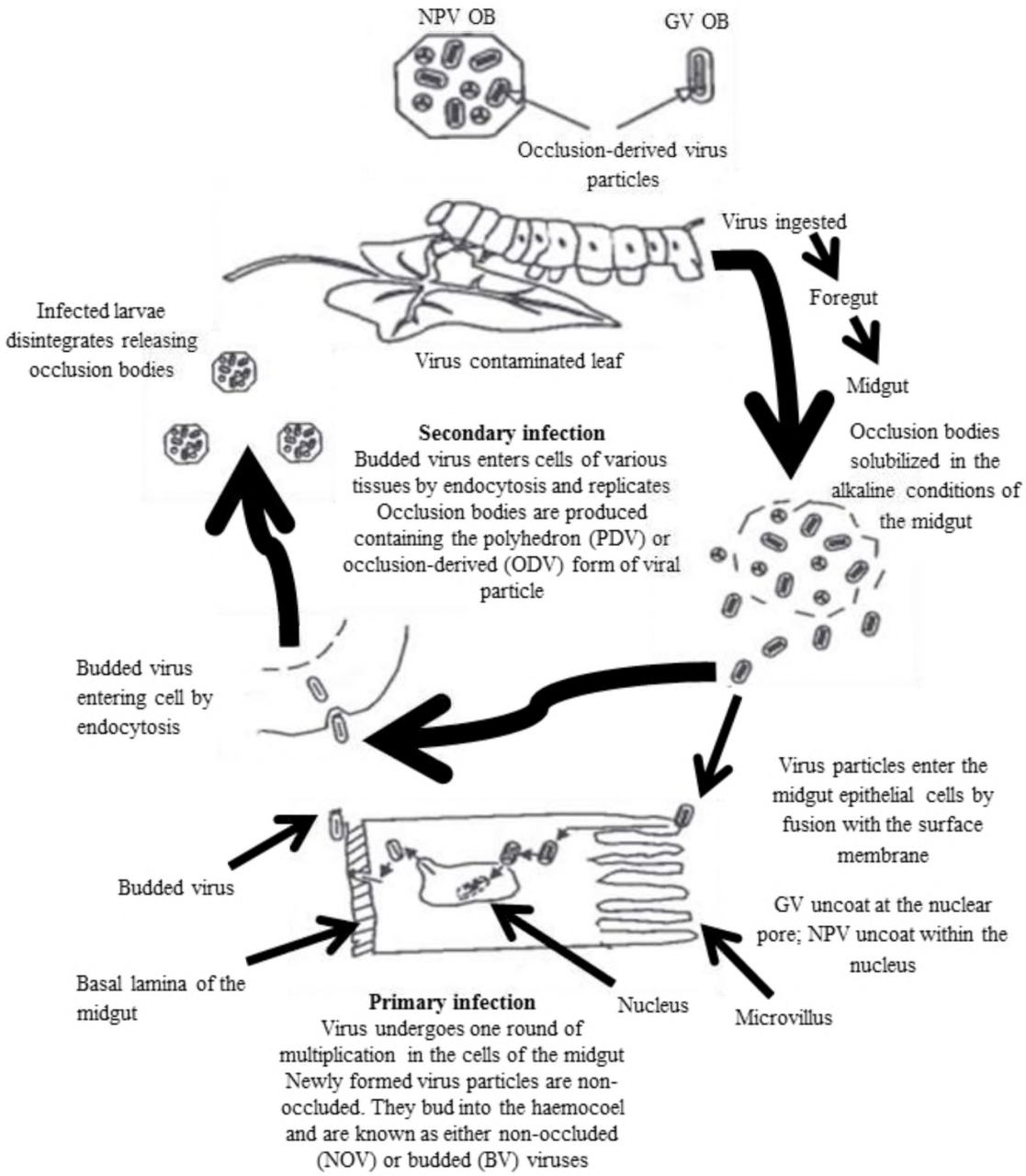


Figure 1.6: Primary and secondary infection cycle of baculoviruses (Hunter-Fujita *et al.* 1998).

Baculoviruses have several favourable characteristics which make them suitable for use in microbial control, such as, being environmentally safe; safe to humans, animals and beneficial insects; host-specific; replicate and persist in the environment, therefore reducing the number of sprays required; and a protein coat which allows for survival outside their host (Cory & Hails 1997; Moscardi 1999; Moscardi *et al.* 2011). Today there are numerous baculoviruses used to control a number of insects, especially in recent times with the improvement in DNA technology allowing for quick isolation, identification and characterisation of viruses (Table 1.4) (Cory & Myers 2003; Knox *et al.* 2015).

Examples of commercially available baculovirus biopesticides that have had great success in South Africa include; CpGV (Madex® and Carpovirusine® (Andermatt-Biocontrol, AG Switzerland)) against *C. pomonella* which is a major pest of apples and pears, CrleGV (Cryptogran® (River Bioscience, South Africa)) against *T. leucotreta* a major pest in citrus and HearNPV (Helicovir® (River Bioscience, South Africa)) against *H. armigera*, a major pest against a wide variety of cultivated crops (Moore *et* al. 2014; Knox *et al.* 2015).

Table 1.4: Baculoviruses that have been developed into successful and commercialised microbial control agents (Inceoglu *et al.* 2001; Moore 2002; Abdulkadir *et al.* 2013; Nawaz *et al.* 2016).

Pest	Crop	Type of virus	Biopesticide
Codling moth, *Cydia pomonella*	Apple, pear	Granulovirus	Cyd-Xe®, Madex®, Granupom®
American bollworm, *Helicoverpa armigera*	Cotton	Nuclear polyhedrovirus	Mamestrin®, Helicovir®
African cotton leafworm, *Spodoptera littoralis*	Cotton, corn, tomatoes	Nuclear polyhedrovirus	Spodopterin®
Tobacco budworm, *Heliothis virescens*	Cotton, vegetables	Nuclear polyhedrovirus	Gemstar LC®, Biotrol®
False codling moth, *Thaumatotibia leucotreta*	Citrus	Granulovirus	Cryptogran®, Cryptex®

1.1.4.1 Identification and characterisation of a baculovirus

In order to identify and isolate a baculovirus, the naturally occurring virus needs to be expressed in the host. In order for the virus to be expressed in the host, it needs to be stressed (Lord 2010). Stressing the host will result in susceptibility to microbial infection (Lord 2010). Overcrowding is one of the most successful and natural stress factors as when larvae are reared at high densities, they will develop at a faster rate in order to reach pupation before food supplies run out (Opoku-Debrah *et al*. 2013). This rapid development proves stressful to the larvae making them susceptible to viral infection (Figure 1.7) as reported by Goulson and Cory (1995).

Figure 1.7: Final instar larvae showing characteristic symptoms of NPV and GV infection (Hunter-Fujita *et al*. 1998).

The initial step of identifying a baculovirus is by the pathology of the insects (Hunter-Fujita *et al*. 1998). Once the baculovirus becomes active, it will utilise the host's physiology and behaviour, causing the insect to climb to the highest point of the vegetation. The insect will then hang from the vegetation and eventually the cuticle will rupture, dispersing the virus for reinfection (Knipe *et al*. 2007). During this process the larvae will begin to swell and change

to a glossy/ milky colour. After this the larvae will liquefy. These are the 'wilting' pathology characteristics of a baculovirus (Knipe *et al.* 2007; Lacey *et al.* 2008).

To confirm whether or not the physical symptoms are caused by a baculovirus, and to obtain initial diagnosis, macerated symptomatic larvae are observed by transmission electron microscopy (TEM). This method is used to confirm the presence of a baculovirus, as baculoviruses have a distinctive appearance. However, the method is insufficient to identify and differentiate the baculovirus from other virus groups (Griffith 1982). Before further identification methods can be used, the virus needs to be purified to reduce or eradicate any contamination such as proteins and insect debris (Shapiro 1982). Occlusion bodies are purified from macerated symptomatic larvae, following a glycerol gradient protocol adapted from Hunter-Fujita *et al.* (1998) and Grzywacz (2007). DNA is extracted from these OBs for further genetic analysis. The final step in the identification of a baculovirus is the use of polymerase chain reaction (PCR), which involves using a small amount of DNA and specific primers to amplify a target characteristic gene of the baculovirus such as the granulin/polh, egt or lef-8 genes. Successful amplification of one or more of these gene regions confirms the presence of the baculovirus (Hunter-Fujita *et al.* 1998).

Once the baculovirus has been identified, it needs to be further characterised in order to be considered for the development into a microbial pesticide. There are two main characterisation techniques: restriction endonuclease analysis (REN) and whole genome sequencing. REN is used to analyse the DNA of the baculovirus, by comparing various viral DNA to the one extracted and creating DNA profiles, also known as genetic "finger prints" of the viruses. This method allows one to determine different isolates of the virus. This technique involves digesting the viral genome into different fragments by using restriction enzymes such as EcoRI, PstI and BamHI, which are then separated by their molecular weight by agarose gel electrophoresis (AGE) (Miller and Dawes 1978; Hunter-Fujita *et al.* 1998). Whole genome sequencing is used as an alternative to REN analysis as it is a more accurate and comprehensive method used to genetically characterise the virus. The whole genome can be sequenced using next-generation sequencing (NGS), specifically the Illumina sequencing method (Mardis 2007, Liu *et al.* 2011). By sequencing the whole genome it gives the researchers a better understanding of the genetic make-up, allows for the manipulation and resistance management of the virus (Mardis 2007).

1.1.4.2 Biological activity

The next step in the characterisation and development of a baculovirus biopesticide is to determine the biological activity. Bioassays are used to analyse the potency of the virus by comparing various strengths of the virus in a controlled environment and same biological stimuli (Finney 1971; Treacy 1999). The results achieved from the bioassays will determine the dosage-response and time-mortality relationship between the virus and host. Various techniques are used for bioassays such as surface dosage, diet incorporation, droplet feeding and egg dipping (Finney 1971; Treacy 1999). The surface dosage technique is used to mimic the natural feeding habits of insect pests that feed on the surface or burrow into the crop (Jones 2002). The diet incorporation technique is most suited for insects that burrow into the fruit. This method is similar to that of surface dosage, however the virus in mixed into an agar based diet (Jones 2002). The droplet feeding technique is peroral method that can be used for insects that do not have the above feeding habits (Hughes & Wood 1981; Jones 2002). Egg dipping involves dipping eggs into a viral suspension and when larvae hatch they will ingest virus found on the chorion of the egg (Jones 2002).

1.1.4.3 Mass production

Once the biological activity of the virus is determined, with the ability to kill majority of the pest in a short period of time at an acceptable dosage, the next step will be to mass produce the virus either *in vivo* or *in vitro*. Mass production of baculovirus biopesticides is commonly done by using the *in vivo* technique either in the homologous host (host that the virus was isolated in) or the heterologous host (a closely related host that the virus can infect) (Ravensberg 2011). *In vivo* production involves the mass rearing of healthy host insects and infecting the host with the virus in order to mass produce infective OBs. *In vivo* production is a simple and inexpensive process that can be adapted at low cost by laboratories and small enterprises (Grzywacz & Moore 2017). The *in vitro* method is used to produce the virus in closely related insect cell lines (Rao *et al.* 2016). *In vitro* production of baculoviruses has various advantages over *in vivo* production such as production at a large scale is more manageable and the process produces high quality OBs. *In vitro* production can be used to produce different baculoviruses that have the ability to infect a single cell line. This will reduce costs, as several insect cultures will not have to be reared to produce each virus. Another advantage is that baculoviruses can be genetically modified and manipulated to improve certain characteristics such as, increasing the

speed of kill (Ravensberg 2011; Grzywacz & Moore 2017). However, the use of cell lines is not yet economically feasible to commercially mass produce a baculovirus biopesticide (Rao *et al.* 2016). Future work on *in vitro* production is required to develop suitable bioreactors for large scale production, low cost medium and the ability to improve yield and virus quality (Grzywacz & Moore 2017). Once these factors have been addressed, *in vitro* production could be used for the mass production of viruses

1.2 RESEARCH AIMS

To date no baculovirus has been identified or characterised for *C. peltastica*. It is thus imperative to attempt to find a microbial control agent, in order to improve the control methods against this pest. Therefore, this study aimed to identify and characterise a baculovirus that can potentially be used as a biopesticide against *C. peltastica*.

The aims were to (1) rear a laboratory culture of *C. peltastica* and determine various biological aspects of *C. peltastica;* (2) collect larvae showing symptoms of baculovirus infection and, using these larvae, isolate and identify a baculovirus using the following methods: OB purification, TEM and PCR; (3) genetically characterise the virus using REN analysis and whole genome sequencing to determine if the virus is novel; (4) determine the biological activity of the virus against the host, *C. peltastica* and two closely related species, *T. leucotreta* and *C. pomonella*, as well as against CpGV resistant European *C. pomonella*; (5) the susceptibility of the isolated baculovirus to the *C. pomonella* cell line, Cp14R; (6) lastly, conduct a preliminary investigation into the mass production of the virus *in vivo*, in the homologous and a heterologous host.

Chapter 2

LABORATORY REARING AND STUDY OF THE BIOLOGY OF *CRYPTOPHLEBIA PELTASTICA*

2.1 INTRODUCTION

Lepidopterans are considered the most damaging pests to agricultural crops worldwide (Simmons *et al.* 2010). This has led to the development of various techniques used in pest control, which include biological, chemical and microbial control (Simmons *et al.* 2010). An important mechanism used to conduct research for the development of products for these control measures, is that of laboratory rearing or mass rearing of insects. The establishment of laboratory cultures has proved to be the foundation for discovery, development and enhancement of the majority of the alternative control measures, including chemical control (Shapiro 1992). Laboratory rearing has undoubtedly increased researchers' knowledge of the biology of pest and beneficial insects, has enabled production and assessment of the efficacy of various microbes, predators and parasites against pest species and has allowed for the development of various other control options, such as sex attractants and sterile insect technique (Gast 1968).

With the wide range of uses for insects, there is also a diverse array of methods in which to rear them, which can either be on natural diets, semi-synthetic diets or artificial diets (Morton 1979; Cohen 2005; Parker 2005). Artificial diets are the more favourable choice for rearing methods as they are simplified, of consistent, known quality, available year round and contain optimal formulations of the insect's natural diet. Artificial diets are however prone to microbial contamination but this can be controlled using various procedures, such as surface sterilisation

of eggs with formaldehyde or sodium hypochlorite, sterilization of working equipment and diet through autoclaving and suitable handling techniques (Waldbauer *et* al. 1984; Webb & Shelton 1988; Toba & Howell 1991; Shapiro 1992; Parker 2005; Dyck 2010).

Laboratory reared insects need to be as representative as possible of the wild population and should be healthy, vigorous and perform optimally. Quality of laboratory reared insects is dependent on the type and quality of diet they are reared on (Bell *et al.* 1980; Cohen 2005). Most artificial diets used to rear Lepidoptera are based on wheat germ (Adkisson *et al.* 1960), but with a few modifications such as Moore's (2014) diet. The ingredients used for Moore's diet are milk powder, wheat germ, brewer's yeast; nipagin, sorbic acid and distilled water; and it is used to rear *T. leucotreta*, *Ectomyelois ceratoniae* (carob moth) (Zeller 1839) (Lepidoptera: Pyralidae) and *C. peltastica* (Moore *et al.* 2014).

Literature on the rearing and biology of *C. peltastica* is limited due to difficulties experienced in the rearing of a laboratory culture (Timm 2005). Attempts have been made to rear *C. peltastica* on FCM's artificial diet as the two species are closely related. Steyn and Schoeman (2007) attempted to rear *C. peltastica* using a similar protocol to *T. leucotreta*, but found that moths were unsuccessful at mating and oviposition and hence the culture collapsed. Hepburn *et al.* (2009), however, managed to culture several generations of litchi moth under laboratory conditions following a similar protocol to *T. leucotreta* rearing. Only low numbers of *C. peltastica* were reared using this method, which was not suitable for research. Opoku-Debrah (unpublished data) managed to improve the protocol developed by Hepburn *et al.* (2009) and in tandem with the culture used in this study, is currently culturing the first successful laboratory culture of *C. peltastica* (Citrus Research International (CRI), Port Elizabeth, Eastern Cape). This method was used to rear a laboratory culture of *C. peltastica* and while doing so determine the biology of the pest and what control measures can be used against it.

The aim of this chapter was to establish a laboratory colony of *C. peltastica* at Rhodes University and determine various biological aspects of the pest, such as optimal rearing conditions, length of life cycle, fecundity and fertility of females, and egg and larval development. The establishment of a laboratory culture of *C. peltastica* will allow for the better understanding of the pest's biology, improvement of the pest's control and allow for mass production of control agents, as well as the possibility to identify a closely related species that will be able to act as a heterologous host for the production of these newly biological control agents.

2.2 MATERIALS AND METHODS

2.2.1 Culture Rearing

A culture of *C. peltastica* was started using larvae collected from infested macadamia nuts and *Caesalpinia pulcherrima* (pride of Barbados) (Swartz) (Fabales: Fabaceae) pods from Nelspruit (25.466°S, 30.985°E) (Mpumalanga Province, South Africa) between 2008 and 2011. These larvae were reared under laboratory conditions at Rhodes University, Grahamstown, Eastern Cape. The rearing protocol for *C. peltastica* was adapted from Ripley *et al.* (1939), Theron (1948), Schwartz (1971) and Moore (2002). The culture was reared in a controlled environmental (CE) room at 27 ± 1°C, 18:6 photoperiod and a relative humidity of 60 – 80%. Adult moths were held in oviposition cages which consisted of an inverted sieve (30cm diameter) placed over a sheet of wax paper (Figure 2.1). The wax paper provided a suitable substrate for oviposition, as well as for easy collection of the eggs (egg sheets). Cotton wool moistened with a 10% sugar suspension in distilled water was placed around the inside of the cage and used as an adult food source. Egg sheets were collected every second day and divided into segments containing approximately 100 eggs. These sheets were surface sterilised to avoid fungal contamination by briefly rinsing the egg sheets through 3% formaldehyde solution. Egg sheets were then placed into glass jars (325 ml honey jars Consol©, Johannesburg, South Africa) containing 50 g of *T. leucotreta* artificial diet (dry mix) (Table 2.1) (Moore *et al.* 2014) and 50 ml distilled water. The jars were sealed with breathable membrane fabric lids to reduce fungal contamination. The jar containing the diet was autoclaved at a 121°C for 18 minutes. Once the diet had cooled, egg sheets were suspended from the lid of the jar. Larvae were left to develop in the diet until the pupal stage was reached. Pupation occurred within the artificial diet. Pupae were collected by removing the artificial diet from the jar and sorting through the diet. Pupae were placed under the inverted sieves, to allow for adult eclosion, followed by mating and oviposition (Figure 2.2). All trials were conducted under the controlled environment conditions described above.

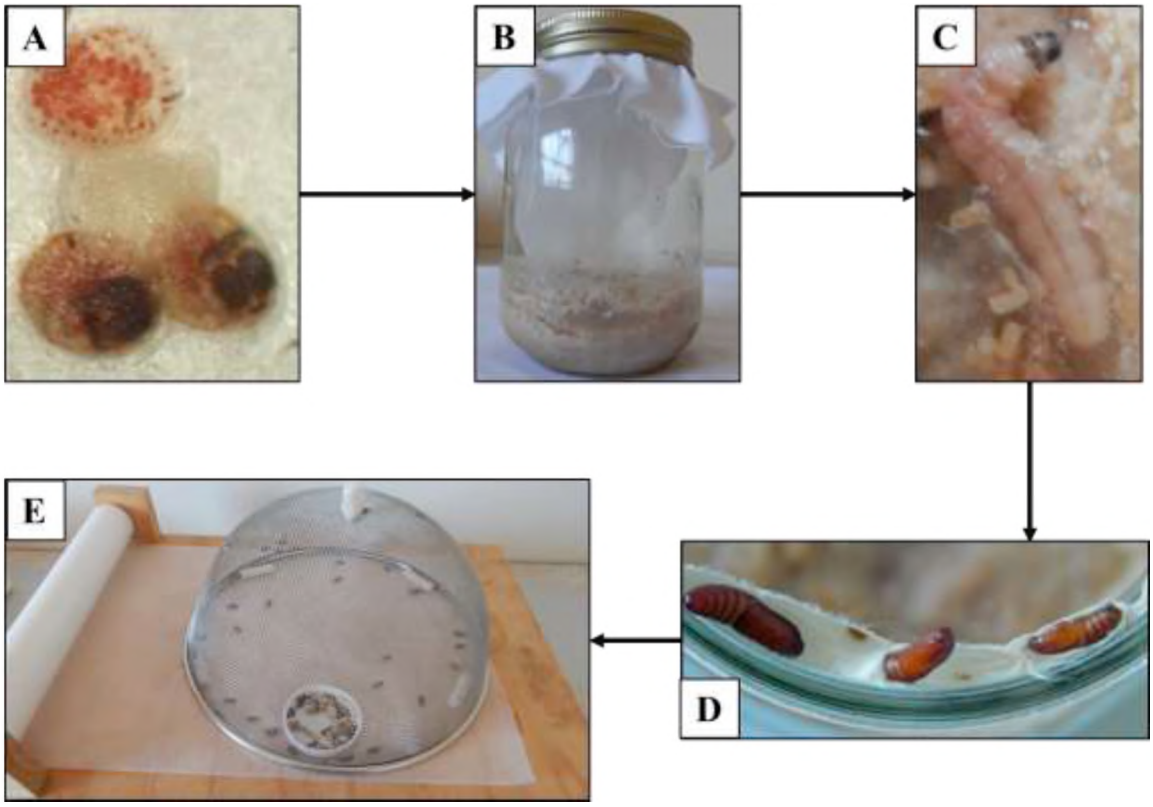


Figure 2.1: Laboratory rearing of *Cryptophlebia peltastica*. (A): egg sheet, (B): egg sheet suspended above the diet in a honey jar, (C) final instar larva feeding within the diet, (D) pupae found around the edges of the glass jar and (E): oviposition cage.

Table 2.1: Recipe for FCM diet used to rear *Cryptophlebia peltastica* larvae (Moore *et al.* 2014)

Ingredients	Bulk dry mix
Maize meal	2000 g
Wheat germ	200 g
Casein or milk powder[1]	36.5 g
Brewer's yeast	100 g
Nipagin	15 g
Sorbic acid	6.5 g
Distilled water	-

[1]Nestlé® Nespray Instant Milk Powder.

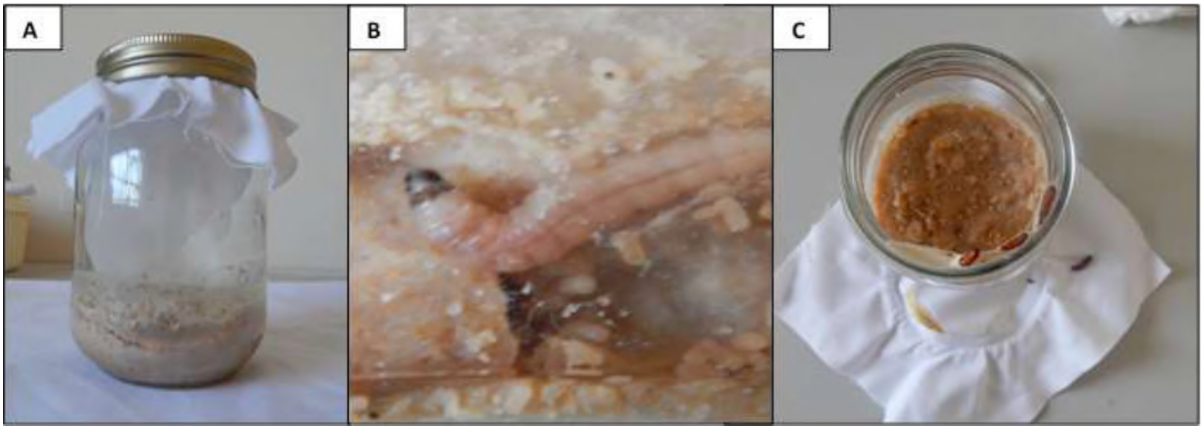


Figure 2.2: (A) Glass jar containing FCM diet for rearing *Cryptophlebia peltastica*, (B) A final instar feeding on the artificially diet, (C) Pupation occurring within the diet and around the edges of the jar.

2.2.2 Fecundity and time to oviposition

Pupae from the main culture were collected and sexed. Male pupae have two small knobs on their ninth segment; a characteristic absent on the female pupae (Daiber 1979; Timm *et al.* 2007). Twelve male pupae and 12 female pupae were obtained and coupled. Each couple was placed underneath a separate sieve, on a sheet of wax paper. Once pupae began to eclose, oviposition was recorded every 12 hours (6:00 and 18:00) by removing the wax paper, counting the number of eggs under a microscope and recording the number of eggs. The removed wax paper was replaced immediately with a clean sheet. Eggs oviposited on the sieve were recorded at the end of the experiment and included in the final fecundity data. Oviposition was recorded until the females died.

2.2.3 Egg development

To record egg development, eggs from the main culture were collected once sufficient moths had eclosed and began ovipositing. A clean sheet of wax paper was then placed underneath the sieve. Adult moths were then allowed to oviposit for approximately 12 hours, after which the egg sheet was removed. The egg sheet was cut into sections containing approximately 30 eggs each. Thirty of these egg sheets were placed into separate Petri dishes, each representing a

replicate. Egg development was recorded every 12 hours by examining the egg sheets under a microscope. Counted eggs were marked with a fine liner marker to prevent being recorded again.

2.2.4 Percentage hatch

Percentage hatch was determined using eggs that were oviposited by multiple females (approximately 20 different females with 15 different males) within a 12 hour period. Egg sheets were inspected to determine whether eggs were clumped or singly oviposited. Egg sheets with an even distribution of singly oviposited eggs were used. These eggs were divided into 18 replicates, with each replicate containing 30 eggs. The egg sheets were left for 5 days, and then viewed under a dissecting microscope to determine the number and hence proportion of hatched eggs. Eggs containing an exit hole were counted as hatched and visible larvae within the chorion were counted as unhatched (Figure 2.3).

Figure 2.3: Difference between a hatched and unhatched egg after six days.

2.2.5 Larval development times

Larval development was measured by collecting egg sheets that were oviposited by multiple females (as in section 2.2.4) within a 12 hour period. Egg sheets were divided into 92 sections containing 30 – 40 eggs each. One egg sheet was placed into a petri dish and inspected for the

first ±120 hours in order to record neonate length. Another egg sheet was placed into a petri dish containing a thin layer of artificial diet to allow for the ease of access of neonate larvae. This petri dish was inspected twice (every 12 hours) for the following 24 hours. The remaining egg sheets were placed into 90 honey jars containing artificial diet and inspected for the remainder study. Inspection occurred every 12 hours and the head capsule width and body length of 20 larvae were recorded A dissecting microscope with a graduated scale on the ocular was used to take measurements. Dyar's rule was then applied to determine a relationship between larval instar and head capsule width (Dyar 1890).

2.2.6 Pupal development

Pupal development and the sex ratio was recorded. For pupal development a jar containing approximately 20 fifth instar larvae was inspected daily to determine when pupation started and ended (adult eclosed). The sex ratio was determined by randomly collecting 732 pupae during pupal collection for the culture rearing. Pupae were sexed using the morphological difference observed by Timm *et al.* (2007), where males were found to have two protrusions on their last abdominal segment, these are absent in females. Male and female pupae were kept separately to allow adults to emerge. Adults were then observed under a dissecting microscope in order to find morphological characteristics that could be used to differentiate between female and male moths.

2.3 RESULTS

2.3.1 Culture rearing, sex ratio and difference between male and female pupae and adults

A laboratory culture of *Cryptophlebia peltastica* was successfully established at Rhodes University in a controlled environment, with a temperature of 27°C ± 1°C and a relative humidity of 60 - 80%. Due to optimal growing conditions and a successful rearing protocol, the culture grew exponentially. During the growth of the culture, various biological tests were completed. In order to carry out these biological tests the difference between male and female pupae and adults needed to be known. Male pupae were found to have two protrusions from their last abdominal segment, these protrusions cannot be seen on the female pupae (Timm *et*

al. 2007). During pupae collection 732 pupae were sexed to determine a sex ratio. A total of 348 male and 384 female pupae were sexed, from this a 1: 1.1 ratio was recorded, however it can be assumed as a 1:1 ratio as a chi-square test indicated no significant difference ($p=0.99$, $p<0.05$) between males and females.

Adult males had a lighter forewing colour around the black triangles, whereas the females had a darker colouring and more prominent black triangles on the forewing. The last abdominal segment of the male possesses anal combs, whereas the female has few or no anal combs. The hind legs of the male possess large amounts of setae on the tibia. Behind the tibia (against abdomen) the setae are black and the front of the tibia are golden brown. The female contains very few setae on the hind leg tibia compared to the male. The female's hind leg is the same colour on both sides (Figure 2.4) (Timm *et al*. 2007).

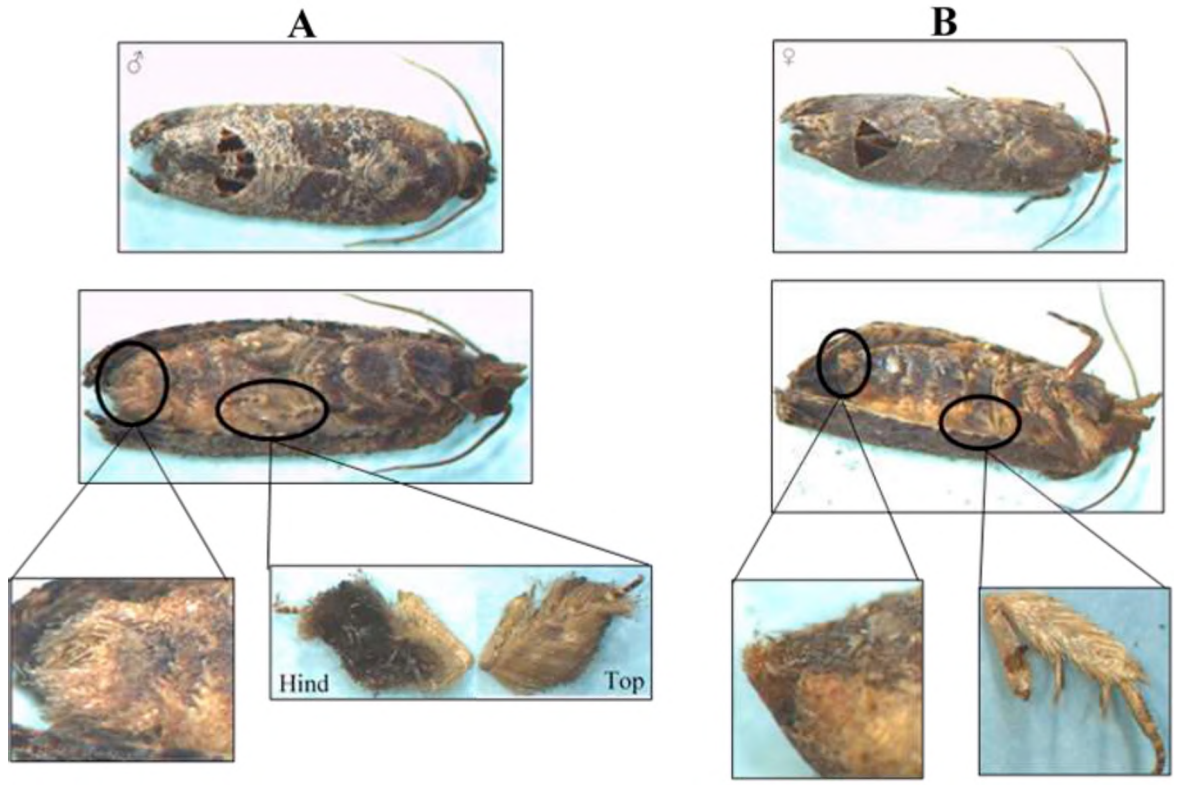


Figure 2.4: Difference between male (A) and female (B) adult *Cryptophlebia peltastica*.

2.3.2 Fecundity and time to oviposition

Once adults eclosed, females had a pre-oviposition period of approximately 2.25 ± 0.3 (mean ± SE) days. The number of eggs oviposited by females ranged from 10 to 506 with a mean of 180.58 ± 47.76 (mean ± SE, n=12) eggs per female moth (Figure 2.5).

Figure 2.5: Oviposition cages used to determine fecundity and time to oviposition.

2.3.3 Egg Development and percentage hatch

Eggs were oval in shape, with colour change as development progressed. Overall development took 4 days from egg to larva. Day 0: eggs were white cream in colour; 12 hours later the eggs appeared to have an orange tinge. Day 1: red spots appeared on the egg surface. Day 2: the red spots began to merge, creating a red appearance. Day 3: The red area moved to one side of the egg; 12 hours later the head capsule of the larva was observed as two black spots. Day 4: the full body of the larva could be observed as well as clear egg sacs, where larvae had already emerged (Figure 2.6). Day 5: majority of eggs had hatched except for those that had stopped developing. Separate eggs were left to develop for 5 days. After 5 days the number of hatched eggs and visible larvae (unhatched) were counted. The percentage of eggs hatched ranged from 80% to 97%, with an average hatch of 90.56% ± 1.22% (mean ± SE).

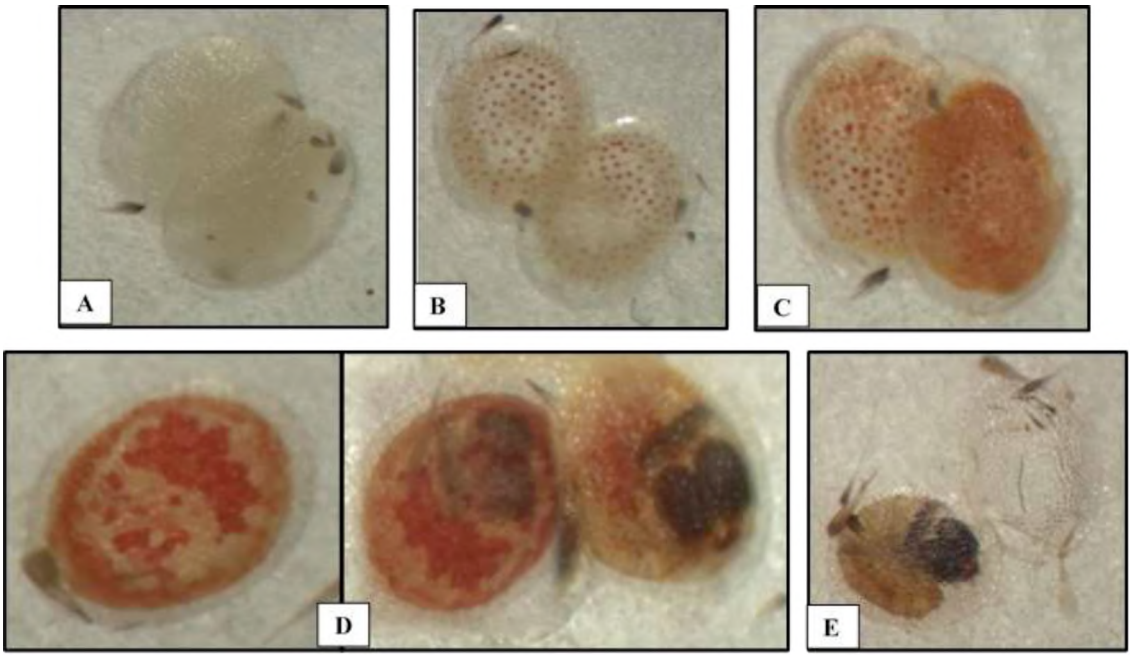


Figure 2.6: Egg development by day (A= day 0, B= day 1, C= day 2, D= day 3 and E= day 4).

2.3.4 Larval duration of development

The larval development (Table 2.2) and length of the life cycle (Figure 2.8) of *C. peltastica* was monitored in a controlled environment, with a temperature of 27 °C ± 1 °C. During larval development head capsule widths were measured. Dyar's rule was then applied to these measurements to determine the number of instars and the range of head capsule widths for each instar.

The mean and standard error obtained from the head capsule widths of each instar conformed to Dyar's rule, producing a regular geometric progression of growth and determining the presence of five larval instars (Figure 2.7) (Dyar & Rhinebeck 1890). Using Dyar's ratio the average increment in head capsule width between each instar was calculated to be 0.2835 mm (Dyar 1890; Hsia & Kao 1987).

Table 2.2: Mean head capsule and body length of five *Cryptophlebia peltastica* larval instars.

		Average measurements (mm)	
Instar	**Range of Head Capsule (mm)**	**Head Capsule (mean ± SE)**	**Body Length (mean ± SE)**
1st	0.3 – 0.5	0.39 ± 0.01	1.26 ± 0.03
2nd	0.5 – 0.7	0.50 ± 0.00	2.66 ± 0.11
3rd	0.7 – 1.2	0.85 ± 0.02	6.49 ± 0.22
4th	1.2 – 1.6	1.28 ± 0.04	11.59 ± 0.35
5th	1.5 – 1.8	1.53 ± 0.01	17.24 ± 0.20

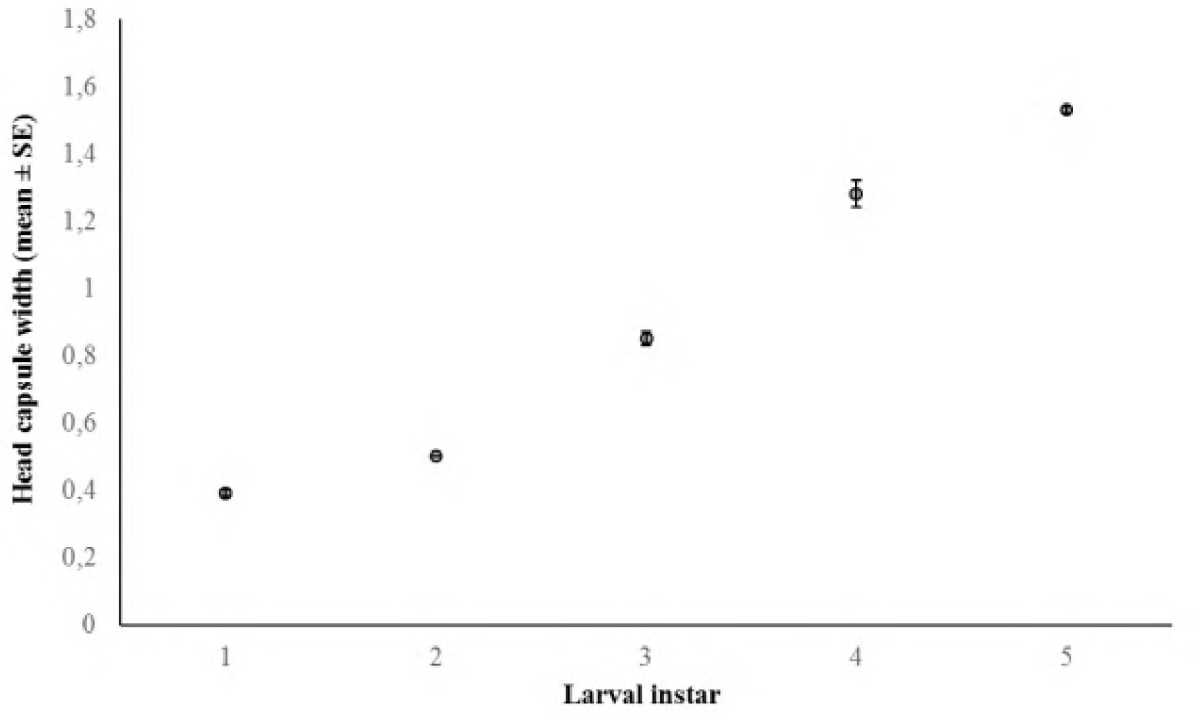


Figure 2.7: Mean head capsule width of five larval instars of *Cryptophlebia peltastica* conforming to Dyar's law.

Eggs are oval in shape and cream in colour, which changes to cream with red speckles, mainly red and then the head capsule of the larvae appears as a black spot (Figure 2.6). It takes an average of four days for the neonates to emerge. First instars are 1.26 mm in length. Their body is cream, with a large black head capsule of 0.39 mm in width. It takes approximately 2 days

for 1st instars to moult to 2nd instar which can be observed by a change in the size and shape of the head capsule (0.39 mm to 0.5 mm) and the length of the body. The next moult is then observed 3 days later, which can again be distinguished by a change in the head capsule and body length. The duration of the 3rd instar was 4 days. Once the larvae reach 4th instar, the colour of the larvae begins to change to pink. The duration of the last moult to 5th instar, was approximately 2 days. Here the larvae are pink in colour with an average body length of 1.53 mm. The larvae remain at 5th instar for approximately 8 days before pupating. Pupation lasts approximately 10 days before adults eclose (Figure 2.8).

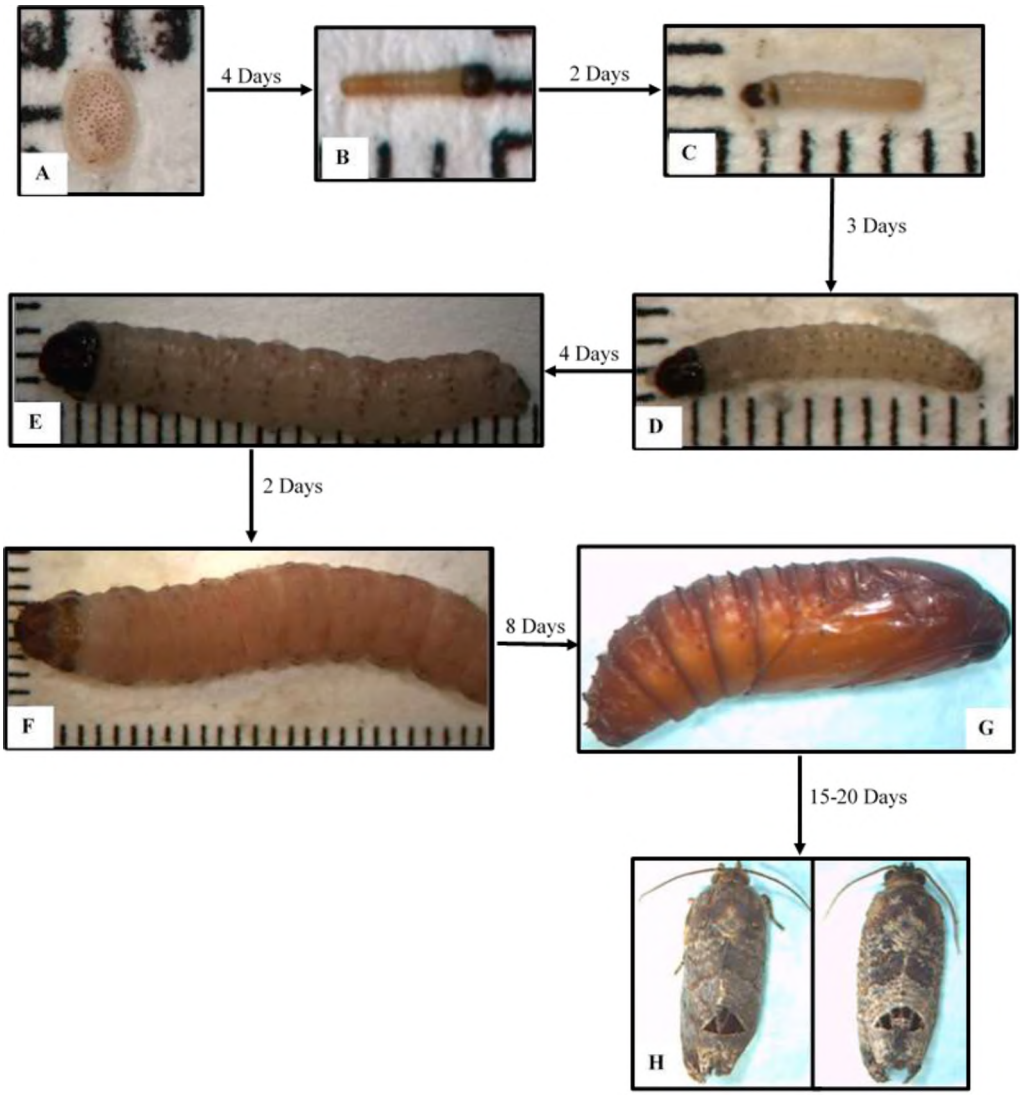


Figure 2.8: Images showing larval development and life cycle length of *Cryptophlebia peltastica*. (A) egg, (B) 1st instar, (C) 2nd instar, (D) 3rd instar, (E) 4th instar, (F) 5th instar, (G) pupae and (H) adults. (Distance between scale bars = 0.5 mm).

2.4 DISCUSSION

This study is the first report of the successful establishment of a laboratory culture of *C. peltastica* using rearing techniques adapted from Ripley *et al.* (1939), Theron (1948), Schwartz (1971) and Moore (2002). With the establishment of a laboratory culture of *C. peltastica* various alternative control options can be developed and improved on. The biology of *C. peltastica* was compared to that of *T. leucotreta* and *C. pomonella*, as they are cryptic pests with overlapping host ranges, specifically on citrus and nuts (walnuts, macadamia nut etc.) (Timm *et al.* 2007; Rentel 2013).

A pre-oviposition period of approximately 2 days was experienced before oviposition occurred, also observed with *T. leucotreta* having an oviposition period of 1 to 2 days (Newton 1998). After this period, females continued to mate and oviposit until death. The fecundity of female *C. peltastica* moths was observed to be an average of 181 eggs and ranging from 10 to 506 eggs. Fecundity of *T. leucotreta* was found to be an average of 500 eggs, whereas *C. pomonella* oviposited an average of 222 eggs (Chambers 2014). It was observed that oviposition was higher at night than during the day, as the majority of eggs were collected from oviposition that occurred between 18h00 and 06h00, and little to no eggs were collected from the period between 06h00 and 18h00. Egg development for *C. peltastica* was observed to be 4 days with 90.56% of the eggs hatching. These values were then compared to *T. leucotreta* and *C. pomonella* which were found to have an average of 3.5 days of egg development with 91.8% egg hatch and 4.2 days egg development with 83.73% egg hatch, respectively (Chambers 2014).

The larval development of the three species were also compared and it was observed that *C. peltastica* and *T. leucotreta* were more similar than *C. pomonella*. The larval development for *C. peltastica* and *T. leucotreta* from first instar to second was 2 and 2.5 days, second to third instar was 5 and 4 days, third to fourth instar was 9 and 7 days and from fourth to fifth instar was 11 and 10 days, respectively (Chambers 2014). The larval development of *C. pomonella* was slightly longer than *C. peltastica* and *T. leucotreta*, as the development from first to second instar was 5 days, second to third instar was 8 days, third to fourth instar was 11 days and from fourth to fifth instar was 17 days (Chambers 2014). Both *T. leucotreta* and *C. pomonella* have been successfully established in a commercial insectary (River Biosciences (Pty) Ltd and ENTOMON) for the mass production of their homologous viruses, CrleGV and CpGV, which are effective commercially available biopesticides. The similar biology between *C. peltastica,*

T. leucotreta and *C. pomonella* and the comparable rearing method of *C. peltastica* to *T. leucotreta* there is a great chance that the mass production of *C. peltastica* and its control agents can be established and commercialised.

Another possibility is the mass production of the various control agents for *C. peltastica* in a heterologous host, specifically *T. leucotreta* due to the similarity between the rearing and biology of the species. A heterologous host is used when it is more cost effective to rear and produces a higher yield of the product compared to the homologous host (Chambers 2014). The use of a heterologous host has been investigated in a study by Chambers (2014). The study compared the biology and CpGV production in *T. leucotreta* and *C. pomonella*. From the results it was shown that *T. leucotreta* could potentially be an ideal candidate as a heterologous host for the production of CpGV, due to the following favourable characteristics: high production of larvae per unit of diet, short development time and therefore more generations per year, and therefore increased virus production and low cost of rearing (Chambers 2014). *Thaumatotibia leucotreta* could potentially be the heterologous host for the possible baculovirus or other control agents of *C. peltastica*, as a mass production facility has already been established (River Bioscience (Pty) Ltd). Not only has a mass production facility been established, but it has also be observed that number of larvae reared per jar is higher for *T. leucotreta* compared to *C. peltastica*. According to a study completed by Chambers (2014) approximately 200 fifth instar *T. leucotreta* larvae can be reared per jar, whereas *C. peltastica* can only be reared with approximately 50 fifth instar larvae per jar. This is due to the larger size of *C. peltastica*, as the average body length of a fifth instar larvae is ±17 mm as compared to *T. leucotreta* fifth instar larvae with an average body length of ±13 mm (Chambers 2014). Therefore one jar of *T. leucotreta* larvae would be equivalent to four jars of *C. peltastica* larvae, making *C. peltastica* more expensive to rear per jar than *T. leucotreta*. Another issue with the rearing of *C. peltastica* is the pupation. Unlike *C. peltastica* which remains in the diet to pupate, fifth instar *T. leucotreta* larvae move up into a cotton wool stopper, which plugs the rearing jars, to pupate. This makes it easier to access *T. leucotreta* pupae for transfer to an eclosion unit (Opoku-Debrah *et al.* 2014) which is not the case for *C. peltastica*. This difficulty in collecting pupae of *C. peltastica* is therefore time consuming and increases the risk of losing pupae during collection. Thus, *T. leucotreta* would be an ideal heterologous host for *C. peltastica* as it is cost effective to rear and due to the higher number of larvae reared per jar, higher yields of the product can be produced.

In conclusion, a successful laboratory culture of *C. peltastica* has been established and continues to flourish. With the development of the culture, the biology of *C. peltastica* could be determined and was observed to be similar to *T. leucotreta*. This validates the potential to successfully mass produce *C. peltastica* and its various control options, as well as make use of *T. leucotreta* as a heterologous host due to its ease of rearing, faster development and low cost of production. However further research needs to be conducted in order to determine whether *T. leucotreta* can be used to produce these control agents, specifically a baculovirus which will be discussed in further chapters.

Chapter 3

MORPHOLOGICAL CHARACTERISATION OF A NOVEL BACULOVIRUS FROM *CRYPTOPHLEBIA PELTASTICA*

3.1 INTRODUCTION

Laboratory rearing and establishment of a healthy insect colony is important for research purposes, production of biological control agents, including microbial control agents (e.g. viruses) and for biological studies for a better understanding of the pest (Morton 1979; Cohen 2005). Laboratory cultures need to be reared under optimal conditions, such as temperature, humidity and photoperiod. However, in some cases the culture is exposed to various stress factors such as quality and quantity of diet, varying temperature, varying humidity, exposure to chemicals and overcrowding (Sørensen *et al.* 2012). These factors can cause an increase in stress to the insect, specifically the larvae, therefore causing an increase in susceptibility to microbial infections, such as baculoviruses (Lord 2010; Opoku-Debrah 2011). A study completed by Opoku-Debrah (2011) found that by exposing insects to high temperatures, feeding was reduced or even stopped, thus increasing their susceptibility to viral infection. Changes in diet quality and quantity have the same stressful effect. Studies by Goulson and Cory (1995) and Opoku-Debrah (2011; 2013) reported that overcrowding was one of the main stress factors. Overcrowding is observed when insects are reared at such high densities that their fitness is compromised through excessive competition, causing them to develop at a faster rate due to limiting food resources. The stress resulting from this increased developmental rate causes these insects to express a baculovirus infection, previously present in an enzootic state.

Overcrowding is one factor that results in laboratory reared insects showing signs of baculovirus infection and is commonly used to isolate baculoviruses from specific insect populations for use as biological control agents. For example, during a study by Abdulkadir (2014), a culture of diamondback moth (DBM), *Plutella xylostella* (Linnaeus) (1758) (Lepidoptera: Plutellidae) became overcrowded and many larvae showed symptoms of a virus infection. It was from these symptomatic larvae that Abdulkadir (2014) was able to isolate a granulovirus from DBM. There are several methods that can be used to isolate and characterise these viral infections.

The initial identification step is that of symptomatology and observation of the insect's morphology to determine whether it shows signs of virus induced 'wilting' disease (Knipe *et al.* 2007; Erlandson & Theilmann 2009). The typical symptoms of viral infection in Tortricidae are a change in integument colour, and pale colouring (early stage) to brown-black (late stage). Once in the late stage the larva's cuticle will rupture, releasing a milky haemolymph. This haemolymph contains numerous OBs which can be used for reinfection or other applications (Andrade & Habid 1984; Fuxa 1991; Barreto *et al.* 2005; Aizawa 2012). When these symptoms are observed a crude extract is prepared. By examining this extract by transmission electron microscopy (TEM), the presence or absence of viral particles can be determined. If a virus is present, the next step is to purify the virus using a glycerol or sucrose purification gradient. These gradients are used to separate OBs from insect and cellular debris using an ultracentrifuge and they are separated according to density, size and shape (Erlandson & Theilmann 2009). Gradients provide highly purified samples which can be observed by TEM to characterise the virus morphologically. The above methods confirm the presence of a baculovirus and allow identification of the type of baculovirus (GV or NPV) using morphological characteristics of OBs (Rohrmann 1986; Kool *et al.* 1995; Possee & Rohrmann 1997; Van Oers *et al.* 2004; Erlandson & Theilmann 2009).

A novel virus is identified genetically through the use of the PCR and sequencing to amplify specific genes (e.g granulin or polyhedrin) using oligonucleotides flanking the target sequence. However, when no sequence data are available, as was the case in this study, universal/degenerate oligonucleotide primers are used. Degenerate primers are used to amplify a number of related and conserved genomic sequences, such as the polyhedrin, granulin, lef-8 and lef-9 genes. Degenerate primers (prPH-1 and prPH-2) for the granulin and polyhedrin gene have been designed by Lange *et al.* (2004). Lange *et al.* (2004) designed these primers through a multiple alignment of 21 Lepidoptera specific baculoviruses (GVs and NPVs). From these

multiple alignments conserved regions within the polyhedrin and granulin gene were identified and used to create a consensus sequence, for which the primers prPH-1 and prPH-2 were designed. These primers amplify a partial sequence of approximately 507 to 510 nucleotides in length and contain the main gene content of the polyhedrin or granulin genes (Lange *et al.* 2004; Jehle *et al.* 2006). This partial sequence can then be subjected to NCBI BLAST to genetically identify the type of baculovirus with the closest match allowing for further characterisation.

A laboratory culture of litchi moth was reared at Rhodes University (Chapter 2). The culture flourished under optimal conditions, leading to high densities and overcrowding. It was during this time that symptomatic larvae were observed and collected. Therefore the aim of this study was to isolate a virus from these symptomatic larvae and to characterise this virus both morphologically and genetically. The specific objectives of the study were to purify OBs through glycerol gradient centrifugation and to analyse them using TEM. PCR amplification and sequencing of the granulin and polyhedrin gene regions was completed by using degenerate primers (prPH-1 and prPH-2) designed by Lange *et al.* (2004).

3.2 METHODS AND MATERIALS

3.2.1 Symptomatology

A laboratory culture of *C. peltastica* was established at Rhodes University (see chapter 2). During rearing larvae showing symptoms of 'wilting' disease were collected in microtubes and placed at -25°C for future use. These symptoms were colour change, milky pink (early stage) or black/ brown appearance (late stage).

3.2.2 Crude baculovirus extraction

In an attempt to isolate a baculovirus from the symptomatic larvae collected, a protocol for a GV crude extract was adapted from Parnell *et al.* (2002). However, no virus was observed using this method and therefore an NPV protocol adapted from Grzywacz *et al.* (2007) was used instead. Insect cadavers were homogenised in 1 ml of 0.1% SDS in a microtube to disrupt the cuticle. The microtube was then vortexed for approximately 2 minutes. The homogenate was then centrifuged at 100 *g* for 10 to 20 seconds to remove insect debris. The supernatant

was collected and placed into a separate microtube. The pellet was re-suspended in 1 ml 0.1% SDS and again centrifuged at 100 *g* for 10 to 20 seconds. The supernatant was pooled and the pellet was discarded. The pooled supernatant was centrifuged at 2 500 *g* for 5 minutes to pellet the virus. The supernatant was discarded and the pellet was re-suspended in 1 ml ddH_2O and centrifuged at 2 500 *g* for 5 minutes. The supernatant was again discarded and the pellet re-suspended in approximately 100 to 200 µl ddH_2O.

3.2.3 Purification of virus occlusion bodies by glycerol gradient centrifugation

To determine the morphology of the baculovirus, occlusion bodies (OBs) are purified using a glycerol gradient. As a NPV was identified from the crude extract, a protocol for an NPV purification was adapted from Grzywacz *et al*. (2007).

A 50 to 60% glycerol gradient was prepared in a microtube following a method adapted from Grzywacz *et al* (2007), by placing 500 µl of 60% glycerol into a 2 ml tube, overlaid with 500 µl of 50% glycerol. The pellet from a crude extract was re-suspended in 500 µl of 20% glycerol. 100 µl of this sample was then placed on top of the gradient and centrifuged at maximum speed for 15 minutes (Figure. 3.1). Both the band and pellet were collected and re-suspended in 1 ml ddH_2O and centrifuged at maximum (12 100 *g*) speed for 15 minutes. The supernatant was discarded and the pellet was re-suspended and centrifuged again as described above. The final pellet was re-suspended in ddH_2O.

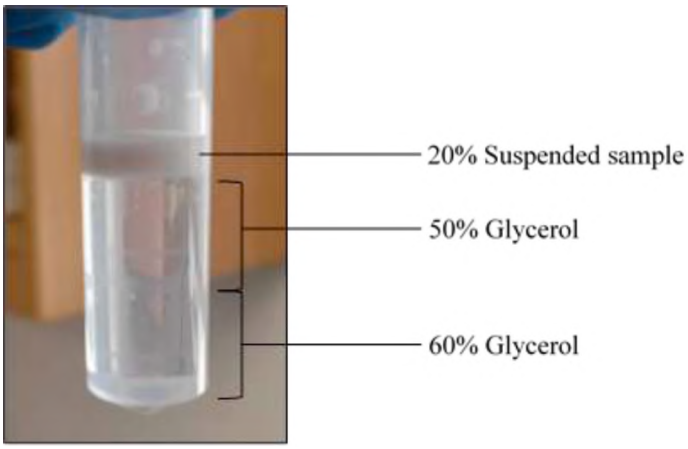


Figure 3.1: Preparation of a partial glycerol gradient in a microtube.

3.2.4 Transmission electron microscopy (TEM)

A droplet of the prepared sample (± 2 µl) was placed on a Forvar carbon coated grid for 30 seconds. Filter paper was used to drain off the excess sample. A droplet of uranyl acetate was then placed on the grid and left for 20 seconds. Filter paper was used to remove the excess stain and the grid was left overnight. The grid was observed using a Zeiss Libra 120 transmission electron microscope at 80 000 kV. The images were analysed using Mega View (G2) Olympus analysis software. The size, width and diameter of the OBs were determined from the images taken by measuring 100 OBs.

3.2.5 Sectioning of occlusion bodies

In order to determine whether the virus was a single NPV (SNPV) or multiple NPV (MNPV) nucleocapsid virus, a sectioning protocol was used to view the ultrastructure of the OB.

A pelleted sample of OBs was obtained using the glycerol gradient purification protocol described in 3.2.3. The sample was then embedded in resin using a protocol adapted from Cross & Pinchuck (1995). The pellet was immersed in a primary fix solution of 2.5% glutaraldehyde in PO_4 overnight. The primary fix was then removed and the pellet was washed by immersing the pellet in 0.1 M PO_4 (pH 7.3) for 10 minutes. This was repeated. The pellet was then immersed in a secondary fix solution of 1% osmium tetraoxide in 0.1 M PO_4 for 90 minutes. The secondary fix solution was removed and the pellet was washed by immersing the pellet in 0.1 M PO_4 (pH 7.3) for 10 minutes. This was repeated. The pellet was then immersed in 30%, 50%, 70%, 80% and 90% cold ethanol consecutively for 10 minutes each, removing the ethanol each time before adding the next percentage. Absolute ethanol was then added to the pellet and left for 10 minutes and removed. This was repeated. Propylene oxide was added to the pellet and left for 20 minutes and then removed, repeated. A solution of 75:25 pro-resin was added to the pellet and left for 60 minutes. This was then removed and a solution of 50:50 pro-resin was added and left for a further 60 minutes. This solution was removed and 25:75 pro-resin was added for another 60 minutes. The solution was removed and the pellet was then submerged in pure resin overnight. The resin was removed, and the pellet was immersed in pure resin and left for 36 hours at 60 °C.

The pellet was then cut into 100 nm thickness sections, using a RMC MT-7 ultramicrotome. The sections were collected on 300 mesh copper grids. These grids were stained for 20 minutes

in 5% uranyl acetate and 2 minutes in lead citrate. The grids were then viewed under a Zeiss Libra TEM at 120 kV.

3.2.6 CTAB DNA extraction

A CTAB DNA extraction protocol was adapted from Aspinall *et al.* (2002). Using 200 µl of purified occlusion bodies, 90 µl of 1M sodium carbonate (Na_2CO_3) was added and incubated at 37°C for 30 minutes. A volume of 120 µl Tris-HCl (1M, pH6.8), 90 µl of 10% SDS and 50 µl proteinase K (25 mg/ml) were added and samples incubated for a further 30 minutes at 37°C. 10 µl RNAse A (10mg/ml) was then added and incubated for another 30 minutes at 37°C. The solution was then centrifuged at 12 100 *g* for 3 minutes. The supernatant was transferred to a new 1.5 ml tube and the pellet was discarded. 400 µl of warm CTAB buffer was then added to the supernatant and incubated at 70°C for 60 minutes. 400 µl of 4°C choloroform was added and centrifuged at 6 500 *g* for 10 minutes. The upper phase was transferred into a new 1.5 ml tube and 400 µl of -25°C isopropanol was added. The sample was then left overnight at -25°C. The sample was then centrifuged at 12 100 *g* for 20 minutes and the supernatant discarded. 1 ml of cold 70% ethanol was added to the pellet and centrifuged at 12 100 *g* for 5 minutes. The supernatant was discarded and the pellet was incubated at 50°C, until dry. The pellet was re-suspended in 20 µl of Tris-HCl (10mM, pH 8.0).

3.2.7 Viral enumeration

The concentration of OBs was determined using the light microscopy protocol adapted from Smith (2009). A stock solution of purified occlusion bodies was well suspended before a 1:5 dilution of one part OBs and four parts ddH_2O was completed. A further 1:5 dilution using 0.07% SDS was achieved using the previous suspension. The SDS suspension was then sonicated for 60 seconds at 15 second time intervals to avoid particle damage. Further dilutions were made from this suspension depending on the OB concentration. Therefore the dilution ranged from 1:10 to 1:80. A Helber counting chamber (0.02 mm depth, Hawksley®, Broomfield, Colorado) was used to count the OBs under the light microscope in dark field illumination at 400× magnification. Before placing the suspension on the counting chamber, the cover slip and counting chamber were cleaned with 70% ethanol and observed under the microscope at 200× magnification to ensure cleanliness. The cover slip was then placed three

quarters over the counting chamber, leaving a small space in which to apply the suspension. 5 µl of the suspension was pipetted onto space left by the cover slip to allow for the suspension to completely fill the counting chamber through capillary action. The cover slip was then slid across to cover the counting chamber. The slide was then left to stand for 5 minutes to allow for Brownian motion of non-virus particles to cease. Five large squares were then chosen (top left, top right, bottom right, bottom left and random middle) and moving OBs were counted. The protocol was repeated four times using the same sample. The mean number of OBs was then calculated to determine the concentration of the virus, using the following formula:

OBs/ml = (D x X) / (N x V) where

D = dilution of suspension; X = number OBs counted; N = number of small squares counted and V = volume in ml (0.00000005 ml)

3.2.8 Polymerase chain reaction (PCR)

Degenerate oligonucleotide primers, prPH-1 and prPH-2 designed by Lange *et* al. (2004) were used to genetically characterise the isolated NPV (Table 3.1). The primers were used to amplify the polyhedrin gene using DNA extracted from the purified OBs.

Table 3.1: Degenerate oligonucleotide primers for the partial amplification of the polh/gran gene region (Lange *et al.* 2004).

Oligonucleotide name	Sequence (5' to 3')
Polh/gran (prPH-1)	TGTAAAACGACGGCCAGTNRCNGARGAYCCNTT
Polh/gran (prPH-2)	CAGGAAACAGCTATGACCDGGNGCRAAYTCYTT

*N= C, A, T or G; R= A or G; Y= C or T; D = A, G or T

PCR reactions were set up for a positive control, negative control and *C. peltastica* NPV (Table 3.2). The positive control consisted of DNA extracted from CrleGV (*Cryptophlebia leucotreta* granulovirus) to ensure correct amplification with the degenerate primers and the negative control contained no DNA in order to determine if there was any contamination. The DNA concentration for *C. peltastica* NPV was determined using a Nanodrop spectrophotometer

(Thermo Scientific®) and which was approximately 84 ng/µl. This was considered when mixing the reagents.

Table 3.2: Quantity of reagents needed for the amplification (PCR) of the *Cryptophlebia peltastica* baculovirus using degenerate primers.

Reagents	Quantities of reagents needed (µl)		
	Control (+ve) FCM	**Control (-ve)**	**Litchi moth**
KAPATaq (Kapa Biosystems®)	12.5	12.5	12.5
prPH-1 (10 µm)	2	2	2
prPH-2 (10 µm)	2	2	2
Template (DNA 168 ng)	2	0	2
dH_2O	6.5	8.5	6.5
TOTAL	25	25	25

PCR parameters used for the amplification of the polyhedrin gene were adapted from Lange *et al.* (2004) in order to obtain a PCR product of 507 to 510 nucleotides for analysis (Table 3.3). The PCR amplification was performed in a thermocycler (BIO-RAD®). The PCR products were analysed by 1% AGE and band sizes estimated using GeneRuler 1 Kbp DNA Ladder (Thermo Scienticfic®), to confirm the presence and size of the virus gene.

Table 3.3: PCR cycles used for the amplification of the polh/gran gene region using degenerate primers (Lange *et al.* 2004).

	Temperature (°C)	**Duration (min)**	**Cycle**
Stage 1 (Denaturing)	95	3:00	
	95	0:30	
Stage 2 (Annealing)	70	1:00	36 Cycles
Stage 3 (Elongation)	50	1:00	
	72	10:00	

3.2.9 Sequencing

The PCR products obtained were sequenced by Inqaba Biotechnology Industries (Pty) Ltd (South Africa). FinchTV® version 1.4.0 (Geospiza Inc. 2004-2006, Seattle, Washington) was used to view the chromatograms in order to clean-up ambiguous nucleotides. Following the clean-up, sequences were subjected to NCBI BLAST to determine a closely matched baculovirus sequence. Sequences were analysed in MEGA® 6 (Tamura *et al.* 2013) to determine single nucleotide polymorphisms (SNPs). Once SNPs were determined, the sequences were translated to determine if the SNPs resulted in amino acid changes.

3.3 RESULTS

3.3.1 Symptomatology

Collection of symptomatic larvae from the culture began once the culture reached high densities. Symptomatic larvae were found randomly during rearing, in medium to low densities depending on how crowded the jars were. Symptomatic larvae were found on the surface of the diet (Figure 3.2C), within the diet and hanging from the sides of the jars. Early symptoms of the virus were observed by a colour change in the insects, i.e. commonly milky pink to a brown-black larvae (Figure 3.2A, B). Once larvae had reached the brown-black stage, they were prone to rupturing, as the cuticle became soft and liquefied. Caution was therefore taken when these larvae were collected to avoid rupturing the cuticle.

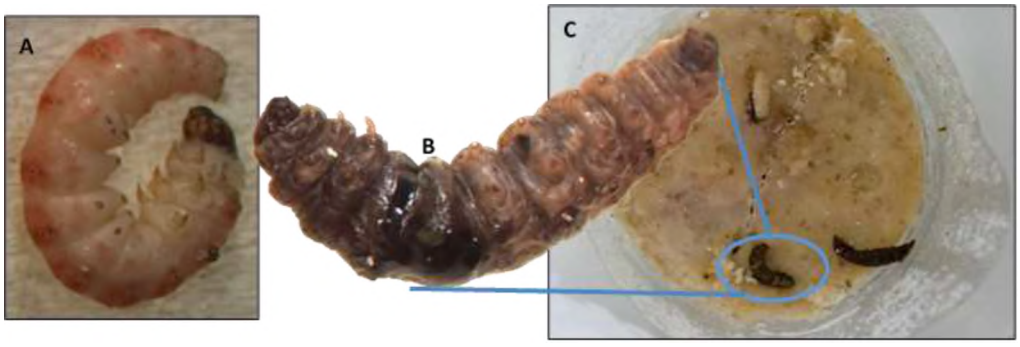


Figure 3.2: Symptoms of a baculovirus infection in final instar larvae of *Cryptophlebia peltastica*. (A) milky pink appearance and engorged body, (B) black-brown appearance and engorged body, (C) symptomatic larvae moving out of the diet.

3.3.2 Crude baculovirus extraction and transmission electron microscopy

Transmission electron microscopy of a crude extract confirmed the presence of a baculovirus. The OBs were observed as amorphous black structures clumped together. The images of the OBs show the characteristic features of an NPV (Figure 3.3). The OBs varied in size between 421 nm to 1263.2 nm. The mean size of 100 NPV OBs was found to be 731.09 ± 15.13 nm.

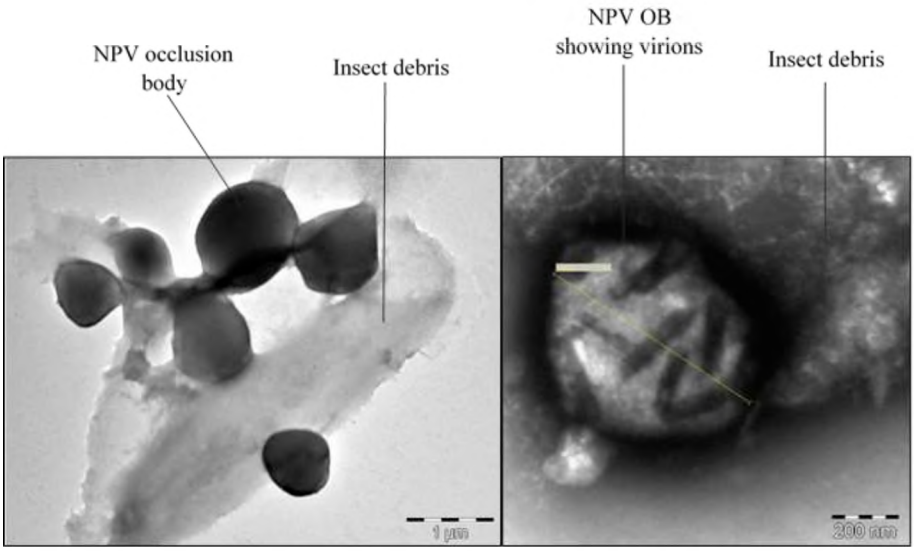


Figure 3.3: Transmission electron micrographs of CrpeNPV occlusion bodies from a crude extract.

3.3.3 Purification of virus occlusion bodies by glycerol gradient centrifugation

The glycerol gradient centrifugation was used to purify OBs from the crude extract. The band forming on top of the gradient and the pellet were collected and placed in separate microtubes (Figure. 3.4). TEM was then completed on both samples. Very few to no OBs were observed in the band, with numerous pure OBs found in the pelleted sample (Figure 3.5).

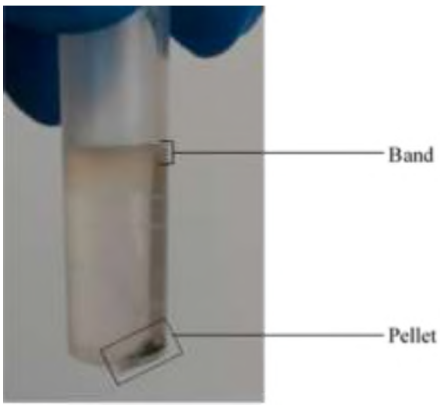


Figure 3.4: A partial glycerol gradient in a microtube, indicating where the pellet and viral band is found.

The bulk of the pellet was made up of NPV OBs. Even though the sample was found in the pellet where the insect debris is usually found, the sample was pure. The purity of the sample could be due to the majority of the insect debris being removed during the crude extract. Occlusion bodies were observed and could be morphologically characterised. The varying sizes of OBs, their circular shapes and clumping together can be observed (Figure.3.5).

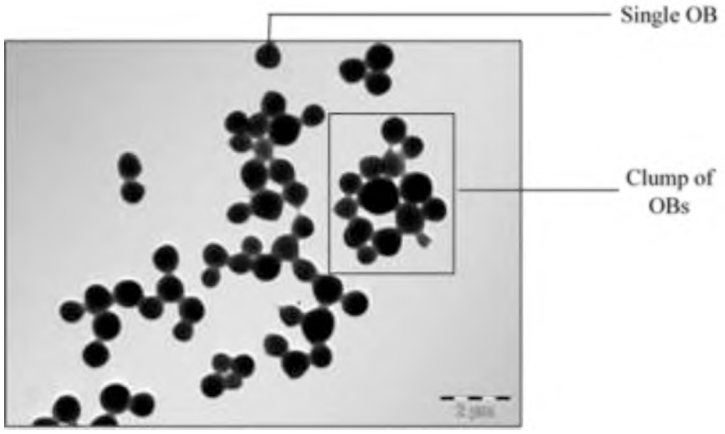


Figure 3.5: Transmission electron micrographs of CrpeNPV OBs purified using the pellet collected from the partial glycerol gradient.

3.3.4 Sectioning of occlusion bodies

To determine whether the isolated NPV was a single or multiple nucleocapsid, the OBs were sectioned and viewed by TEM. From the images of the OBs, occlusion derived viruses (ODV) can be clearly seen (Figure 3.6). When observed closely, each ODV contains a single capsid which confirms that the isolated NPV is an SNPV.

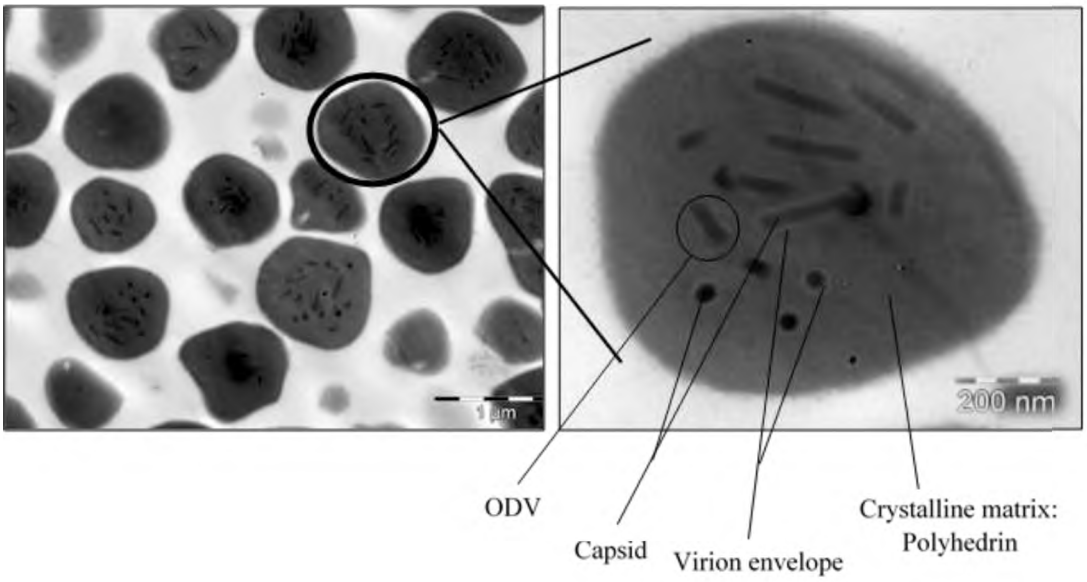


Figure 3.6: Transmission electron micrographs of OBs after sectioning of resin-embedded samples.

3.3.5 Viral enumeration

The concentration of OBs was calculated using a 2.75 g sample of symptomatic larvae. This sample was purified using glycerol gradient centrifugation. The concentration of OBs was enumerated using a counting chamber observed under a light microscope using dark field illumination. The average concentration was determined to be 3.16×10^{10} OBs/ml in a 1:40 dilution and 2.16×10^{10} OBs/ml in a 1:50 dilution. This enumeration was repeated for biological assays as it is important to have a known concentration of OBs in order to determine the correct dosage for the assays (See Chapter 5).

3.3.6 DNA extraction using NPV genomic DNA

Genomic DNA was successfully extracted from *C. peltastica* NPV crude extract of OBs. The DNA was analysed on 0.7% agarose gel electrophoresis and found to be of high molecular weight (>10 000 bp) (Figure 3.7). From the gel it was observed that the genomic DNA was of good quality as it produced a pure band and no degradation was observed. The DNA extraction protocol was conducted twice to ensure there was no contamination or false result.

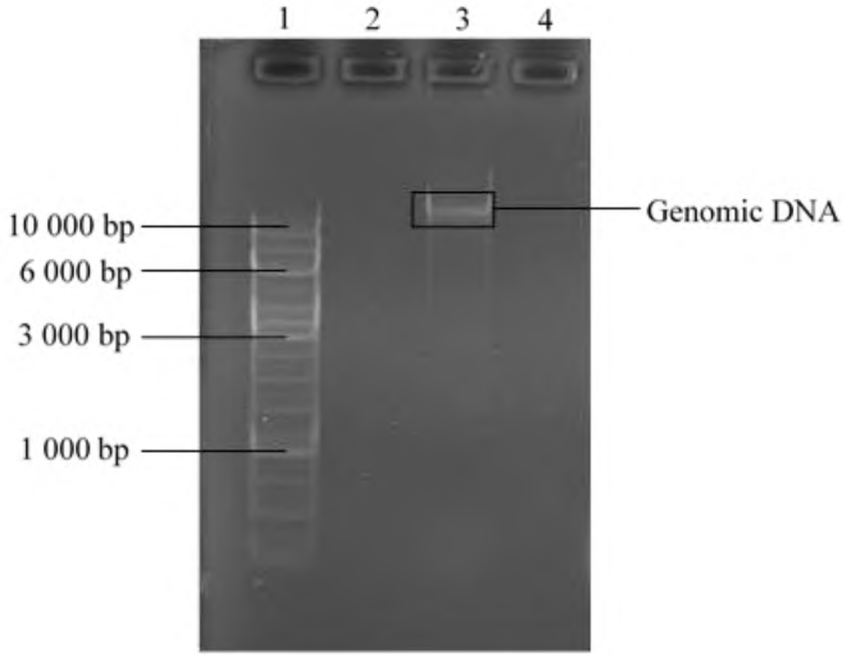


Figure 3.7: 0.7% Agarose gel electrophoresis used to observe genomic DNA of CrpeNPV. Lane 1: GeneRuler 1 Kbp DNA Ladder (Thermo Scienticfic®), Lane 3: CrpeNPV genomic DNA.

3.3.7 PCR amplification of the polyhedrin gene using degenerate primers

Genomic DNA obtained was used to amplify the polyhedrin gene using degenerate primers. The amplified product was analysed on 1% agarose gel electrophoresis. There was no evidence of contamination as no band was present in the negative control (lane 3). The positive control of CrleGV was successful as a ± 500 base pair product was amplified (Lane 5 & 6). The polyhedrin gene of *C. peltastica* was successfully amplified, as a PCR product of

approximately 500 base pairs was obtained (Lane 8 & 9). All products were of good quality, producing pure, tight bands (Figure. 3.8).

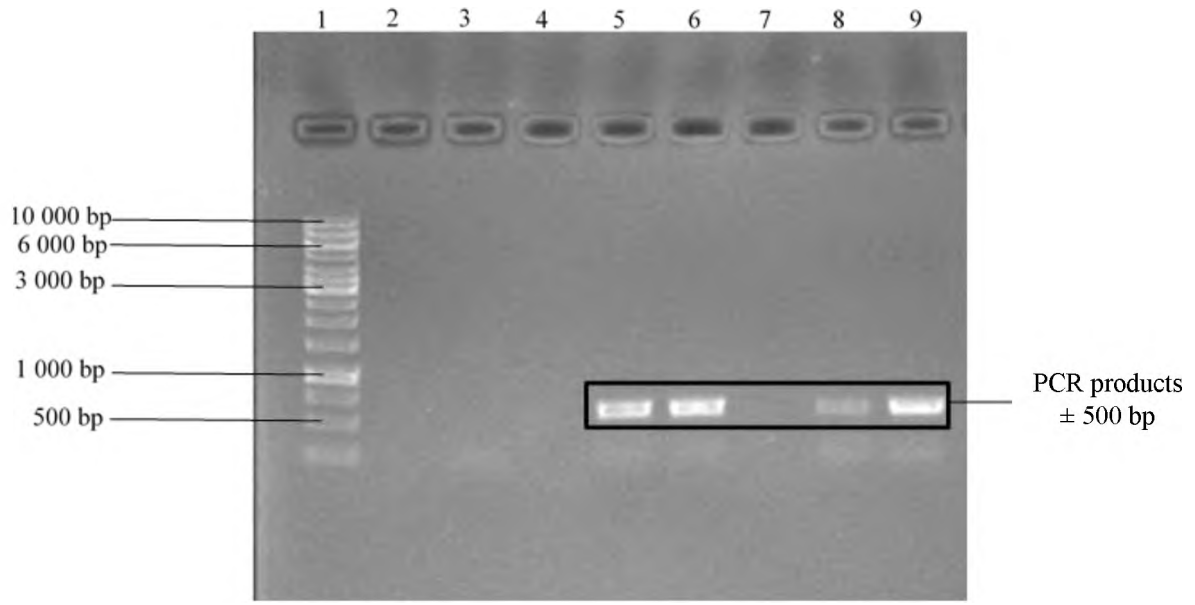


Figure 3.8: PCR amplification of the polyhedrin gene of *Cryptophlebia peltastica* analysed on 1% Agarose gel electrophoresis. Lane 1: GeneRuler 1 kKbp DNA Ladder (Thermo Scientific®), Lane 3: negative control and Lane 5: ± 500 bp positive control PCR product of concentrated CrleGV DNA, Lane 6: ± 500 bp PCR product of 1:10 dilution of CrleGV DNA, Lane 8: concentrate DNA of *Cryptophlebia peltastica* NPV and Lane 9: 1:10 dilution of *C. peltastica* NPV DNA.

3.3.8 Sequencing analysis

Sequences obtained were subjected to a BLAST, using a blastn analysis where a closest match of 93% was observed with a partial sequence of *Epinotia granitalis* polyhedrin gene region (501 nucleotides). After sequence clean-up the sequence for *C. peltastica* NPV was 507 nucleotides in length. Following translation and alignment between the two sequences, several SNPs were observed. Four of these SNPs resulted in amino acid changes: Isoleucine to Valine (nucleotide position: 169), Phenylalanine to Tyrosine (nucleotide position: 203), Serine to Alanine (nucleotide position: 358-360) and Valine to Isoleucine (nucleotide position: 433). The remaining SNPs were minor and did not result in any changes (Table 3.4).

Table 3.4: Single nucleotide polymorphisms (SNPs) found between *Cryptophlebia peltastica* and *Epinotia granitalis* NPV (93 % match) and the resulting amino acid changes.

Isolate		Amino Acid
***E. granitalis* NPV**	***C. peltastica* NPV**	
CC**A**	CC**C**	Proline
AC**G**	AC**A**	Threonine
CT**C**	CT**A**	Leucine
AC**C**	AC**T**	Threonine
GA**A**	GA**G**	Glutamic acid
AG**T**	AG**C**	Serine
TT**C**	TT**T**	Phenylalanine
GA**T**	GA**C**	Aspartic acid
GT**T**	GT**A**	Valine
ATT	**G**TT	Isoleucine - Valine
TG**T**	TG**C**	Cysteine
T**T**C	T**A**C	Phenylalanine - Tyrosine
CTG	**T**TG	Leucine
GC**G**	GC**T**	Alanine
CA**G**	CA**A**	Glutamine
GC**C**	GC**T**	Alanine
CC**A**	CC**G**	Proline
AG**T**	AG**C**	Serine
AA**A**	AA**G**	Lysine
GG**T**	GG**C**	Glycine
GG**T**	GG**A**	Glycine
AA**C**	AA**T**	Asparagine
CT**T**	CT**G**	Leucine
TCC	**G**CT	Serine - Alanine
AC**T**	AC**C**	Threonine

TT**T**	TT**C**	Phenylalanine
CC**T**	CC**C**	Proline
GTA	GT**G**	Valine
TA**T**	TA**C**	Tyrosine
GTT	**A**TT	Valine - Isoleucine
CT**T**	CT**A**	Leucine
TTG	**C**TG	Leucine

3.4 DISCUSSION

The laboratory culture of *C. peltastica* flourished under optimal conditions. The high density of *C. peltastica* larvae being reared possibly lead to overcrowding, presenting symptoms of baculovirus infection. This was also shown in a study by Fuxa (2004) where host insects were more susceptible to NPVs at high densities due to overcrowding. Other factors that contribute to the host's susceptibility are nutritional stress, changing temperature and high humidity (Goulson and Cory 1995; Fuxa 2004; Opoku-Debrah 2011; Opoku-Debrah *et al.* 2013), which the *C. peltastica* larvae were subjected to during rearing.

Larvae observed with these symptoms were collected and stored for further characterisation. Typical symptoms observed were slight to no movement, change in appearance; cuticle changing from a pink to pale pink-white (early stage) and brown-black (late stage). Once the larva's cuticle turned brown-black, the cuticle was highly sensitive to rupturing with minor contact (liquefaction). Once the cuticle ruptured, turbid/milky haemolymph was discharged, releasing multiple OBs for reinfection of other larvae (Andrade & Habid 1984; Fuxa 1991; Barreto *et al.* 2005; Aizawa 2012).

Occlusion bodies were purified from the symptomatic larvae collected using 50 to 60% glycerol gradient centrifugation to obtain pure OBs for analysis. Generally a glycerol gradient ultracentrifugation protocol would be used to obtain a high purity of OBs, however it has been found that 50 to 70% of NPV OBs can be lost within the gradient (Grzywacz *et al.* 2007). Therefore the ultracentrifugation protocol was unnecessary as pure OBs were obtained from the gradient centrifugation. A unique characteristic was observed with the gradient

centrifugation, as purified OBs are generally found in the band formed in the middle of the gradient and insect debris or other contaminants are found in the pellet. However, in this study numerous OBs were found in the pellet. The samples found in the pellet were pure and no contaminants were observed when observing transmission electron micrographs. A reason for finding OBs in the pellet could be due to clumping of the OBs, as it has been observed that pure OBs clump together which could have perhaps led to an increase in density resulting in the OBs being found in the pellet (Grzywacz *et al.* 2007; Erlandon & Theilmann 2009).

Occlusion bodies were observed by TEM to confirm and identify the presence and type of baculovirus collected from symptomatic larvae. The morphology of the virus is used to distinguish between an NPV and GV. One of the main morphological characteristics of an NPV is its irregular large circular shape and variable size (Allen & Knell 1977; Ishii *et al.* 2003; Kumar *et al.* 2011). Several NPVs isolated from the family Tortricidae, *Adoxophyes orana* (Fischer von Röslerstamm) (1834) (Lepidoptera: Tortricidae), *Adoxophyes reticulana* (Fischer von Röslerstamm) (1834) (Lepidoptera: Tortricidae), *Choristoneura fumiferana* (Clemens) (1865) (Lepidoptera: Tortricidae), *Pandemis heparana* (Denis & Schiffermüller) (1775) (Lepidoptera: Tortricidae), *Epiphyas postvittana* (Walker) (1863) (Lepidoptera: Tortricidae), *Epinotia granitalis* (Butler) (1881) (Lepidoptera: Tortricidae) and *Platynota idaeusalis* (Walker) (1859) (Lepidoptera: Tortricidae) have the similar irregular shape and vary in size from 700 to 2000 nm in diameter (Bilimoria 1991; Ishii *et al.* 2003; Takatsuka 2007). The *C. peltastica* NPV OBs observed had the same irregular morphology similar to the NPVs listed above. They also varied in size between 421 and to 1263.2 nm with a mean width of 731.09 ± 15.13 nm, which falls into the reported range for Tortricidae NPVs (Andrade & Habid 1984; Ishii *et al.* 2003; Kumar *et al.* 2011). NPVs are characterised by their polyhedra, as described above. The polyhedra contain another important characteristic of NPVs, that of nucleocapids which can either be found as single (SNPV) or multiple (MNPV) capsids within the ODV (Rohrmann 2013). *Cryptophlebia peltastica* NPV samples were sectioned to determine whether it was an SNPV or MNPV and the TEM images showed that each ODV contained a single nucleopcapsid, therefore confirming an SNPV in *C. peltastica* infected larvae.

To identify the virus genetically, PCR amplification and sequencing of the polh/gran gene using degenerate primers described by Lange *et al*, (2004) was performed. The 507 bp sequence was subjected to BLAST to determine the closest matched baculovirus sequence. The closest match was with a partial *Epinotia granitalis* polyhedrin gene sequence, with a similarity of 93%. When the sequences were analysed there were 34 SNPs, out of which only five resulted

in amino acid changes. *Epinotia granitalis*, the cypress bark moth, is native to Japan and is a pest of Japanese cedar and Japanese cypress (Takatsuka 2007). There is limited research on *E. granitalis* and only a partial polh/gran sequence is available for comparison. The reasons as to why the polyhedrin gene sequences of these viruses are so closely matched are unknown because they infect different insect species that are not found in the same geographic region and they do not share the same hosts.

In conclusion, a baculovirus recovered from symptomatic *C. peltastica* larvae has been identified morphologically as an SNPV, and will now be referred to as CrpeSNPV. Sequencing of the polh/gran gene followed by BLAST analysis showed that the closest match was a baculovirus from *E. granitalis*. This is the first identification of an SNPV infecting *C. peltastica* and further characterisation through whole genome sequencing and REN analysis of genomic DNA, as described in Chapter 4, is required to investigate the novel status of this virus.

Chapter 4

GENOMIC ANLAYSIS OF *CRYPTOPHLEBIA PELTASTICA* NUCLEOPOLYHEDROVIRUS

4.1 INTRODUCTION

Chapter three described the morphological and genetic characterisation of a novel nucleopolyhedrovirus for *C. peltastica*, CrpeNPV. Further genetic characterisation of the isolated NPV is required using restriction endonuclease (REN) analysis and sequencing of the full genome. REN analysis is a technique used extensively in molecular biology for genetic engineering, genome mapping and DNA sequence analysis (Roberts 1979; Bikandi *et al.* 2004). REN analysis is also an important technique that can be used to compare the genotypes of different virus isolates for biocontrol purposes (Miller & Dawes 1978; de Moraes & Manruniak 1997; Erlandson & Theilmann 2009).

Restriction endonuclease analysis is used to create a genetic 'fingerprint' for isolated viruses. In order to produce this 'fingerprint' viral DNA is digested with the use of restriction enzymes. These enzymes cut the DNA strands at certain nucleotide sequences, resulting in several pieces of DNA strands. The fragments are then analysed by agarose gel electrophoresis, which separates them according to size, creating a DNA profile. It is from these 'fingerprints' that viral isolates can be characterised in terms of genotype (Miller & Dawes 1978; Possee & Rohrmann 1997; Erlandson & Theilmann 2009; Mayrand, unpublished). REN profiles are primarily used to compare isolates of the same or similar viruses, in order to determine the novelty of the isolate. If full genome sequences are available, DNA profiles can also be generated in silico, using a variety of restriction enzymes. In this case, the band sizes are

accurate and can be used to compare DNA profiles produced by REN analysis of either the same or different virus isolate (Bikandi *et al.* 2004), here a novel baculovirus that has been isolated from *C. peltastica* (Chapter 3). This is a first report world-wide, of an NPV being isolated from this genus of host, *Thaumatotibia*, or the closely related tortricid genera of *Cryptophlebia* or *Cydia*. Therefore, no equivalent or comparable baculovirus isolates are available for comparison with CrpeNPV. Due to the novelty of CrpeNPV, a more comprehensive genetic analysis is required.

The most accurate and comprehensive method used to genetically characterise a virus is that of whole genome sequencing and analysis, using next generation sequencing (NGS). NGS has the ability to process millions of parallel sequence reads at once, using a high throughput approach producing short reads (35 – 250 bp) that are obtained from a fragment 'library'. These short reads are then used in a *de novo* assembly to create a whole genome sequence (Mardis 2007; Liu *et al.* 2011). There are three types of NGSs available; 454 pyrosequencing (454 Life Science), Illumina (Solexa) sequencing and Solid sequencing (ABI Biosystems). Illumina sequencing is the more widely used sequencing method and the one used in this study.

There are approximately 64 complete baculovirus genomes available on GenBank, 41 of these belong to the genera Alphabaculovirus (lepidopteran NPVs), 13 Betabaculoviruses (lepidopteran GVs), three Gammabaculoviruses (hymenopteran NPVs) and one Deltabaculovirus (dipteran NPVs) (Garavaglia *et al.* 2012; Ferrelli *et al.* 2012; Rohrmann 2014; Craveiro *et al.* 2015). Baculovirus genomes vary from 80 000 bp to 180 000 bp which code for 90 to 180 genes. A limited number of these genes are shared by all baculoviruses. These genes are known as the core genes and are responsible for crucial functions such as virion structure, the process of host gut infection and expression of late genes. Core genes include late expression factor (lef)-2, lef-1, DNA polymerase, DNA helicase, alkaline exonuclease, lef-8, lef-9, p47, lef-4, very late factor (VLF)-1, lef-5, vp1054, ac66, gp41, vp91, vp39 capsid, p6.9, ODV-e25, ac109, ac142, ODV-e18, ac144, pif-2, pif-6, pif-4, pif-3, pif-1, p74, ODV-e56, ubiquitin, sulfhydryl oxidase, 38k, ac78, ac81, ac93, ac101 and ac103 (Garavaglia *et al.* 2012; Ferrelli *et al.* 2012; Rohrmann 2014). Lepidopteran specific baculoviruses contain these core genes as well as additional genes which include polyhedrin/granulin, pk-1, ac13, F-protein, DNA binding protein (dbp), lef-6, ac29, fgf, 39k, lef-11, ODV-e66, ADP-ribose phosphate, lef-10, few polyhedral (fp), lef-3, ac75, ac76, telokin-like, ac102, ac106/107, ac110, Me53, ac145 and immediately early (ie)-0 (Rohrmann 2014). In some cases these genes may be present in genomes as homologs or functional analogs

(identified under another name) (Rohrmann 2014). The remaining genes differ among viruses, creating variation between isolates.

The aim of this chapter was to further characterise CrpeNPV by comprehensively analysing the virus genetically through the use of REN profiles and whole genome sequencing. The specific objectives were to create REN profiles using a variety of restriction enzymes and comparing these profiles to in silico profiles created from the whole genome sequence of the isolated CrpeNPV.

4.2 METHODS AND MATERIALS

4.2.1 Restriction Endonuclease Analysis

Genomic DNA was extracted (see Section 3.2.6) and used to create REN profiles, using the following fast digest restriction enzymes *Bam*HI, *Eco*RI, *Hind*III, *Kpn*I, *Pst*I, *Sal*I, *Sma*I, *Xba*I and *Xho*I (Thermo Scientific®, USA). A 30 µl reaction was set up, comprising of 200 ng/µl of template DNA, 3 µl fast digest restriction enzyme, 3 µl fast digest buffer and the remaining volume made up with ddH_2O. The reaction was incubated at 37°C for 15 minutes. REN profiles were visualised using 0.6% agarose gel electrophoresis at 30 volts for 16 hours in 1X TAE buffer and stained with ethidium bromide. Images of the REN profiles were captured using UVIpro chemi (UVItec) UV trans-illuminator. To determine the band sizes two DNA ladders were used, a GeneRuler high range ladder (Thermo Scientific®, USA) and GeneRuler 1 Kb DNA ladder (Thermo Scientific®, USA). UviBand software (UVItec) was used to estimate the band and genome sizes.

4.2.2 Whole genome analysis

4.2.2.1 Genome sequencing

The full genome of CrpeNPV using approximately 100 ng of DNA extracted from purified OBs (see Section 3.2.3) was sequenced by Inqaba Biotechnical Industries (Pty) Ltd (South Africa) using a MiSeq Desktop Sequencer (Illumina). The Illumina sequencing of the full genome of CrpeNPV produced a total of 3 871 946 paired reads. Geneious (New Zealand) version R7 (Kearse *et al.* 2012) was used to complete a *de novo* assembly. In order to produce

a *de novo* assembly, the ends of the paired reads were trimmed using the soft trimming function in Geneious. Once the paired reads were trimmed a *de novo* assembly was run using 20% of the data and numerous contigs were produced. The longest contig was selected as a consensus sequence. This consensus sequence was used to create a full genome sequence for CrpeNPV.

4.2.2.2 ORF identification

Open reading frames were identified using the 'annotate data from reference file' function in Geneious. This reference file was created using 18 complete NPV genome sequences (Table 4.1), 15 sequences obtained from the BLAST results in chapter 3, section 3.3.8, and 3 random sequences. Reference sequences were compared to the CrpeNPV genome sequence. From the comparison, genes from the reference sequences were aligned to a position with a similar sequence within the CrpeNPV genome. Out of the 18 references, the gene containing the highest percentage of transferred similarity was chosen. The ORF in the CrpeNPV genome was determined by identifying the start (ATG) and stop (TAG, TAA, TGA) codons within or around the highlighted gene sequence. The ORF was then annotated according to the gene with the highest transferred similarity. This process was followed for all the genes identified in the CrpeNPV genome. All genes were assigned an ORF number with the polyhedrin gene labelled ORF 1, and other ORFs labelled consecutively in a clockwise direction.

Table 4.1: Reference sequences used to annotate and identify ORFs in the CrpeNPV genome.

Name	Accession Number	BLAST similarity
Plusia orichalcea (Fabricius) (1775) (Lepidoptera: Noctuidae) NPV	Af019882	83%
Agrotis segetum (Denis & Schiffermüller) (1775) (Lepidoptera: Noctuidae) NPV	DQ123841	NA
Plutella xylostella (Linnaeus) (1758) (Lepidoptera: Plutellidae) NPV	DQ457003	83%
Clanis bilineata (Walker) (1866) (Lepidoptera: Sphingidae) NPV	DQ504428	81%

Anticarsia gemmatalis (Hübner) (1818) (Lepidoptera: Noctuidae) NPV	DQ813662	NA
Chrysodeixis chalcites (Esper) (1789) (Lepidoptera: Noctuidae) NPV TF1-C	JX560539	84%
Chrysodeixis chalcites (Esper) (1789) (Lepidoptera: Noctuidae) NPV TF1-G	JX560541	84%
Chrysodeixis chalcites (Esper) (1789) (Lepidoptera: Noctuidae) NPV TF1-H	JX560542	84%
Hemileuca species (Walker) (1855) (Lepidoptera: Saturniidae) NPV	KF158713	81%
Autographa californica (Speyer) (1875) (Lepidoptera: Noctuidae) NPV	L22858	83%
Lymantria dispar (Linnaeus) (1758) (Lepidoptera: Erebidae) NPV	NC001973	NA
Spodoptera exigua (Hübner) (1808) (Lepidoptera: Noctuidae) NPV	NC002169	NA
Helicoverpa armigera (Hübner) (1809) (Lepidoptera: Noctuidae) NPV	NC00309	80%
Adoxophyes honmai (Yasuda) (1998) (Lepidoptera: Tortricidae) NPV	NC004690	81%
Chrysodeixis chalcites (Esper) (1789) (Lepidoptera: Noctuidae) NPV	NC007151	81%
Trichoplusia ni (Hübner) (1800-1803) (Lepidoptera: Noctuidae)	NC007383	83%
Adoxophyes orana (Fischer von Rölerstamm) (1834) (Lepidoptera: Tortricidae) NPV	NC011423	82%
Apocheima cinerarium (Erschov) (1874) (Lepidoptera: Geometridae) NPV	NC018504	82%

4.2.2.3 In silico profiles

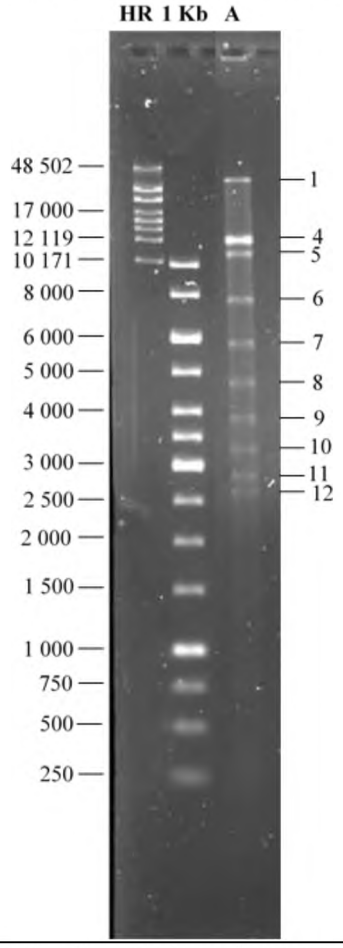
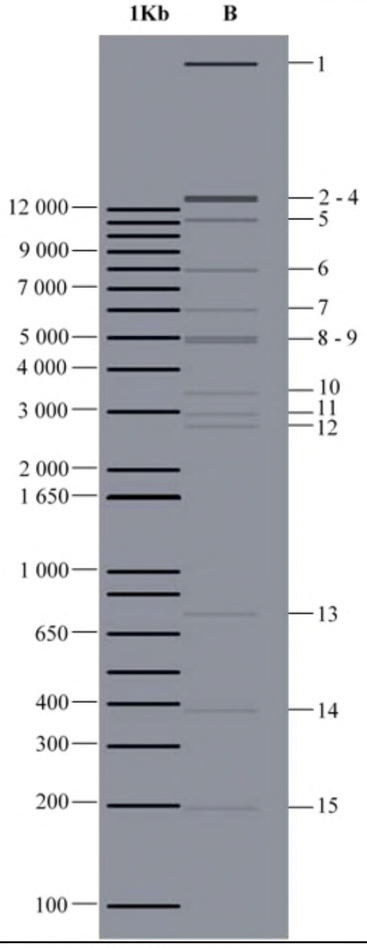
A Plasmid Editor (ApE) v2.0.47 software (USA) was used to create in silico profiles of the CrpeNPV genome. The following enzymes were used to create the profiles: *Bam*HI, *Eco*RI, *Hind*III, *Kpn*I, *Pst*I, *Sal*I, *SmaI*, *Xba*I and *Xho*I. The in silico and REN profiles were then compared against one another for accuracy.

4.3 RESULTS

4.3.1 Restriction Endonuclease Analysis Profiles

*4.3.1.1 Bam*HI *REN Profile*

The comparison between the REN and in silico *Bam*HI profiles generated for CrpeNPV is shown in Figure 4.1. A total of 10 bands were detected for the REN profiles (A), whereas 15 were observed in the in silico digest (B). The total estimate size of the genome was 84 403 bp for the REN profile and 116 646 for the in silico profile. The bands detected in the REN profile could be compared to similar bands produced by the in silico digest. However, a few bands were not present in the REN profile: bands 2, 3, 13, 14 and 15. Band 4 in the REN profile was of greater intensity than the rest of the bands, which could indicate the presence of a doublet or triplet band, resulting in the missing bands 2 and 3. Bands 13, 14 and 15 could not be detected due to low molecular weight. Due to the above factors and inaccurate sizing of bands the genome size according to the REN profile is less than the in silico profile.

BamHI				
C. peltastica NPV (in vitro)	**A**	**Band**	**B**	*C. peltastica* NPV (in silico)
	30 404	1	32 690	
		2	13 056	
		3	12 802	
	12119	4	12 759	
	10 700	5	11 212	
	7 900	6	7 887	
	5 900	7	6 019	
	4 800	8	4 963	
	3 886	9	4 866	
	3 300	10	3 398	
	2 832	11	2 952	
	2 562	12	2 714	
		13	748	
		14	385	
		15	195	
	84 403	**Size**	116 646	
	10	**Bands**	15	

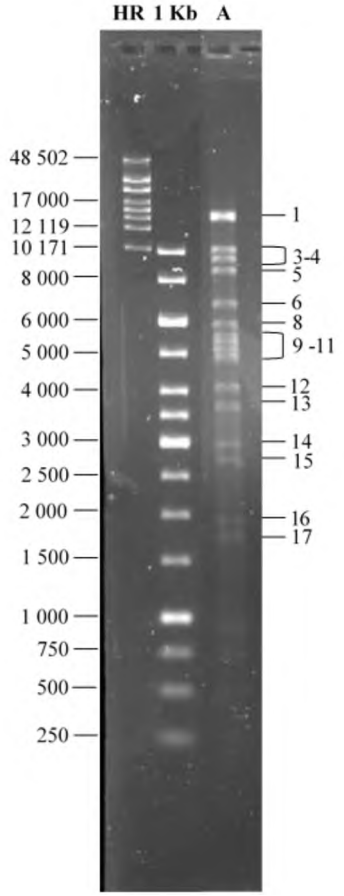
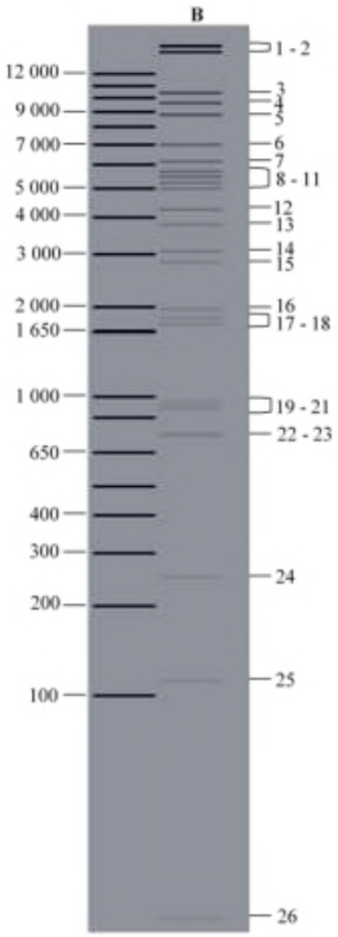
HR= GeneRuler High Range DNA ladder, 1Kb= GeneRuler 1Kb DNA ladder

Figure 4.1: *Bam*H1 restriction profiles comparing in vitro and in silico profiles of *Cryptophlebia peltastica* NPV genomic DNA.

*4.3.1.2 Eco*RI *REN Profile*

The comparison between the REN and in silico *Eco*RI profiles generated for CrpeNPV is shown in Figure 4.2. A total of 16 bands were detected for the REN profiles (A) whereas 26 were observed in the in silico digest (B). The total estimate size of the genome was 87 939 bp for the REN profile and 116 646 for the in silico profile. Like the *Bam*HI profile, the bands detected in the REN profile could be referred to similar bands produced by the in silico profile. Bands not present in the REN profile are bands 2, 7 and 18 - 26. Band one in the REN profile was of greater intensity than the rest of the bands, which could indicate the presence of a

doublet or triplet band, resulting in the missing bands. Bands 18 - 26 could not be detected due to low molecular weight. Due to the above factors and inaccurate sizing of bands the genome size according to the REN profile is less than the in silico profile.

***Eco*RI**				
C. peltastica NPV (in vitro)	**A**	**Band**	**B**	*C. peltastica* NPV (in silico)
	14 118	1	14 932	
		2	14 294	
	10 118	3	10 391	
	9 527	4	9 628	
	8 616	5	8 803	
	6 832	6	6 997	
		7	6 111	
	5 950	8	5 678	
	5 509	9	5 449	
	5 318	10	5 211	
	4 881	11	4 979	
	4 098	12	4 253	
	3 713	13	3 785	
	2 916	14	3 068	
	2 727	15	2 820	
	1 903	16	1 973	
	1 713	17	1 832	
		18	1 753	
		19	964	
		20	934	
		21	914	
		22	753	
		23	744	
		24	250	
		25	112	
		26	18	
	87 939	**Size**	116 646	
	16	**Bands**	26	

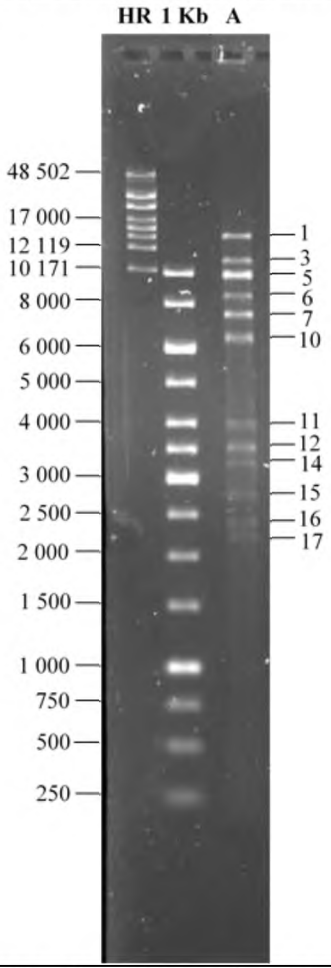
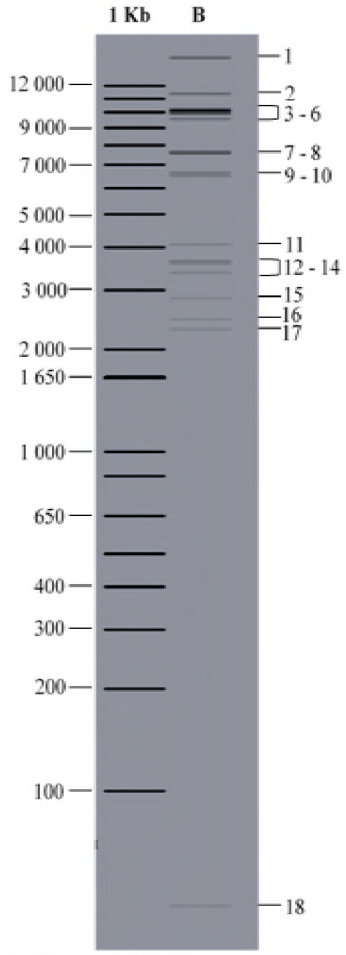
HR= GeneRuler High Range DNA ladder, 1Kb= GeneRuler 1Kb DNA ladder

Figure 4.2: *Eco*RI restriction profiles comparing in vitro and in silico profiles of *Cryptophlebia peltastica* NPV genomic DNA.

*4.3.1.3 Hind*III *REN Profile*

The comparison between the REN and in silico *Hind*III profiles generated for CrpeNPV is shown in Figure 4.3. A total of 12 bands were detected for the REN profiles (A) whereas 18

were observed in the in silico digest (B). The total estimate size of the genome was 74 772 bp for the REN profile and 116 646 for the in silico profile. Like the *Eco*RI profile, the bands detected in the REN profile could be referred to similar bands produced by the in silico profile. Bands not present in the REN profile are bands 2, 4, 8, 9, 13 and 18. Band 5 in the REN profile was of greater intensity than the rest of the bands, which could indicate the presence of a doublet or triplet band, resulting in the missing bands. Band 18 could not be detected due to low molecular weight. Due to the above factors and inaccurate sizing of bands the genome size according to the REN profile is less than the in silico profile.

		***Hind*III**		
C. peltastica NPV (in vitro)	**A**	**Band**	**B**	*C. peltastica* NPV (in silico)
	14 040	1	14 516	
		2	11 357	
	10 579	3	10 226	
		4	10 128	
	9 900	5	9 993	
	8 299	6	9 564	
	7 500	7	7 659	
		8	7 609	
		9	6 679	
	6 250	10	6 510	
	4 000	11	4 093	
	3 552	12	3 663	
		13	3 614	
	3 359	14	3 383	
	2 727	15	2 852	
	2 381	16	2 455	
	2 185	17	2 299	
		18	46	
	74 772	**Size**	116 646	
	12	**Bands**	18	

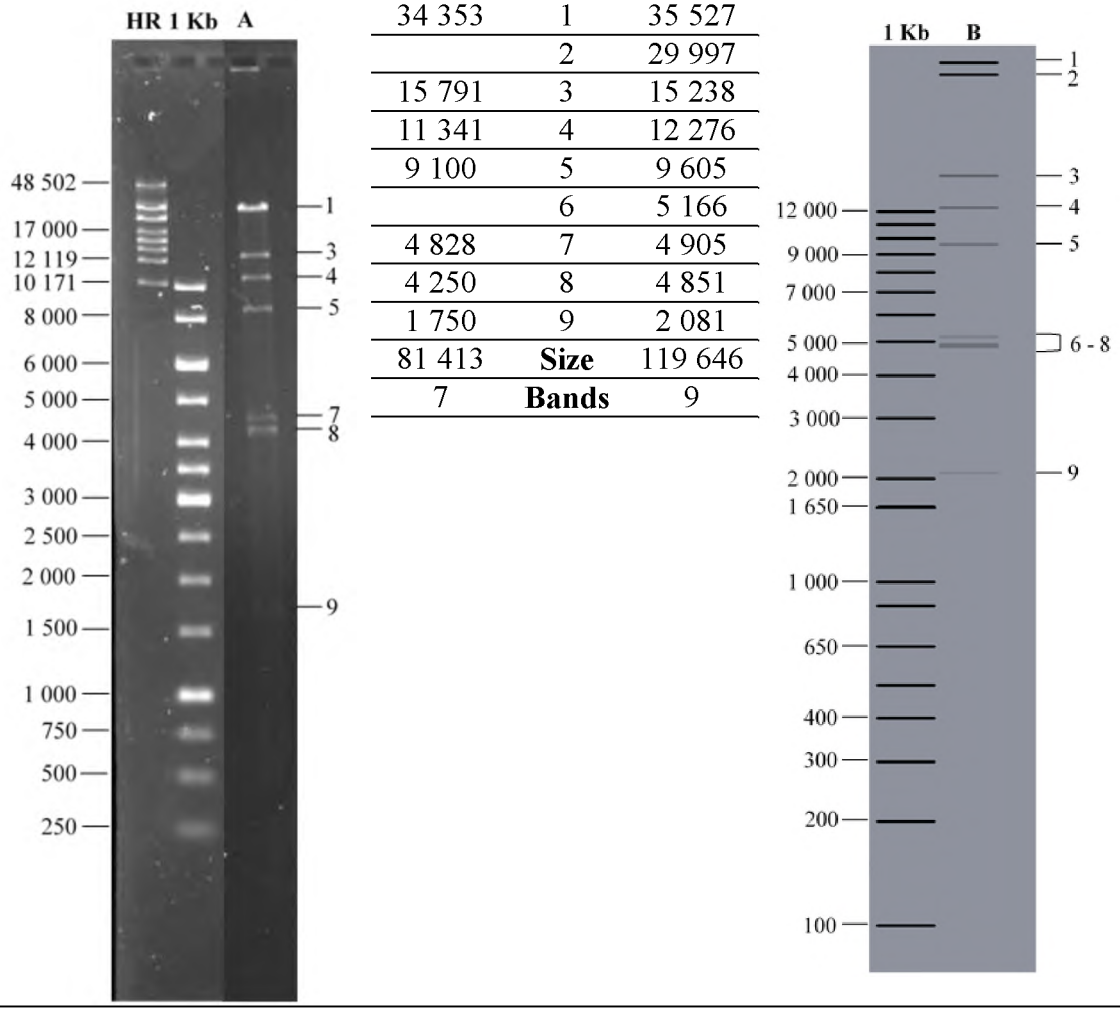
HR= GeneRuler High Range DNA ladder, 1Kb= GeneRuler 1Kb DNA ladder

Figure 4.3: *Hind*III restriction profiles comparing in vitro and in silico profiles of *Cryptophlebia peltastica* NPV genomic DNA.

*4.3.1.4 Kpn*I *REN Profile*

The comparison between the REN and in silico *Kpn*I profiles generated for CrpeNPV is shown in Figure 4.4. A total of 7 bands were detected for the REN profiles (A) whereas 9 were observed in the in silico digest (B). The total estimate size of the genome was 81 413 bp for the REN profile and 119 646 for the in silico profile. Like the *Hind*III profile, the bands detected in the REN profile could be referred to similar bands produced by the in silico profile. Bands not present in the REN profile are bands 2 and 6. Band 1 in the REN profile was of greater intensity than the rest of the bands, which could indicate the presence of a doublet or triplet band, resulting in the missing bands. Due to doublet and triplet bands, low molecular weight and inaccurate sizing of bands the genome size according to the REN profile was less than the in silico profile.

*Kpn*I			
C. peltastica NPV (in vitro)	**A**	**Band**	**B**
	34 353	1	35 527
		2	29 997
	15 791	3	15 238
	11 341	4	12 276
	9 100	5	9 605
		6	5 166
	4 828	7	4 905
	4 250	8	4 851
	1 750	9	2 081
	81 413	**Size**	119 646
	7	**Bands**	9



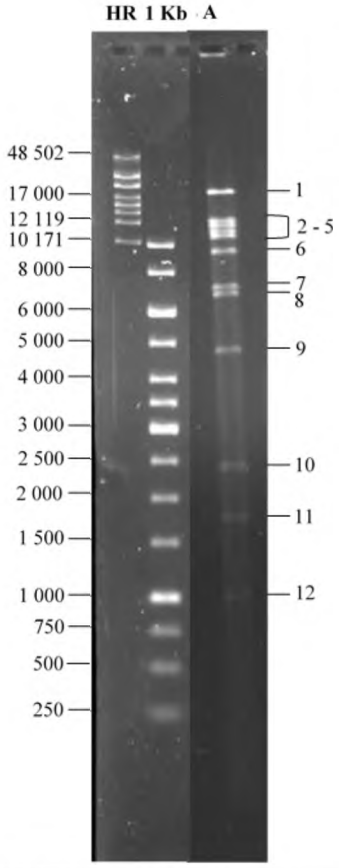
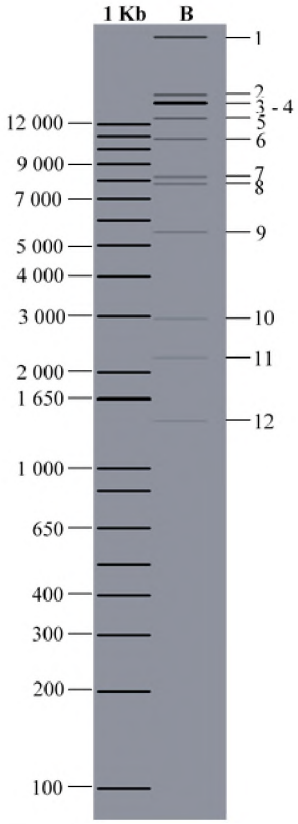
HR= GeneRuler High Range DNA ladder, 1Kb= GeneRuler 1Kb DNA ladder

Figure 4.4: *Kpn*I restriction profiles comparing in vitro and in silico profiles of *Cryptophlebia peltastica* NPV genomic DNA.

*4.3.1.5 Pst*I *REN Profile*

The comparison between the REN and in silico *Pst*I profiles generated for CrpeNPV is shown in Figure 4.5. A total of 12 bands were detected for the REN profiles (A) and 12 bands were also observed in the in silico digest (B). The total estimate size of the genome was 102 859 bp for the REN profile and 116 646 for the in silico profile. Like the *Kpn*I profile, the bands detected in the REN profile could be referred to similar bands produced by the in silico profile.

Due to inaccurate sizing of bands, as sizing in REN profiles are determined by estimation, the genome size according to the REN profile is less than the in silico profile.

		***Pst*I**		
C. peltastica NPV (in vitro)	**A**	**Band**	**B**	*C. peltastica* NPV (in silico)
	24 035	1	22 489	
	12 119	2	14 787	
	11 350	3	14 039	
	10 900	4	13 955	
	10 160	5	12 493	
	10 010	6	10 800	
	7 235	7	8 204	
	6 950	8	7 806	
	4 900	9	5 492	
	2 450	10	2 938	
	1 750	11	2 231	
	1 000	12	1 412	
	12	**Size**	12	
	102 859	**Bands**	116 646	

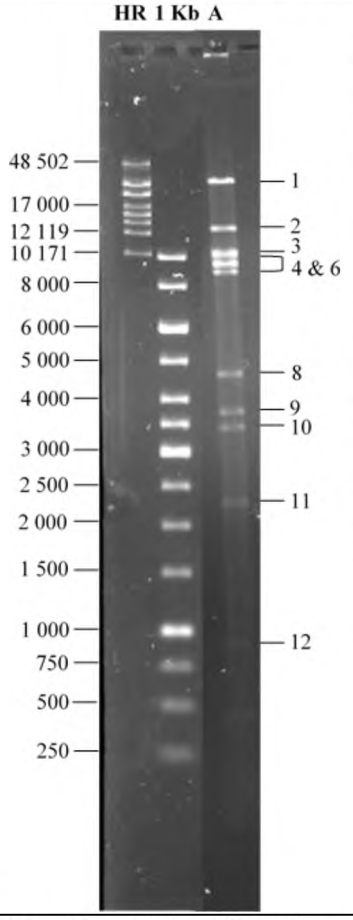
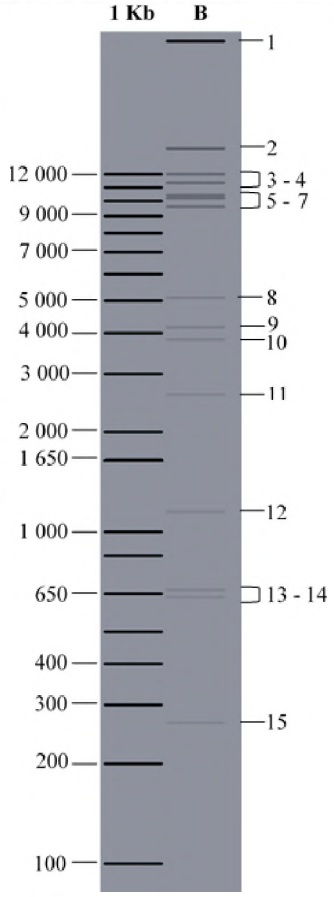
HR= GeneRuler High Range DNA ladder, 1Kb= GeneRuler 1Kb DNA ladder

Figure 4.5: *Pst*I restriction profiles comparing in vitro and in silico profiles of *Cryptophlebia peltastica* NPV genomic DNA.

*4.3.1.6 Sal*I *REN Profile*

The comparison between the REN and in silico *Sal*I profiles generated for CrpeNPV is shown in Figure 4.6. A total of 10 bands were detected for the REN profiles (A) whereas 15 were observed in the in silico digest (B). The total estimate size of the genome was 95 176 bp for the REN profile and 116 646 for the in silico profile. Like the *Pst*I profile, the bands detected

in the REN profile could be referred to similar bands produced by the in silico profile. Bands not present in the REN profile are 5, 7, 13 - 15. Band 3 and 4 in the REN profile were of greater intensity than the rest of the bands, which could indicate the presence of a doublet or triplet band, resulting in the missing bands. Band 13 - 15 could not be detected due to low molecular weight. Due to these factors and inaccurate sizing of bands the genome size according to the REN profile was less than the in silico profile.

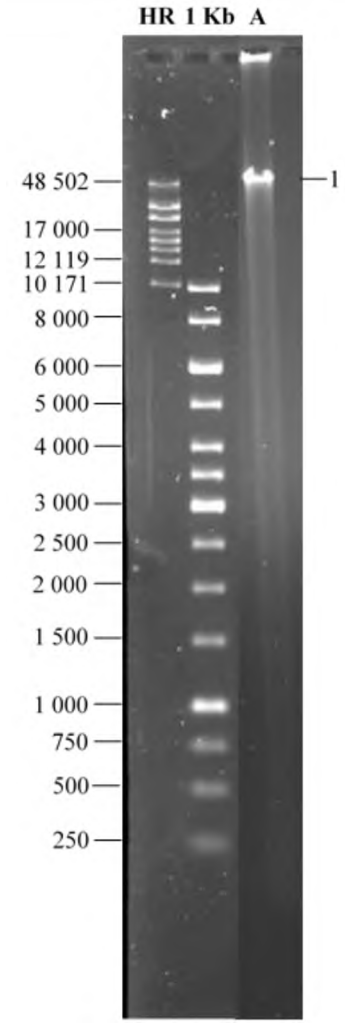
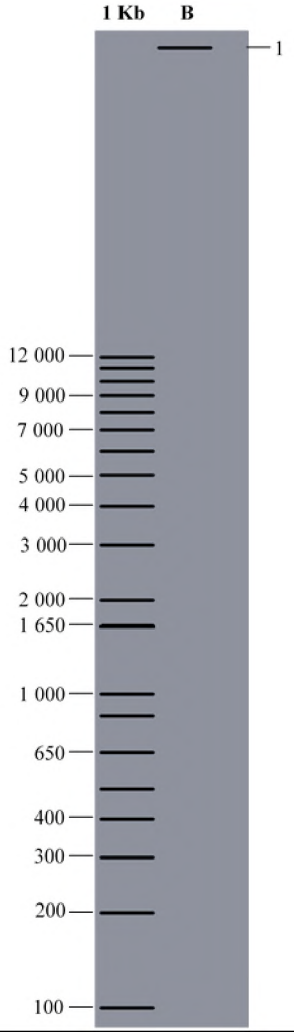
		***Sal*I**		
C. peltastica NPV (in vitro)	**A**	**Band**	**B**	*C. peltastica* NPV (in silico)
	38 502	1	30 356	
	12 243	2	14 350	
	10 341	3	11 999	
	10 110	4	11 366	
		5	10 401	
	8 945	6	10 201	
		7	9 625	
	4 804	8	5 085	
	3 731	9	4 147	
	3 470	10	3 803	
	2 210	11	2 594	
	820	12	1 148	
		13	670	
		14	635	
		15	266	
	95 176	**Size**	116 646	
	10	**Bands**	15	

HR= GeneRuler High Range DNA ladder, 1Kb= GeneRuler 1Kb DNA ladder

Figure 4.6: *Sal*I restriction profiles comparing in vitro and in silico profiles of *Cryptophlebia peltastica* NPV genomic DNA.

*4.3.1.7 Sma*I *REN Profile*

The comparison between the REN and in silico *Sma*I restriction profiles generated for CrpeNPV is shown in Figure 4.7. A single band was detected for both the REN (A) and in silico (B) profiles. The total estimate size of the genome was 80 494 bp for the REN profile and 116 646 for the in silico profile.

***Sma*I**				
C. peltastica NPV (in vitro)	**A**	**Band**	**B**	*C. peltastica* NPV (in silico)
	80 494	1	116 646	
	80 494	**Size**	116 646	
	1	**Bands**	1	

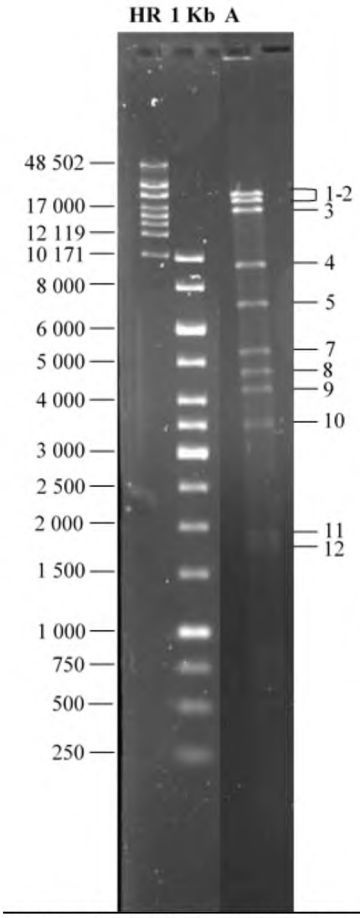
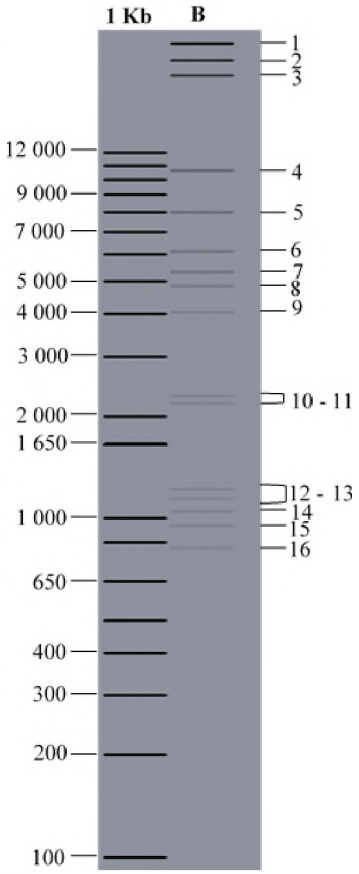
HR= GeneRuler High Range DNA ladder, 1Kb= GeneRuler 1Kb DNA ladder

Figure 4.7: *Sma*1 restriction profiles comparing in vitro and in silico profiles of *Cryptophlebia peltastica* NPV genomic DNA.

The band detected in the REN profile was substantially smaller than that of the in silico profile. This is due to inaccurate estimation of band size in the REN profile. The profile produced by *Sma*I is a diagnostic feature for CrpeNPV, due to the single band and can be used in the future as a distinguishing feature between viral isolates.

*4.3.1.8 Xba*I *REN Profile*

The comparison between the REN and in silico *Xba*I profiles generated for CrpeNPV is shown in Figure 4.8. A total of 11 bands were detected for the REN profiles (A) whereas 16 were observed in the in silico digest (B). The total estimate size of the genome was 96 472 bp for the REN profile and 116 646 for the in silico profile. Like the *Kpn*I profile, the bands detected in the REN profile could be referred to similar bands produced by the in silico profile. Bands not present in the REN profile are bands 6 and 13 - 16. These bands could not be detected due to low molecular weight. Due to this and inaccurate sizing of bands the genome size according to the REN profile was less than the in silico profile.

XbaI				
C. peltastica NPV (in vitro)	**A**	**Band**	**B**	*C. peltastica* NPV (in silico)
	20 650	1	25 180	
	19 870	2	22 457	
	17 235	3	20 308	
	9 980	4	10 659	
	7 150	5	7 994	
		6	6 123	
	5 230	7	5 330	
	4 860	8	4 854	
	4 178	9	4 071	
	3 570	10	2 295	
	1 950	11	2 188	
	1 800	12	1 219	
		13	1 147	
		14	1 048	
		15	953	
		16	820	
	96 472	**Size**	116 646	
	11	**Bands**	16	

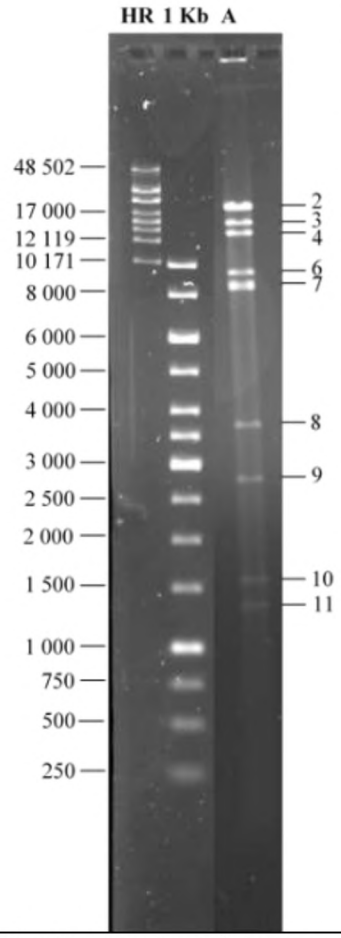
HR= GeneRuler High Range DNA ladder, 1Kb= GeneRuler 1Kb DNA ladder

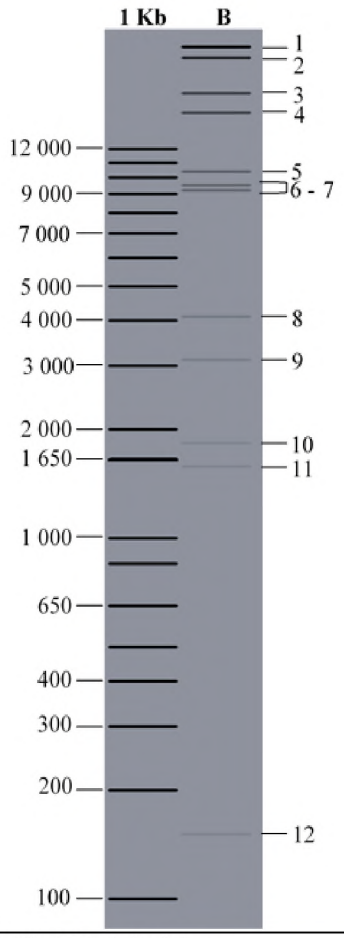
Figure 4.8: *Xba*1 restriction profiles comparing in vitro and in silico profiles of *Cryptophlebia peltastica* NPV genomic DNA.

*4.3.1.9 Xho*I *REN Profile*

The comparison between the REN and in silico *Xho*I profiles generated for CrpeNPV is shown in Figure 4.9. A total of 9 bands were detected for the REN profiles (A) whereas 12 were observed in the in silico digest (B).

XhoI				
C. peltastica NPV (in vitro)	**A**	**Band**	**B**	*C. peltastica* NPV (in silico)
		1	23 004	
	20 490	2	21 421	
	15 200	3	17 188	
	13 532	4	15 148	
		5	10 380	
	9 990	6	9 487	
	8 530	7	9 229	
	3 850	8	4 111	
	2 900	9	3 114	
	1 650	10	1 835	
	1 435	11	1 578	
		12	151	
	77 577	**Size**	116 646	
	9	**Bands**	1	





HR= GeneRuler High Range DNA ladder, 1Kb= GeneRuler 1Kb DNA ladder

Figure 4.9: *Xho*I restriction profiles comparing in vitro and in silico profiles of *Cryptophlebia peltastica* NPV genomic DNA.

The total estimate size of the genome was 77 577 bp for the REN profile and 116 646 for the in silico profile. Like the *Xba*I profile, the bands detected in the REN profile could be referred to similar bands produced by the in silico profile. Bands not present in the REN profile are 1, 5 and 12. Bands 2 and 7 in the REN profile were of greater intensity and thicker than the rest of the bands, which could indicate the presence of doublet or triplet bands, resulting in the apparently missing bands. Band 12 could not be detected due to low molecular weight. Due to these factors and inaccurate sizing of bands, the genome size according to the REN profile was less than the in silico profile.

4.3.2 Analysis of the CrpeNPV genome

A full genome sequence for CrpeNPV was obtained from an Illumina MiSeq desktop sequencer. A total of 3 871 946 paired reads were produced from the Illumina sequencing. Of the three million reads 760 297 were used to complete a *de novo* assembly, which produced 104 532 contigs. The largest contig, contig 1, was used to create a consensus sequence of 116 646 bp. The consensus sequence had a coverage of 731.6 ± 390.8 (Mean ± StDev) and a GC content of 37.1%.

The full genome of CrpeNPV was annotated using a database of 18 NPV full genome reference sequences. A total of 105 genes with complete coding regions (stop to start condons) were identified in the genome. The 105 genes were labelled according to ORF numbers, ORF1 starting at the polyhedrin gene. These were then colour coded according to core gene function: structural, replication, transcription, homologous, auxiliary and unknown functions (Figure 4.10).

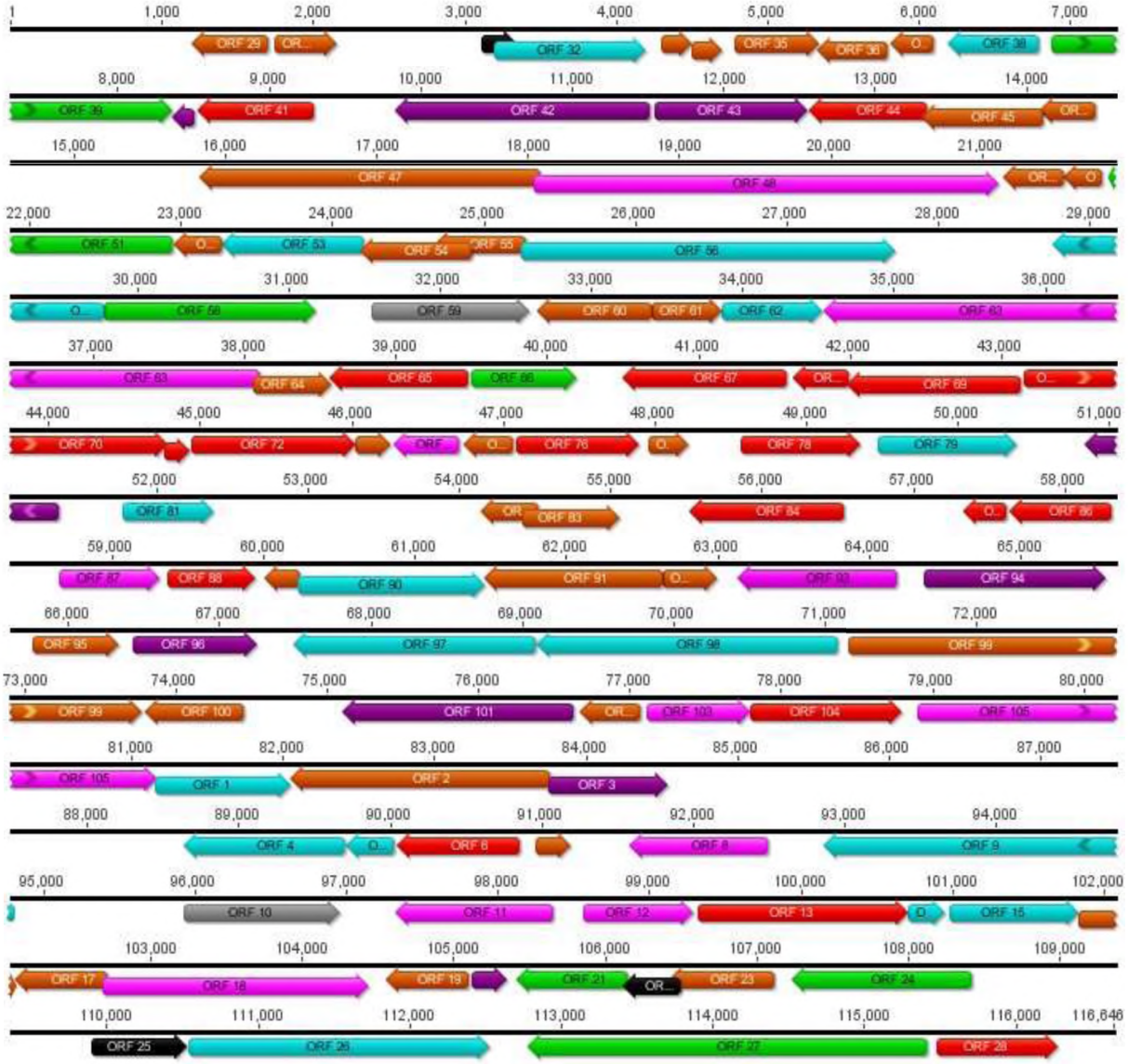


Figure 4.10: Representation of the full genome sequence of CrpeNPV. Core genes are represented by different coloured arrows; pink = Replication, green = Transcription, blue = Structural, purple = Auxiliary, black = Regulation of gene expression, grey = Bro genes, orange = hypothetical regions and red = unknown functions.

Core genes involved in replication

Eleven core genes involved in DNA replication (Table 4.11, pink arrows [figure 4.1]) were identified in the CrpeNPV genome. Five of the six essential replication genes were identified: lef-1, lef-2, DNA polymerase, helicase and immediately-early (ie) -1. Lef-3 was not found in the CrpeNPV genome. All core genes involved in nucleotide metabolism were identified:

dUtPase, ribonucleotide reductase (rr) 1 and rr 2. One non-essential stimulatory replication gene was recognized and the following were not found ie-2; lef-7 and pe38.

Transcriptional core genes

All Seven transcriptional core genes (Table 4.11, green arrows [Figure 4.10]) were identified in the CrpeNPV gene and are common to all baculoviruses.

Auxiliary core genes

Nine of the 15 auxiliary genes (Table 4.2, purple arrows [Figure 4.10]) present in baculoviruses were identified in the CrpeNPV genome. The following auxiliary genes were not found: protein tyrosine phosphatase (ptp) -1, ptp-2, arif, viral enhancing factor (vef) and proliferating cell nuclear antigen (pcna).

Regulation of gene expression

The regulation of gene expression core genes (Table 4.2, black arrows [Figure 4.10]) share six genes with the transcriptional genes; lef-4, lef-5, lef-8, lef-9, 39k and p47. Gene lef-10, lef-11 and lef-12 are the only genes not shared and identified in the CrpeNPV genome. The lef-6 gene was not found.

Structural genes

Eighteen structural core genes (Table 4.2, blue arrows [Figure 4.10]) were recognized in the CrpeNPV genome. Common baculovirus structural genes not identified in the genome include basic DNA binding protein p6.9, odvp-6e, p95, gp64, vp80/87, gp67 and p87 capsid.

Table 4.2: Core genes identified in CrpeNPV genome with respective opening reading frame (ORF) numbers for replication (pink), transcription (green), auxiliary (purple), structural (blue) and regulation of gene expression (black), as well as hypothetical regions (orange), bro regions (grey) and unknown functions (red).

Function	Gene	ORF		Gene	ORF
REPLICATION	DNA binding protein	ORF 8		PKIP	ORF 88
	Me53	ORF 11		38.7k	ORF 104
	Ie-0	ORF 12	STRUCTURAL	polh	ORF 1
	Ie-1	ORF 18		Odv-e56	ORF 4
	DNA polymerase	ORF 48		P10	ORF 5
	Helicase	ORF 63		P74	ORF 9
	dUtPase	ORF 74		Odv-e18	ORF 14
	Lef-2	ORF 87		Odv-e27	ORF 15
	rr-2	ORF 93		Odv-e66	ORF 26
	Lef-1	ORF 103		Vp1045	ORF 32
	rr1	ORF 105		Fp25k	ORF 38
TRAN-SCRIPTION	39kDa	ORF 21		Gp41	ORF 53
	P47	ORF 24		Vp91	ORF 56
	Lef -8	ORF 27		Vp 39	ORF 57
	Lef-9	ORF 39		Odv-e25	ORF 62
	Vlf-1	ORF 51		Pep	ORF 79
	Lef-4	ORF 58		Pif-3	ORF 81
	Lef-5	ORF 66		Pif-2	ORF 90
AUXILIARY	Protein kinase	ORF 3		Pif-1	ORF 97
	Viral ubiquitin	ORF 20		Fusion protein	ORF 98
	Conotoxin	ORF 40	GENE EXPRESSION	Lef-11	ORF 22
	Chitinase	ORF 42		Lef-12	ORF 25
	v-cathespin	ORF 43		Lef-10	ORF 31
	SOD	ORF 80	HYPOTHETICAL REGIONS	Hypothetical	ORF 2, 7, 16, 17, 19, 23, 29, 30, 33-37, 45-47, 49-50, 52, 54, 55, 60, 61, 64, 73, 75, 77, 82, 83, 89, 91, 92, 95, 99, 100, 102
	Alkaline exonuclease	ORF 94			
	fgf	ORF 96			
	egt	ORF 101			
BRO GENES	bro	ORF 10			
	bro	ORF 59			
UNKNOWN FUNCTION	P26	ORF 6			
	P49	ORF 13			
	j-domain	ORF 28			
	Gp37	ORF 41			
	Iap-2	ORF 44			
	38k	ORF 65			
	P40	ORF 67			
	P12	ORF 68			
	P48	ORF 69			
	Capsid	ORF 70			
	Ac110	ORF 71			
	Odv-ec43	ORF 72			
	P13	ORF 76			
	Iap-3	ORF 78			
	nrk-1	ORF 84			
	Gp16	ORF 85			
	P24	ORF 86			

4.4 DISCUSSION

To determine the genetic 'fingerprint' of CrpeNPV, genomic DNA was used to produce restriction profiles for *Bam*HI, *Eco*RI, *Hind*III, *Kpn*I, *Pst*I, *Sal*I, *Sma*I, *Xba*I and *Xho*I. This is the first report of a baculovirus for *C. peltastica* and no restriction profiles or whole genome sequences are available for comparison. Therefore, the REN profiles generated were compared to in silico profiles created using the CrpeNPV whole genome sequence obtained during this study. From the results, it was shown that the in silico method is a more accurate method to create restriction profiles, as REN analysis using gel electrophoresis is an estimation of the genome size. Nonetheless, the profiles can be used in the future for comparisons of other potential isolates of CrpeNPV. A diagnostic feature for future comparisons is the profile created by the restriction enzyme *Sma*I. A single band was produced in the profile of *Sma*I, unlike the other profiles which produce a range of bands. This unique profile can be used to differentiate virus isolates. As no other profiles were available for comparison, REN analysis could not be used to accurately characterise CrpeNPV. Therefore the whole genome was sequenced for a more accurate method of genetic characterisation.

Nucleopolyhedroviruses have been isolated from 28 lepidopteran families, compared to 19 GVs (Lapointe *et al.* 2012). Sequences of baculovirus genomes range from 90 000 bp to 180 000 bp. The largest alphabaculovirus (lepidoptera NPV specific) genome is *Leucania separata* NPV (LeselNPV) at 168 041 bp and the smallest genome is *Adoxophyes orana* NPV (AdorNPV) at 111 724 bp (Ferrelli *et al.* 2012; Rohrmann 2014). The full genome of CrpeNPV was sequenced and assembled into a contiguous sequence of 116 646 bp. The CrpeNPV genome falls into the NPV genome range. Baculovirus genomes have a GC content of less than 50%; the lowest GC content is that of *Neodiprion lecontei* NPV (NeleNPV) at 33.3% (Ferrelli *et al.* 2012). The CrpeNPV genome had a GC content of 37.1%.

A total of 105 methionine-initiated ORFs were identified with little to no overlap. The first ORF of the sequence was the polyhedrin gene and labelled consecutively in a clockwise direction. The CrpeNPV ORFs displayed no preference to orientation, 50% forward and 50% reverse. The majority of the 31 core genes identified in all baculovirus genome were recognised in the CrpeNPV genome with the exception of p6.9, odvp-6e, p95, gp64, vp80/97, gp67, p87, lef-6, ptp-2, ptp-2, arif, vef, pcna, ie-2, lef-7, pe38 and lef-3. The gp64 gene is a distinguishing feature for NPVs, as NPVs are divided into two groups, group 1 and group 2. Group 1 NPVs express the gp64 gene and fusion protein, whereas group 2 NPVs lack the gp64 gene but

contain the fusion protein. Therefore, the CrpeNPV is a group 2 NPV (Rohrmann 2014). The missing genes ie-2, lef-7 and pe38 are non-essential genes used for the stimulation of DNA replication (de Jong *et al.* 2005). The lef-7 gene plays a role in the host range of baculoviruses, ie-2 is involved in binding to the host's transcriptional factors, and pe-38 stores the host's multi-protein complexes of G-actin in the nucleus (Ferrelli *et al.* 2012; Nguyen *et al.* 2013). The essential lef-3 gene involved in DNA synthesis and stimulation of apoptosis was not identified in the CrpeNPV genome. This has also been observed in the genome of CumiNPV (Afonso *et al.* 2001; Ferrelli *et al.* 2012; Nguyen *et al.* 2013).

All essential genes involved in transcription were identified in the CrpeNPV genome. Five auxiliary genes were not identified in the CrpeNPV genome: ptp-1, ptp-2, arif, vef and pcna. The only gene that is conserved amongst all baculovirus genomes is the alkaline exonuclease gene which was identified in the CrpeNPV genome. The missing genes ptp 1 and 2 are involved in growth and development, particularly the hyperactive behaviour of the host and the arif gene is associated with the cytoskeleton, storing F-actin by the plasma membrane. The remaining auxiliary genes are mainly involved in virus replication (Herniou *et al.* 2003; Nguyen *et al.* 2013). Structural genes not present in the CrpeNPV genome include DNA binding protein p6.9, odvp-6e, p95, gp64, vp80/87, gp67 and p87. The DNA binding protein p6.9 is used for nucleocapsid structure, VP80 is involved with the transport of nucleocapsids in the cytoplasm and gp-64 interacts with the host cell receptors (Herniou *et al.* 2003; Nguyen *et al.* 2013). Another gene not identified in the CrpeNPV genome is lef-6 which is involved in the regulation of gene expression, more specifically it contains a TIP associating domain that interacts with nuclearporins, which allows for the export of mRNA to the cytoplasm (Nguyen *et al.* 2013). Genes IAP-2, IAP-3, p49 and 38k are thought to have an unknown function, however it has been found that IAP and p49 are involved in apoptosis, mainly interacting with caspases whilst the 38k gene is involved in inhibiting RNA elongation (Nguyen *et al.* 2013). It is assumed that the hypothetical genes identified in CrpeNPV could possibly be associated with these missing genes. Therefore, the hypothetical genes could possibly be homologs or functional analogs of the missing genes.

In conclusion, the novel CrpeNPV isolated was genetically characterised by means of REN analysis using genomic DNA and gel electrophoresis, and in silico analysis using the whole genome and genome sequencing using NGS technology. As this is the first world-wide isolate of a baculovirus for *C. peltastica* and a nucleopolyhedrovirus from this and any closely related species, there are no isolates available for comparison, thus these results will be used for future

comparison of potential new isolates. The analysis of the whole genome sequence further genetically characterised the virus and placed the isolate into group 2 NPVs. The whole genome sequence completed in this study is a preliminary assembly and requires more characterisation in the future. The following chapter involves developing biological assays and determining the virulence of CrpeNPV under laboratory conditions.

Chapter 5

BIOLOGICAL ACTIVITY OF CrpeNPV AGAINST TORTRICID PESTS

5.1 INTRODUCTION

Baculovirus characterisation was described in chapters 3 and 4. The next objective was to evaluate the potency of *Cryptophlebia peltastica* NPV as a potential microbial control agent. This screening is made possible through the use of biological assays (bioassays) (Cory & Bishop 1997). As stated by Saha (2002) "biological assays are methods used for the estimation of nature, constitution, or potency of a material (or of a process) by means of the reaction that follows its application to living matter". Bioassays are used to screen baculoviruses mainly focused with the virus-host relationship. This information is then used to determine the virulence (rate of kill and mortality) of the virus to determine suitable application rates in the field (Carter 1984; Cory & Bishop 1997).

The potency and efficacy of bioassays are dependent on various factors: (1) purity of the sample to be used (purity of occlusion bodies (OBs)), this can be determined through morphological studies using transmission electron microscopy or scanning electron microscopy, (2) contamination of the sample i.e. whether there is bacteria or fungi present in the sample, and (3) reproducibility of the bioassays (Vail 1975; Whitlock 1978). In order to obtain this information, the dosage mortality (the amount of pathogen required) and time-mortality (the amount of time required to kill the host) using bioassay techniques such as diet incorporation, surface-contamination (surface dosage), diet plugs and droplet feeding has to be determined (Farrar & Ridgway 1998; Van Frankenhuyzen *et al.* 2007).

Dosage-response (mortality) bioassays enable the calculation of the LC_{50} and LC_{90}, the concentration of the viral product needed to cause 50% and 90% mortality of the organisms

within a sample (Kooijman 1981; Hughes & Wood 1981), whereas time-response (mortality) bioassays measure the speed of kill i.e. the amount of time required to cause 50% and 90% mortality of the organisms in a sample (LT_{50} and LT_{90}). These methods are analysed using the probit and logit models, respectively (Hamilton *et al.* 1977; Van Beek & Hughes 1998).

A favourable characteristic of a NPV is that it is often not host (species) specific and can have the ability to infect more than one species within a genus or family (Ishii *et al.* 2003; Szewczyk *et al.* 2006; Rao *et al.* 2015). Therefore, the NPV isolated from the litchi moth, *C. peltastica*, may have the potential to be a commercially successful biopesticide for controlling not only its homologous host, *C. peltastica*, but other pests within the family Tortricidae. Thus the aim of this chapter was to determine the virulence of CrpeNPV against three closely related species with overlapping host ranges *C. peltastica, Thaumatotibia leucotreta* and *Cydia pomonella* using the surface dosage bioassay method (Timm *et al.* 2006). The first objective of this study was to determine the LC_{50} and LC_{90} by using a range of dosages against all three species. The second objective was to determine the speed of kill to cause 50% and 90% mortality of the population using the LC_{90} value obtained from the dosage-response assays.

5.2 MATERIALS AND METHODS

5.2.1 Surface dosage-response bioassays with Cryptophlebia peltastica *neonate larvae*

Surface dosage bioassays were conducted in glass vials. Each glass vial was filled with 2 g of FCM artificial diet (Moore *et al.* 2014) and 2 ml of distilled water. Diet was autoclaved at 121°C for 13 min. Six five-fold dilutions of purified OBs of CrpeNPV (described in section 3.2.3) in sterilised distilled water were used for dosages and sterile distilled water (dH_2O) was used as a control (Figure 5.1). Forty-eight larvae were treated per dosage and assays were replicated three times. A volume of 100 µl of each viral dilution and of the dH_2O control was pipetted onto the centre of the diet surface. The fluid was spread evenly over the diet surface by rotating the vial at an angle. Inoculated glass vials were left for ± 30 min, until the diet had dried. *Cryptophlebia peltastica* egg sheets were obtained from the Rhodes University culture. First instar larvae hatching at approximately the same time were used. One neonate larva was placed into each vial. Vials were then closed with a lid and left upside down for approximately 12 hours to prevent larvae from escaping. Vials were kept in a CE room at 27°C and a relative

humidity of 60 – 80%. Vials were then turned upright. Approximately 24 hours later, lids were replaced with cotton wool.

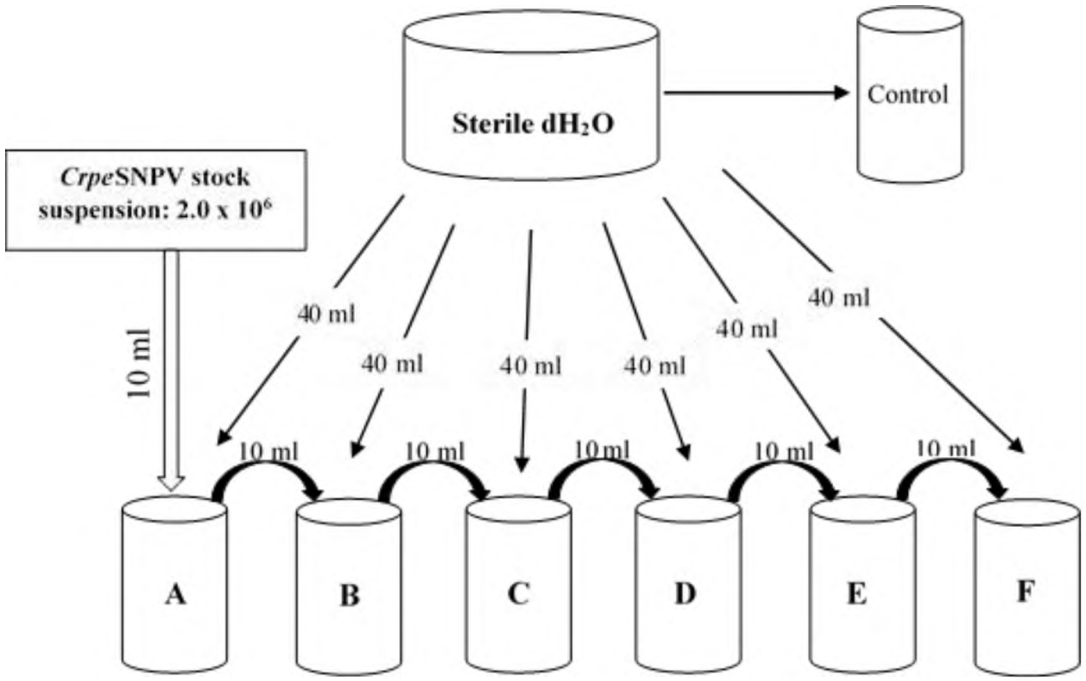


Figure 5.1: Five-fold dilution series of CrpeNPV for surface dosage-response bioassays with neonate larvae of *Cryptophlebia peltastica*, *Thaumatotibia leucotreta* and *Cydia pomonella*.

After 7 days, vials were opened and diet was inspected. Larvae were recorded as dead (when touched with fine forceps, the cuticle would rupture or larva did not move) or alive (when touched with fine forceps larva would move away). The dosage-response curve was calculated using PROBAN (Van Ark 1995) software used for probit analysis (Finney 1971). Mortality of control larvae was taken into consideration by PROBAN and corrected using Abbott's formula (Abbott 1925). From the analysis, LC_{50} and LC_{90} were calculated for each replicate. An average LC_{50} and LC_{90} were obtained from the means from the three replicates of each assay.

5.2.2 Surface dosage-response bioassays with Thaumatotibia leucotreta *neonate larvae*

Surface dosage bioassays were conducted in 24 well plates. Diet was prepared by adding 250 g of FCM artificial diet to a pyrex dish, and 300 ml of dH_2O was added to the diet. The diet

was cooked (and sterilised) in an oven at 180°C for 30 mins. Diet plugs were then cut out using a glass vial and then pressed into the wells of the plate using a sterile glass rod. Six five-fold dilutions of purified OBs of CrpeNPV (described in section 3.2.3) in sterilised distilled water were used for dosages. Sterile distilled water was used for the control (Figure 5.1). Forty-eight larvae were treated per dosage and assays were replicated three times. A volume of 50 µl of each viral dilution and control was pipetted onto the centre of the diet surface. The fluid was spread evenly over the diet surface by rotating the plates. Inoculated plates were left for ± 30 mins, until diet had dried. *Thaumatotibia leucotreta* egg sheets were obtained from River Bioscience (Pty) Ltd. (Hermitage, South Africa). First instar larvae hatching at approximately the same time were used. One neonate larva was placed into each well. Assay plates were sealed with Parafilm M® and kept in a CE room at 27°C and a relative humidity of 60 – 80%. After 7 days, plates were opened and the diet was inspected. Larvae were recorded as dead or alive. The dosage-response curve was calculated using PROBAN (Van Ark 1995) software used for probit analysis (Finney 1971). Mortality of control larvae were taken into consideration by PROBAN and corrected using Abbott's formula (Abbott 1925). From the analysis, LC_{50} and LC_{90} were calculated for each replicate. An average LC_{50} and LC_{90} were obtained from the means from the three replicates for each assay.

5.2.3 Surface dosage-response bioassays with Cydia pomonella *neonate larvae*

Surface dosage bioassays were conducted in 24 well plates. Diet was prepared by autoclaving 13 g of agar in 400 ml of distilled water. A total of 235.27 g of pre-mixed bollworm diet was sterilised in an oven at 180 °C for 10 mins. Propionic acid (1527 µl) and 154 µl of phosphoric acid were mixed into the sterilised diet. Agar was then added to the diet, and 200 to 400 ml of boiling water was added and mixed to form a uniform paste. Diet was then piped into the 24 well plates. Five five-fold dilutions of purified OBs of CrpeNPV (described in section 3.2.3) in sterilised distilled water was used for dosages and sterile distilled water was used as a control (Figure 5.1). Forty-eight larvae were treated per dosage and assays were replicated three times. A volume of 50 µl of each viral dilution and dH_2O control was pipetted onto the centre of the diet surface. The fluid was spread evenly over the diet surface by rotating the plates. Inoculated plates were left for ± 30 mins, until diet had dried. *Cydia pomonella* egg sheets were obtained from ENTOMON Technologies (Pty) Ltd. (Stellenbosch, South Africa). First instar larvae hatching at approximately the same time were used. One neonate larva was placed into each

well. Wells were sealed with Parafilm M® and kept in a CE room at 27°C and a relative humidity of 60 – 80%.

After 7 days, plates were opened and diet was inspected. Larvae were recorded as dead or alive. The dosage-response curve was calculated using PROBAN (Van Ark 1995) software used for probit analysis (Finney 1971). Mortality of control larvae was taken into consideration by PROBAN and corrected using Abbott's formula (Abbott 1925). From the analysis, LC_{50} and LC_{90} were calculated for each replicate. An average LC_{50} and LC_{90} were obtained from the means from the three replicates.

5.2.4 Time-response bioassays with Cryptophlebia peltastica *neonate larvae*

Time response bioassays were conducted in glass vials. The diet described above in section 5.2.1 was used. Fifty glass vials were inoculated with 100 µl of sterile distilled water and 50 vials were inoculated with the LC_{90} (calculated from the dosage-response bioassays) suspension of purified CrpeNPV. The diet was left for ± 30 mins, until the diet had dried adequately. One neonate larva was placed into each vial. Vials were sealed with a lid and the same process repeated as above (section 5.2.1.1) and kept in a CE room at 27°C and a relative humidity of 60 – 80%. Bioassays were replicated three times.

After 16 hours, the vials were inspected for any dead larvae. Vials were checked every 8 hours thereafter until no more mortality was recorded. The diet was then dissected to determine whether the remaining vials contained dead or live larvae.

Time-response was analysed using a logit regression in STATISTICA version 12 (StatSoft 2013). From the results obtained, the LT_{50} and LT_{90} were calculated.

5.2.5 Time-response bioassays with Thaumatotibia leucotreta *neonate larvae*

Time response bioassays were conducted in glass vials. The diet described above in section 5.2.1.1 was used. Fifty glass vials were inoculated with 100 µl of sterile distilled water and 50 vials were inoculated with the LC_{90} (calculated from the dosage-response bioassays) suspension of purified CrpeNPV. The diet was left for ± 30 mins, until the diet had dried adequately. One neonate larva was placed into each vial. Vials were sealed with a lid and the

same process repeated as above (section 5.2.1.1) and kept in a CE room at 27°C and a relative humidity of 60 – 80%. Bioassays were replicated three times.

After 16 hours, the vials were inspected for any dead larvae. Vials were checked every 8 hours thereafter until mortality had ceased. The diet was then dissected to determine whether the remaining vials contained dead or live larvae.

Time-response was analysed using a logit regression in STATISTICA version V12 (StatSoft 2013). From the results obtained, the LT_{50} and LT_{90} were calculated.

5.2.6 Time-response bioassays with Cydia pomonella *neonate larvae*

Time response bioassays were conducted in glass vials. The diet described above in section 5.2.1.3 was used. Fifty glass vials were inoculated with 100 µl of sterile distilled water and 50 vials were inoculated with the LC_{90} (calculated from the dosage-response bioassays) suspension of purified CrpeNPV. The diet was left for ± 30 mins, until the diet had dried adequately. One neonate larva was placed into each vial. Vials were sealed with a lid and the same process repeated as above (section 5.2.1.1) and kept in a CE room at 27°C and a relative humidity of 60 – 80%. Bioassays were replicated three times.

After 16 hours, the vials were inspected for any dead larvae. Vials were checked every 8 hours thereafter until mortality had ceased. The diet was then dissected to determine whether the remaining vials contained dead or live larvae.

Time-response was analysed using a logit regression in STATISTICA version V12 (StatSoft 2013). From the results obtained, the LT_{50} and LT_{90} were calculated.

5.3 RESULTS

5.3.1 Surface dosage-response bioassays with Cryptophlebia peltastica *neonate larvae*

Surface dosage bioassays were carried out against neonate *C. peltastica* larvae in glass vials. The regression lines fitted to the data in Table 5.1 for three replicates with CrpeNPV are as follows; replicate one y = 1.0122 (SE of slope = 0.1353) x + 1.6020, replicate two y = 0.7965 (SE of slope = 0.1065) x + 1.9529 and replicate three y = 0.7800 (SE of slope = 0.1009) +

1.7291 (Figure 5.2). Deviations for the lines for all replicates were estimated to be homogenous.

Table 5.1: Mortality of neonate *Cryptophlebia peltastica* larvae in dosage-response bioassays with six dosages of CrpeNPV.

Treatment (OBs/ml)	Replicate 1			Replicate 2			Replicate 3		
	Larval Mortality (%)	Corrected Mortality (%)	Empirical Probit	Larval Mortality (%)	Corrected Mortality (%)	Empirical Probit	Larval Mortality (%)	Corrected Mortality (%)	Empirical Probit
Control	21.00			15.00			8.00		
1.28×10^2	29.17	10.34	3.735	22.92	9.31	3.631	10.42	2.63	3.373
6.4×10^2	33.33	15.61	4.442	25.00	11.76	4.188	20.83	13.95	3.918
3.2×10^3	72.92	65.72	5.150	56.25	48.53	4.745	37.50	32.07	4.463
1.6×10^4	91.67	89.45	5.858	70.83	65.69	5.301	60.42	56.97	5.008
8.0×10^4	91.67	89.45	6.565	79.17	75.49	5.858	62.50	59.24	5.554
4.0×10^5	97.92	97.36	7.273	93.75	92.65	6.415	91.67	90.94	6.099

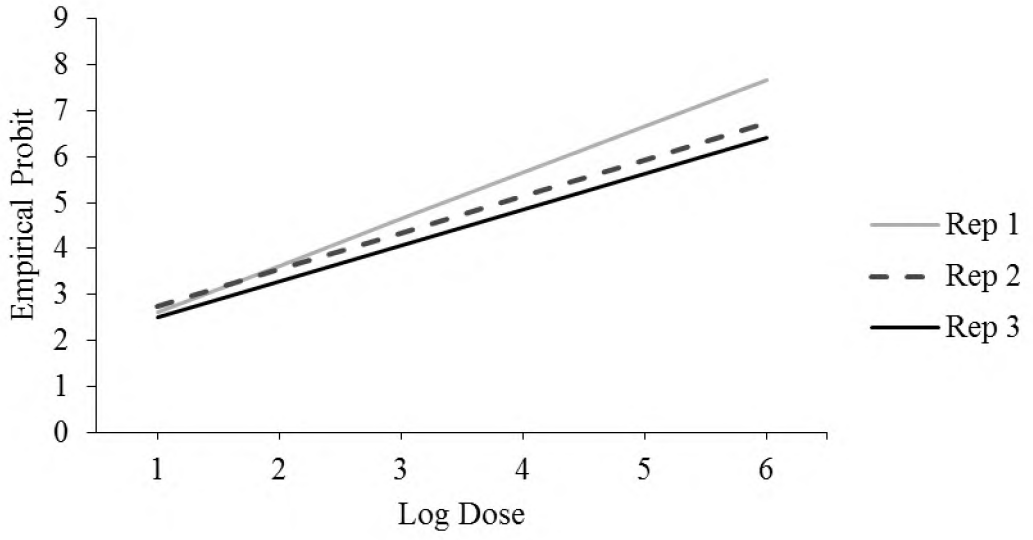


Figure 5.2: Dosage-mortality probit lines for CrpeNPV with *Cryptophlebia peltastica* neonate larvae.

5.3.2 Surface dosage-response bioassays with Thaumatotibia leucotreta *neonate larvae*

Surface dosage bioassays were carried out against neonate *T. leucotreta* larvae in 24 well plates. The regression lines fitted to the data in table 5.2 for three replicates with CrpeNPV are as follows: replicate one y = 0.6808 (SE of slope = 0.0938) x + 2.8282, replicate two y = 0.9900 (SE of slope = 0.1139) x + 1.5438 and replicate three y = 0.7392 (SE of slope = 0.0857) +

2.5280. Deviations for the lines for all replicates were estimated to be homogenous (Figure 5.3).

Table 5.2: Mortality of neonate *Thaumatotibia leucotreta* larvae in dosage-response bioassays with six dosages of CrpeNPV.

Treatment (OBs/ml)	Replicate 1			Replicate 2			Replicate 3		
	Larval Mortality (%)	Corrected Mortality (%)	Empirical Probit	Larval Mortality (%)	Corrected Mortality (%)	Empirical Probit	Larval Mortality (%)	Corrected Mortality (%)	Empirical Probit
Control	15.00			8.00			4.00		
1.28×10^2	35.42	24.02	4.263	22.92	16.21	3.630	18.75	15.36	4.086
6.4×10^2	56.25	48.53	4.739	25.00	18.48	4.322	43.75	41.41	4.602
3.2×10^3	60.42	53.43	5.214	50.00	45.65	5.014	54.17	52.26	5.119
1.6×10^4	68.75	63.24	5.690	81.25	79.62	5.706	70.83	69.62	5.636
8.0×10^4	93.75	92.65	6.166	93.75	93.21	6.398	89.58	89.15	6.152
4.0×10^5	97.92	97.55	6.642	97.92	97.74	7.090	95.83	95.66	6.669

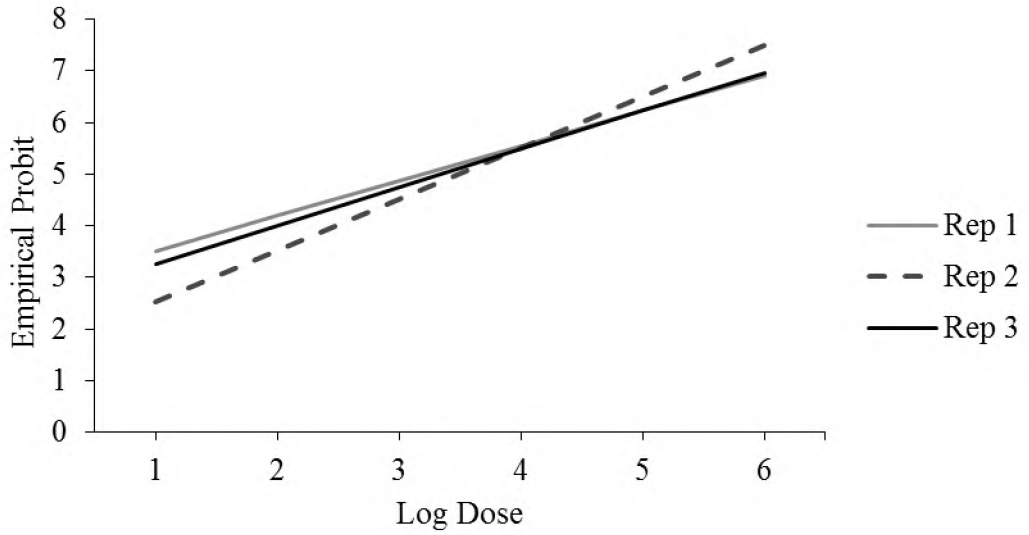


Figure 5.3: Dosage-mortality probit lines for CrpeNPV with *Thaumatotibia leucotreta* neonate larvae.

5.3.3 Surface dosage-response bioassays with Cydia pomonella *neonate larvae*

Surface dosage bioassay were carried out against *C. pomonella* neonate larvae in two 24 well plates. The regression lines fitted to the data in table 5.3 for three replicates with CrpeNPV are as follows: replicate one y = 1.5768 (SE of slope = 0.2556) x – 0.4309, replicate two y = 1.2083 (SE of slope = 0.1473) x + 1.3226 and replicate three y = 1.0416 (SE of slope = 0.1449) +

2.2721. Deviations for the lines for all replicates were estimated to be homogenous (Figure 5.4).

Table 5.3: Mortality of *Cydia pomonella* neonate larvae in dosage-response bioassays with five dosages of CrpeNPV.

Treatment (OBs/ml)	Replicate 1			Replicate 2			Replicate 3		
	Larval Mortality (%)	Corrected Mortality (%)	Empirical Probit	Larval Mortality (%)	Corrected Mortality (%)	Empirical Probit	Larval Mortality (%)	Corrected Mortality (%)	Empirical Probit
Control	21.00			6.00			8.00		
1.28×10^2	22.92	2.43	2.892	25.00	20.21	3.869	33.33	27.54	4.467
6.4×10^2	27.08	7.70	3.994	29.17	24.65	4.713	60.42	56.97	5.195
3.2×10^3	68.75	60.44	5.096	79.17	77.84	5.558	87.50	86.41	5.923
1.6×10^4	91.67	89.45	6.198	93.75	93.35	6.402	95.83	95.47	6.651
8.0×10^4	97.92	97.36	7.300	97.92	97.78	7.247	97.92	97.74	7.379

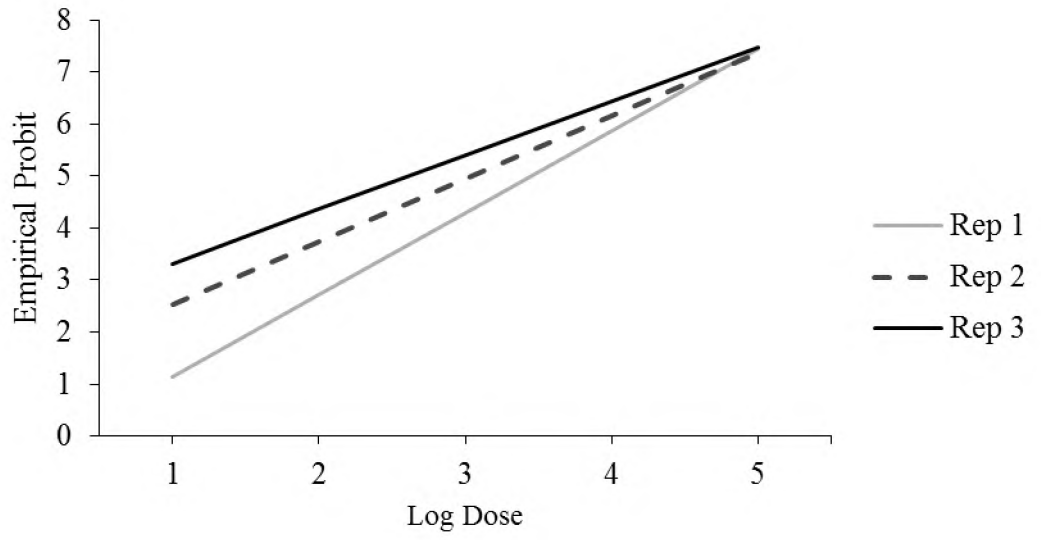


Figure 5.4: Dosage-mortality probit lines for CrpeNPV with *Cydia pomonella* neonate larvae.

5.3.4 Surface dosage-response comparison for the three Tortricidae pests

The residual variance of the regression lines of the three *C. peltastica* replicates were compared and found to be homogenous, with a chi squared value of 0.351 with two degrees of freedom and approximate probability of 0.8380. The G for fiducial limits was calculated to be 0.0686,

0.0687 and 0.0642 for the three respective bioassay replicates with *C. peltastica*. The results obtained from the three replicates were used to calculate the mean LC_{50} and LC_{90} (Table 5.4).

The residual variance of the regression lines of the three *T. leucotreta* replicates were compared and found to be homogenous, with a chi squared value of 0.929 with two degrees of freedom and approximate probability of 0.6340. For the three bioassay replicates for *T. leucotreta* the G for fiducial limits were calculated as 0.0729, 0.0509 and 0.0517. The results obtained from the three replicates were used to calculate the mean LC_{50} and LC_{90} (Table 5.4).

The residual variance of the regression lines of the three *C. pomonella* replicates were compared and found to be homogenous, with a chi squared value of 0.782 with two degrees of freedom and approximate probability of 0.6820. For the three bioassay replicates for *T. leucotreta* the G for fiducial limits were calculated as 0.1009, 0.0571 and 0.0743. The results obtained from the three replicates were used to calculate the mean LC_{50} and LC_{90} (Table 5.4).

Table 5.4: Comparison of the mean fiducial limits, LC_{50} and LC_{90} values for *Cryptophlebia peltastica, Thaumatotibia leucotreta* and *Cydia pomonella*.

		Mean Dosage (OBs/ml)	SE	Fiducial limits	
				Upper ± SE	Lower ± SE
C. peltastica	LC_{50}	8.19×10^3	2.41×10^3	1.46×10^4	4.43×10^3
	LC_{90}	3.33×10^5	1.81×10^5	1.32×10^6	1.38×10^5
T. leucotreta	LC_{50}	2.29×10^3	6.63×10^2	3.94×10^3	1.22×10^3
	LC_{90}	9.97×10^4	4.78×10^4	3.32×10^5	4.53×10^4
C. pomonella	LC_{50}	1.43×10^3	3.37×10^2	2.21×10^3	8.37×10^2
	LC_{90}	1.26×10^4	4.05×10^3	2.84×10^4	7.42×10^3

5.3.5 Time-response bioassays with Cryptophlebia peltastica *neonate larvae*

Larval mortality was observed 64 hours after treatment and continued until 136 hours (6 days) post treatment until 100% mortality was observed for three replicates. Control mortality was 4% for replicates one and two and 6% for replicate three.

Table 5.5: Mortality of neonate *Cryptophlebia peltastica* larvae in time-response bioassays using the LC_{90} concentration of CrpeNPV.

Time after treatment		Larval mortality (%)		
Days	Hours	Rep 1 (n=47)	Rep 2 (n=48)	Rep 3 (n=49)
0	16	0	0	0
	24	0	0	0
1	32	0	0	0
	40	0	0	0
	48	0	0	0
2	56	0	2	0
	64	13	6	4
	72	68	58	37
3	80	87	73	57
	88	94	83	71
	96	96	94	88
4	104	98	94	92
	112	98	94	92
	120	98	100	98
5	128	98		100
	136	100		

During the first 16 hours of each replicate, a small number of larvae were recorded as dead. These deaths were not symptomatic of viral infection and were therefore recorded as non-responsive. A total of three, two and one larvae were recorded as non-responsive in replicates one, two and three, respectively. These larvae were omitted from the replicates, thus having a total number of 47 larvae for replicate one, 48 larvae for replicate two and 49 larvae for replicate three. This is an acceptable practice, known as biological truncation (Moore *et al.* 2011).

The time-mortality relationship (Figure 5.5) for three replicates of neonate *C. peltastica* larvae were analysed using a logit regression analysis (Table 5.6). From the logit regression, the LT_{50} and LT_{90} for each replicate were determined. The mean for the three replicates was calculated as LT_{50} = 73.44 hours and LT_{90} = 89.21 hours.

Table 5.6: Logit regression data for mortality of neonate *Cryptophlebia peltastica* larvae at the LC_{90} concentration of CrpeNPV.

Replicate	Logit regression data for *Cryptophlebia peltastica*							
	Regression Coefficient	**SE**	**P-value**	**Chi-square**	**P-value**	**X-intercept**	**SE**	**P-Value**
1	-10.14	0.64	<0.0001	1742.8	<0.0001	0.158	0.0098	<0.0001
2	-10.72	0.64	<0.0001	1767.0	<0.0001	0.142	0.0084	<0.0001
3	-9.88	0.55	<0.0001	1684.2	<0.0001	0.122	0.0067	<0.0001

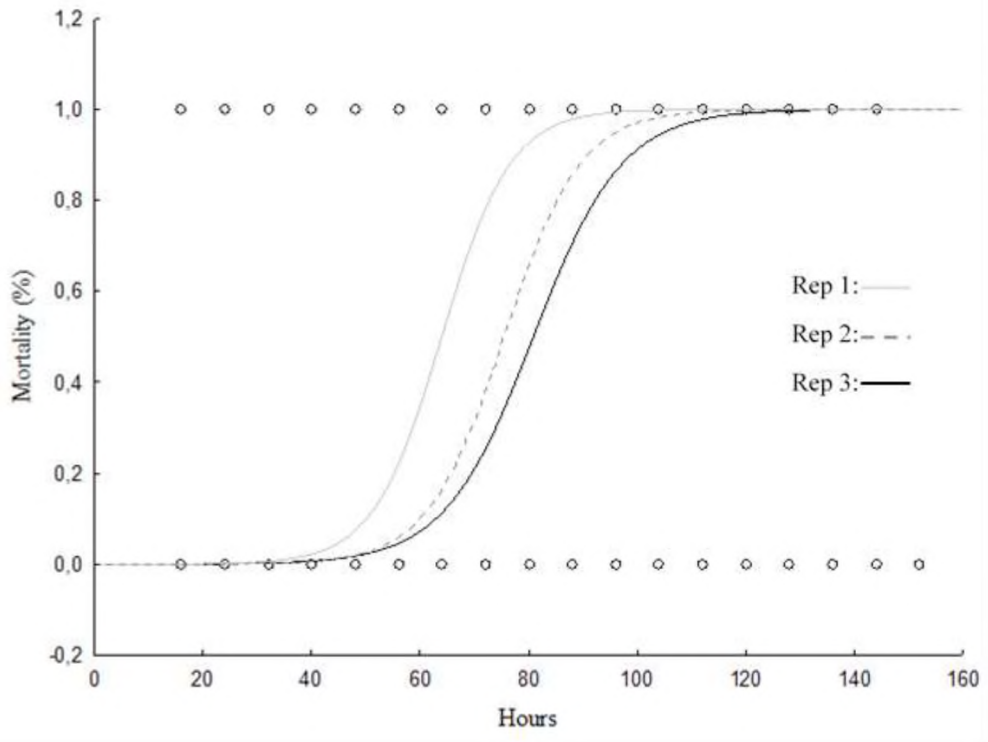


Figure 5.5: Logit regression model for time-response of neonate *Cryptophlebia peltastica* larvae to the LC_{90} of CrpeNPV.

5.3.6 Time-response bioassays with Thaumatotibia leucotreta *neonate larvae*

Larval mortality was observed 64 hours after treatment and continued until 104 hours post treatment until 100% mortality was observed for all replicates. Control mortality for replicate one was 0% and 6.25% and 8.16% for replicates two and three.

Table 5.7: Mortality of neonate *Thaumatotibia leucotreta* larvae in time-response bioassays using the LC_{90} concentration of CrpeNPV.

Time after treatment		Larval mortality (%)		
		Rep 1 (n=47)	Rep 2 (n=47)	Rep 3 (n=49)
Days	Hours			
0	16	0	0	0
	24	0	0	0
1	32	0	0	0
	40	0	0	0
	48	0	0	0
2	56	11	0	0
	64	19	4	6
	72	26	30	16
3	80	34	60	59
	88	57	81	80
	96	87	87	96
4	104	100	91	100
	112		100	

In replicate one, a total of 3 larvae, replicate two a total of 3 larvae and replicate three a total of 1 larva showed no response to the treatment. As in the trial with *C. peltastica,* all larvae showing no response were omitted, thus having a total number of 47 larvae for replicate one, 47 larvae for replicate two and 49 larvae for replicate three (Table 5.7).

The time-mortality relationship (Figure 5.6) for the three replicates of neonate *T. leucotreta* larvae were analysed using a logit regression analysis (Table 5.8). From the logit regression, the LT_{50} and LT_{90} values for each replicate were determined. The mean for the three replicates was calculated as LT_{50} = 80.69 hours and LT_{90} = 96.37 hours.

Table 5.8: Logit regression data for mortality of neonate *Thaumatotibia leucotreta* larvae at the LC_{90} concentration of CrpeNPV.

Replicate	Logit regression data for *Thaumatotibia leucotreta*							
	Regression Coefficient	**SE**	**P-value**	**Chi-square**	**P-value**	**X-intercept**	**SE**	**P-Value**
1	-9.86	0.55	<0.0001	1369.0	<0.0001	0.1212	0.0067	<0.0001
2	-11.74	0.72	<0.0001	1496.4	<0.0001	0.1472	0.0089	<0.0001
3	-16.83	1.22	<0.0001	1636.2	<0.0001	0.2050	0.0147	<0.0001

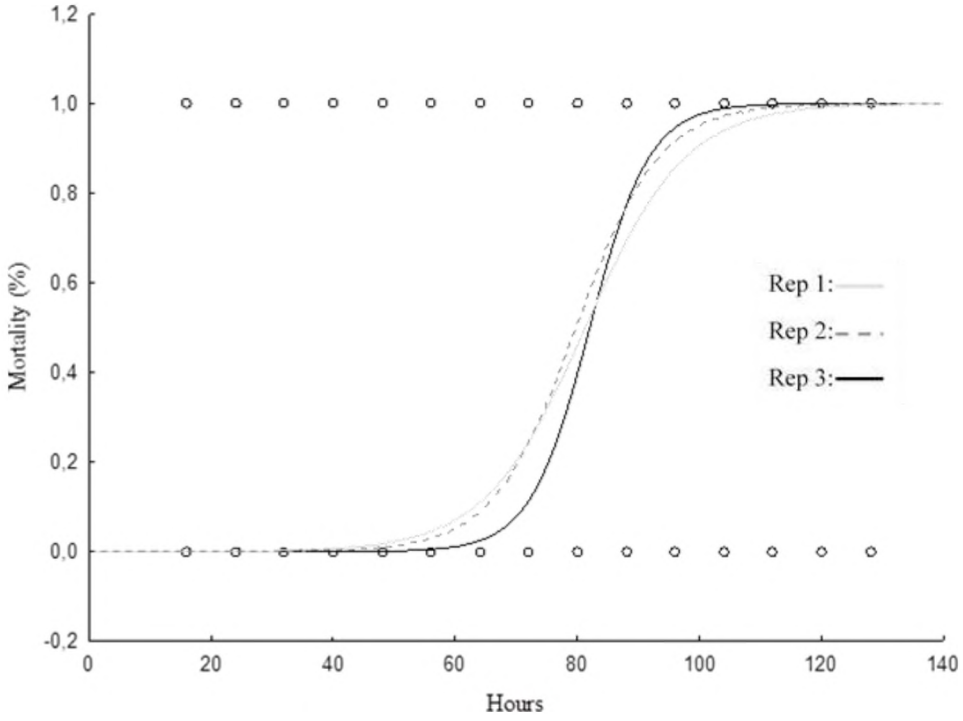


Figure 5.6: Logit regression model for time-response of neonate *Thaumatotibia leucotreta* larvae to the LC_{90} of CrpeNPV.

5.3.7 Time-response bioassays with Cydia pomonella *neonate larvae*

Larval mortality was observed 80 hours after treatment and continued until 152 hours post treatment until 100 % mortality was observed for all replicates. There was no control mortality for any of the three replicates.

Table 5.9: Mortality of *Cydia pomonella* neonate larvae in time-response bioassays using the LC_{90} concentration of CrpeNPV.

Time after treatment		Larval mortality (%)		
Days	Hours	Rep 1 (n=50)	Rep 2 (n=48)	Rep 3 (n=49)
0	16	0	0	0
	24	0	0	0
1	32	0	0	0
	40	0	0	0
	48	0	0	0
2	56	0	0	0
	64	0	0	0
	72	0	0	0
3	80	2	4	0
	88	20	23	2
	96	38	48	6
4	104	46	56	18
	112	58	71	29
	120	76	88	53
5	128	92	92	71
	136	94	98	94
	144	100	98	96
6	152		100	100

In replicate two a total of two larvae and replicate three a total of one larva showed no response to the treatment. In the first replicate, no larvae were regarded as unresponsive. As in the previous two trials, all larvae showing no response were omitted, thus having a total number of 50 larvae for replicate one, 48 larvae for replicate two, and 49 larvae for replicate three (Table 5.9).

Table 5.10: Logit regression data for mortality of neonate *Cydia pomonella* larvae at the LC_{90} concentration of CrpeNPV.

Replicate	Logit regression data for *Cydia pomonella*							
	Regression Coefficient	**SE**	**P-value**	**Chi-square**	**P-value**	**X-intercept**	**SE**	**P-Value**
1	-11.21	0.59	<0.0001	1754.1	<0.0001	0.1058	0.0055	<0.0001
2	-11.31	0.60	<0.0001	1833.0	<0.0001	0.1110	0.0058	<0.0001
3	-14.41	0.83	<0.0001	1836.0	<0.0001	0.1300	0.0074	<0.0001

The time-mortality relationship (Figure 5.7) for three replicates with neonate *C. pomonella* larvae were analysed using a logit regression analysis (Table 5.10). From the logit regression, the LT_{50} and LT_{90} for each replicate was determined. The mean for the three replicates was calculated as LT_{50} = 106.09 hours and LT_{90} = 125.22 hours.

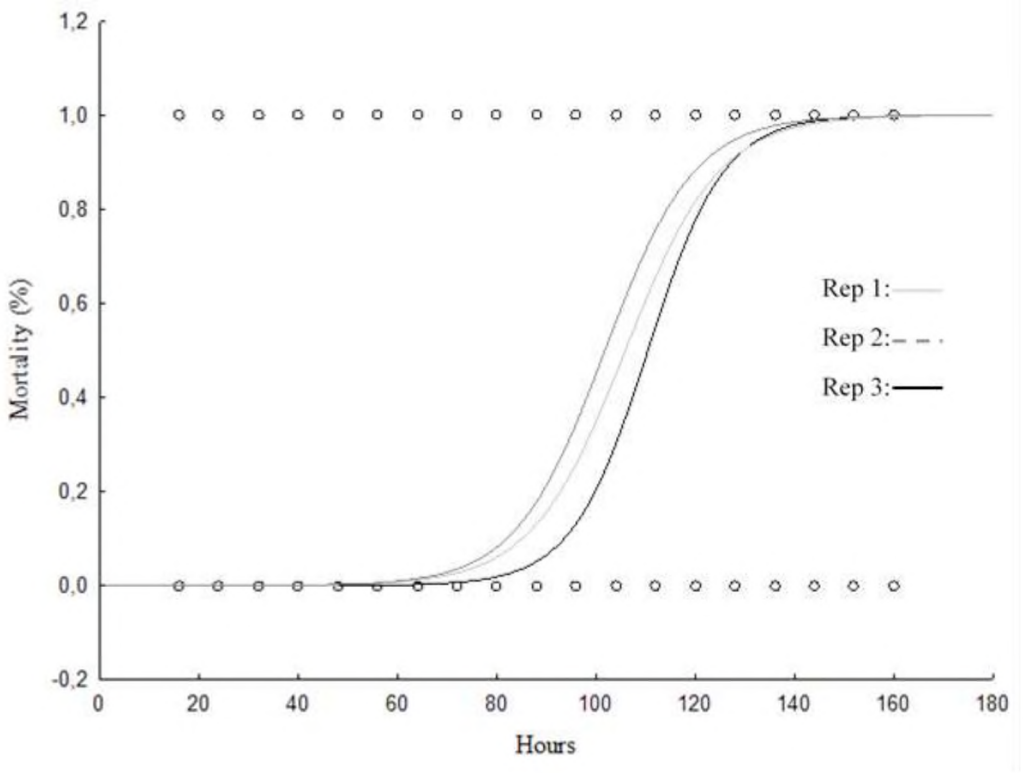


Figure 5.7: Logit regression model for time-response of neonate *Cydia pomonella* larvae to the LC_{90} of CrpeNPV.

5.4 DISSCUSSION

This chapter focused on the biological activity of CrpeNPV against *C. peltastica*, *T. leucotreta* and *C. pomonella* neonate larvae, as they are closely related species with overlapping host ranges (Timm *et al.* 2006). To determine the dosage and time response relationships for each of the species, the surface dosage method was the chosen method to administer various virus concentrations. The surface dosage method was most suited for all three species as they are all internal (cryptic) feeders and penetrate the surface of the fruit first; therefore the surface dosage method mimics feeding in the field (Jones 2002; Grové *et al.* 2015; Grout & Moore 2015; Pringle *et al.* 2015).

The dosage-response curves were used to determine the lethal concentrations (LC_{50} and LC_{90}) for each of the three species. The results obtained from this study were compared to *T. leucotreta* and *C. pomonella* homologous baculoviruses, CrleGV and CpGV (Table 5.11). From the comparison it was observed that CrpeNPV was slightly more virulent against *T. leucotreta* and *C. pomonella* than against its own host, *C. peltastica*. *Cryptophlebia peltastica* NPV was also more virulent against *T. leucotreta* and *C. pomonella* than their own homologous viruses.

Table 5.11: Comparison of dosage response results of the homologous and heterologous viruses for *Cryptophlebia peltastica*, *Thaumatotibia leucotreta* and *Cydia pomonella* (Moore *et al.* 2011; Motsoeneng 2016).

		CrpeNPV	**CrleGV**	**CpGV**
C. peltastica	LC_{50}	8.19×10^3	-	-
	LC_{90}	3.33×10^5	-	-
T. leucotreta	LC_{50}	2.29×10^3	4.09×10^3	-
	LC_{90}	9.97×10^4	1.18×10^5	-
C. pomonella	LC_{50}	1.43×10^3	-	1.63×10^3
	LC_{90}	1.26×10^4	-	1.16×10^5

The time response curves were used to determine the lethal concentrations (LT_{50} and LT_{90}) for each of the three species. The results obtained from this study were compared to *T. leucotreta*

and *C. pomonella* homologous baculoviruses, CrleGV and CpGV (Table 5.12). From the comparison it was observed that CrpeSNPV had a slightly faster speed of kill against *C. peltastica,* compared to *T. leucotreta* and *C. pomonella*. However, CrpeNPV had a faster speed of kill against *T. leucotreta* and *C. pomonella* than their own homologous viruses.

Table 5.12: Comparison of time response results of the homologous and heterologous viruses for *Cryptophlebia peltastica*, *Thaumatotibia leucotreta* and *Cydia pomonella* (Moore *et al.* 2011; Motsoeneng 2016).

		CrpeNPV	**CrleGV**	**CpGV**
C. peltastica	LT_{50}	73.44 hours	-	-
	LT_{90}	89.21 hours	-	-
T. leucotreta	LT_{50}	80.69 hours	118 hours	-
	LT_{90}	96.37 hours	176 hours	-
C. pomonella	LT_{50}	106.09 hours	-	135 hours
	LT_{90}	125.52 hours	-	-

From the above results it was established that CrpeNPV was more virulent against neonate *T. leucotreta* and *C. pomonella* larvae, specifically with a faster speed of kill for both species. The mean LC and LT values were slightly less than for their own homologous baculovirus, CrleGV and CpGV-SA. A study by Abdul Kadir *et al.* (1999) also observed this with *Plutella xylostella* (Linnaeus) (Lepidoptera: Yponomeutidae) and its interaction between its own homologous virus (PxGV) and two other NPVs, *Galleria mellonella* NPV (GmNPV) and *Autographa californca* NPV (AcNPV). From this study they found that the NPV was relatively more virulent than PlxyGV, especially with a faster speed of kill. Abdul Kadir *et al.* (1999) explained that this is because NPVs have more than one virion per OB, whereas GVs have a single virion per OB. Thus, the starting number of infecting cells for an NPV is greater than that of a GV, enabling NPV to have a higher virulence and faster speed of kill (Payne 1986; Adul Kadir *et al.* 1999; Lacey *et al.* 2002).

The ability of NPV to infect a wider host range has been reported. For example, in the case of AcNPV, 43 species in 11 families of Lepidoptera are susceptible to the virus (Payne 1986;

Copping & Menn 2000). However, the virulence of AcNPV against all 43 hosts varies greatly. Majority of the species susceptible to AcNPV have extremely high viral doses, making them biologically irrelevant (Payne, 1986; Grzywacz 2017). Another example of a NPV with a broad spectrum is HearNPV and HzSNPV. These viruses have the ability to infect a range of species in the genus *Heliothine* (Grzywacz 2017). A specific benefit of NPV with a wide host range is when multiple related (and hence susceptible) lepidopterans infest the same host plant and can thus be controlled with one treatment. The other lepidopteran pests may only become obvious once the key pest has been suppressed with a highly selective pesticide. The advantage of NPV with a wide host range is that it may not only control the key pest but also the secondary pests, as long as they are closely related and thus potentially susceptible to the same virus. An example of this is the interaction between *C. pomonella*, the key pest and three secondary apple orchard pest species; *Choristoneura rosaceana*, *Pandemis pyrusana* and *Grapholitha molesta*. When specific control of *C. pomonella* is necessary through the use of mating disruption using specific pheromones, the secondary pests increase above their economic threshold (Lacey *et al.* 2002; Walker & Walter 2001; Lacey & Unruh 2005). A wide host range baculovirus such as AcMNPV will be useful in this situation, as it will have the ability to suppress both the key pest and the secondary pests. This could be a possibility for CrpeNPV and the three species used in this study where overlapping host ranges occur, such as in macadamias and litchis.

In conclusion, CrpeNPV was found to be more virulent against the heterologous hosts than its own host, specifically with a faster speed of kill. This indicates that CrpeNPV has the potential to be commercially developed into a biopesticide and used for the control of *C. peltastica, T. leucotreta* and *C. pomonella*. Additionally, CrpeNPV could also be tested against other lepidopteran pests in the Tortricidae family, such as: *Thaumatotibia batrachopa, Cydia molesta* and *Epichoristodes accerbella*.

Chapter 6

SUSCEPTIBILITY OF RESISTANT *CYDIA POMONELLA* AND Cp14R CELL LINE TO CrpeNPV

6.1 INTRODUCTION

The previous chapter dealt with the virulence of the newly isolated CrpeNPV against three economically important pests with overlapping host ranges: *Cryptophlebia peltastica*, *Thaumatotibia leucotreta* and *Cydia pomonella* (Timm *et al.* 2006; Timm *et al.* 2010; van den Berg *et al.* 2010). From the results obtained it was observed that the NPV was highly virulent against all three species, with the possibility of infecting several other species within the genus or family. As CrpeNPV was found to be virulent against *C. pomonella,* this chapter will focus on populations of *C. pomonella* in Europe which have developed resistance to various isolates of the *C. pomonella* granulovirus (CpGV) (Asser-Kaiser *et al.* 2007; Schmitt *et al.* 2013).

Cydia pomonella is a pest of pome fruit, which includes important agricultural crops such as: apples, pears and quince, as well as walnuts (Moscardi 1999; Lacey *et al.* 2008). However, *C. pomonella* has the greatest economic impact on apples (Jehle 2008). Various control measures were implemented against *C. pomonella*, however effective control was not achieved until CpGV products were applied, providing efficient control of the pest on apples (Jehle 2008). The CpGV products were commercialised in all European countries, North America, New Zealand and South Africa (Jehle 2008). However, in 2005 there were reports of *C. pomonella* developing resistance to CpGV products in Germany and France (Asser-Kaiser *et al.* 2007; Lacey *et al.* 2008; Jehle 2008). *Cydia pomonella* resistance was observed in orchards where CpGV products had been applied intensively and multiple times over several years (Lacey *et*

al 2008; Schmitt *et al.* 2013). Research was then conducted to determine the genetic basis of resistance inheritance to CpGV in *C. pomonella.* Studies completed by Eberle & Jehle (2006) and Asser-Kaiser *et al.* (2007) found that the resistance of *C. pomonella* was due to autosomal and incomplete dominant inheritance of resistance which is sex-linked and found on the Z-chromosome (Jehle 2008). During the investigation of how the resistance to CpGV was inherited, two cultures of *C. pomonella*, CpRR1 (type 1 resistance: sex-linked inheritance on Z-chromosom) and CpR5M (type 2 resistance: dominant autosomal inheritance), were developed by mass crossing, single crossing and back crossing of a culture susceptible to CpGV (CpS) and a culture resistant to CpGV (CpR) (Jehle 2008; Asser-Kaiser *et al.* 2010; Jehle *et al.* 2016). Thus with CrpeNPV being highly virulent against susceptible *C. pomonella,* it could potentially be virulent against resistant *C. pomonella* and possibly used as an alternative control option in the resistance management programme of *C. pomonella* in Europe.

In addition to resistant laboratory cultures of *C. pomonella*, a *C. pomonella* cell line, Cp14R has been established (Winstanley & Crook 1993; Gebhardt et al., 2014). Using cell lines to study baculoviruses has numerous advantages such as studying virus-host interactions, genetic manipulation, production of various recombinant proteins and the commercialisation and production of biopesticides (Hu 2005; Smagghe *et al.* 2009). The Julius Kühn Institute (Darmstadt, Germany) has inherited a Cp14R cell line culture which is permissive to CpGV (Jehle, personal communication). Thus, with the high virulence of CrpeNPV against *C. pomonella* there is a possibility that CrpeNPV could infect the Cp14R cell lines. This would be of great advantage as it will allow for the further characterisation and manipulation of the virus and provides the opportunity for the virus to be mass produced *in vitro.*

The aim of this chapter was to determine whether CrpeNPV is also virulent against CpGV resistance and susceptible *C. pomonella* in Europe. The second aim of this study was to determine the susceptibility of CrpeNPV to the Cp14R cell line. The specific objectives of this study were to determine the virulence of CrpeNPV against resistant and susceptible European *C. pomonella* using diet incorporation dosage-response bioassays. A further objective was to test whether the Cp14R cell lines are permissive for CrpeNPV infection through transfection with viral DNA and infection with budded virus by determining a tissue culture infectious dosage ($TCID_{50}$) and the expression of genes using real time qPCR.

6.2 MATERIALS AND METHODS

6.2.1 Biological activity of CrpeNPV against CpS, CpRR1 and CpR5M

6.2.1.1 Infectivity bioassays with CpS, CpRR1 and CpR5M neonate larvae

Bioassays were performed in autoclavable boxes (LICEFA, Bad Salzuflen, Germany) containing 50 separate wells (1.5 × 1.5 × 2 cm). *Cydia pomonella* diet was prepared by autoclaving 32 g of maize meal and 5 g of agar in 500 ml of H_2O for 20 minutes at 121°C. In a large mixing bowl, 33 g of wheat and 15 g of yeast were added. The autoclaved mixture was added to this large bowl and mixed with an electronic beater. Ascorbic acid (2.85 g) was added to 10 ml of H_2O and 1.15 g of benzoic acid and nipagin were mixed in 10 ml of ethanol. Once the contents in the large beaker cooled to 50°C, the ascorbic acid, benzoic acid and nipagin mixtures were added. To each autoclavable box, 50 ml of artificial diet was added. The viral concentrations or ddH_2O (control) was incorporated into this 50 ml of artificial diet. Two discriminating viral concentrations of CrpeNPV were used; 5.8×10^4 OBs/ml and 5.8×10^5 OBs/ml. Boxes were left overnight to dry. One first instar larva was placed into each cell, with a total of 50 larvae per treatment. The boxes were wrapped in polyethylene (cling wrap), covered with tissue paper and a hard plastic sheet, which was held in place with two rubber bands. Bioassays were incubated at 26°C with a relative humidity of 60 – 70% and 18:6 photoperiod. After 24 hours, larval mortality was recorded and these larvae were excluded from the experiments, as these larvae would have died from handling. Mortality was then recorded on the seventh day to determine infectivity of CrpeNPV.

6.2.1.2 Dosage response bioassays with CpS, CpRR1 and CpR5M neonate larvae

Dosage response bioassays were performed in 50 well autoclavable boxes (LICEFA, Bad Salzuflen, Germany). *Cydia pomonella* diet was prepared as described above (section 6.2.1.1). Five six-fold virus concentrations ranging from 1.28×10^2 OBs/ml to 8.0×10^4 OBs/ml were incorporated into the codling moth diet. The various mixtures were placed into the boxes, including an untreated control. Boxes were left overnight to dry. One first instar larva was placed into each cell, with a total of 50 larvae per treatment. The boxes were wrapped in polyethylene (cling wrap), covered with tissue paper and a hard plastic sheet, which was held in place with two rubber bands. Bioassays were incubated at 26°C with a relative humidity of 60 – 70% and 18:6 photoperiod. After 24 hours, larval mortality was recorded and these larvae

were excluded from the experiments. Mortality was then recorded on the seventh and fourteenth day. The dosage-response curve was calculated using ToxRat® Solutions GmbH 2001-2015, standard version 3.2.1 software. Mortality of control larvae was taken into consideration by ToxRat® and corrected using Abbott's formula. From the analysis, LC_{50} and LC_{90} were calculated for each replicate. This method was repeated for each culture: CpS, CpRR1 and CpR5M, and replicated three times for each.

6.2.1.3 Time response bioassays against CpS and CpR5M neonate larvae using CrpeNPV and three CpGV isolates (CpGV-S, CpGV-E2 and CpGV-M)

Time response bioassays were conducted in 50 well autoclavable boxes (LICEFA, Bad Salzuflen, Germany). *Cydia pomonella* diet was prepared as above in section 6.2.1.1. Virus concentrations as seen in table 6.1 were incorporated into the *C. pomonella* diet. The various mixtures were placed into the boxes for CpS and CpR5M for each of the four viral isolates (CrpeNPV, CpGV-S, CpGV-E2 and CpGV-M) and controls. Boxes were left overnight to dry. One first instar larva was placed into each cell, with a total of 50 larvae per treatment. The boxes were wrapped in polyethylene (cling wrap), covered with tissue paper and hard plastic, which was held in place with two rubber bands. Bioassays were incubated at 26°C with a relative humidity of 60 – 70% and 18:6 photoperiod. After 24 hours, larval mortality was recorded and these larvae were excluded from the experiments. Mortality was then recorded every 8 hours, until mortality had ceased. Only one replicate was completed due to time constraints.

Table 6.1: Dosages used for time response analysis for each viral isolate against CpS and CpR5M

	CpS	**CpR5M**
CpGV-S	2.86×10^4 OBs/ml	2.32×10^4 OBs/ml
CpGV-E2	2.86×10^4 OBs/ml	2.32×10^4 OBs/ml
CpGV-M	2.86×10^4 OBs/ml	2.32×10^4 OBs/ml
CrpeNPV	2.86×10^4 OBs/ml	2.32×10^4 OBs/ml

Time-response data were analysed using a logit regression in STATISTICA version V12 (StatSoft 2013). From the results obtained, the LT_{50} and LT_{90} were calculated.

6.2.2 Susceptibility of CrpeNPV to the Cp14R cell line

6.2.2.1 Maintenance of Cp14R cells

Cydia pomonella cells (Cp14R) were maintained in IZDO4 medium supplemented with 10% foetal calf serum (FCS) and antibiotics (100 units/ml penicillin and 50µg/ml streptomycin) at 22°C (Winstanley & Crook 1993). Cells were passaged every 7-10 days by washing with 1 to 2 ml phosphate buffered saline (PBS). After washing, 1 to 2 ml of trypsin was added to lift the cells off the bottom of the flask, allowing for resuspension (trypsinisation). Cells were then resuspended in complete medium (IZD04, 10% FCS and antibiotics) and placed into new flasks using a 1:3 ratio of suspension and complete medium.

6.2.2.2 Transfection of Cp14R cells with CrpeNPV DNA

A T75 cm^2 confluent flask of Cp14R cells was used for the transfection using CrpeNPV DNA. Cells were trypsinised as in section 6.2.2.1 and counted using a Neubauer counting chamber (0.1 mm depth). A six-well plate was seeded with 1×10^5 cells/well in 2 ml of complete medium. The plate was incubated at 22°C for three to four days to allow for attachment of cells. Once the cells had attached they were used for a transfection. Transfection medium (medium without antibiotics and FCS) was used for the transfection. A negative control (without DNA), positive control (CpGVbac) and CrpeNPV DNA were used for the transfection. Transfection medium (200 µl) was added to three polyethylene centrifuge tubes. To each tube, except the negative control, 1 µg of the required DNA was added. Lipofectamin (Merck, Germany) (7 µl) was added to each polyethylene centrifuge tube and left at room temperature for 20 minutes. Cells in the six-well plate were then prepared by removing medium and washing the cells twice with PBS. After 20 minutes, 800 µl of transfection medium was added to each polyethylene centrifuge tube. Each tube was added to a separate well by dropping the solution onto the cells. The plate was then incubated overnight at 22°C. The transfection medium was removed and 2 ml of complete medium was added to each well. The plate was then incubated at 22°C until occlusion bodies were observed by light microscopy.

6.2.2.3 Infection of Cp14R cells with budded virus

Fourth instar larvae from the susceptible *C. pomonella* culture (CpS) were used to collect budded virus. Approximately 50 larvae were starved overnight. Larvae were then fed a 2 mm cube piece of diet containing 2 µl of 1×10^8 OBs/ml. Larvae were allowed to feed overnight. Larvae were then placed onto fresh diet and left for four days. Larvae were collected and placed onto ice to be numbed. Once larvae were numb, the last proleg was cut with sterile scissors and the haemolymph was collected and placed into an eppendorf containing 500 µl of complete cell culture medium and one crystal of N-Phenylthiourea to prevent melanisation. Using a confluent T25 cm^2 flask of Cp14R cells, medium was removed and 200 µl of the budded virus suspension mixed with 500 µl of medium was added to the cells. The flask was left at room temperature for three hours, and rotated every 30 minutes to spread the suspension. After three hours, 4 ml of complete medium was added to the flask and incubated at 22°C. The flask was checked every day until OBs were observed.

6.2.2.4 End point dilution assay (tissue culture infectious dosage$_{50}$ (TCID$_{50}$))

In order to complete the $TCID_{50}$, a stock suspension of budded virus was required. A T75 cm^2 flask of almost confluent cells was used. The medium was removed and 1 ml of the budded virus supernatant was added to the cells. The flask was left at room temperature for three hours and rotated every 30 minutes to spread the suspension. Complete medium (11 ml) was then added and the flask was incubated at 22°C. A 10 ml volume of the supernatant was collected when OBs were observed. The supernatant was placed in a polyethylene centrifuge tube and stored at 4°C. A further 7 ml of complete medium was added to the flask, and incubated for another two to three days. The cells and supernatant were collected in a polyethylene centrifuge tube. The tube was centrifuged at 1000 rpm for 10 minutes. The supernatant was removed and pooled with the previous supernatant. Cells (used in section 6.2.2.5) and supernatant were stored at 4°C.

A ten-fold serial dilution, from 10^{-1} to 10^{-10}, of the budded virus supernatant was prepared. A 60-well plate was seeded with 2×10^3 cells/well in complete medium. Each column was then infected with 5 µl of each budded virus dilution (Figure 6.1). The plates were incubated at 22°C in a plastic container with moistened paper towel to prevent the wells from drying out. The wells were scored at 3, 5, 7 and 9 days. A positive score was given to a well containing two or

more cells with OBs. The $TCID_{50}$ was calculated using the spreadsheet provided by O'Reilly *et al.* (1992).

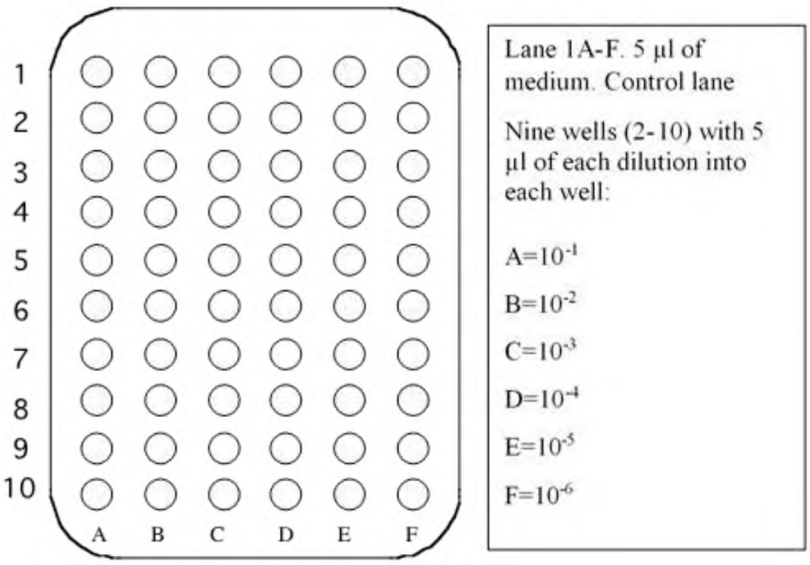


Figure 6.1: Inoculation of a sixty well plate for a $TCID_{50}$

6.2.2.5 Virulence of OBs collected from Cp14R cells

Occlusion bodies were purified from Cp14R cells collected in section 6.2.2.4. Cells were re-suspended in 1.5 ml of 0.5% SDS and centrifuged at 13 000 rpm for 10 minutes. Supernatant was removed and the pellet was re-suspended in 1.5 ml of 0.5M NaCl. The sample was centrifuged at 13 000 rpm for 10 minutes and the supernatant was removed. The pellet was re-suspended in ddH_2O and cells were disrupted using a micropestle. A sucrose gradient purification was not required as cells were pure enough for use in bioassays. OBs were enumerated using a Petroff-Hauser counting chamber (depth 0.02 mm) using a light microscope under dark field with 400X magnification.

Bioassays were then conducted against first instar larvae of CpS and CpR5M cultures using the LC_{50} values obtained from the results from section 6.2.1.2. *Cydia pomonella* diet was prepared as above in section 6.2.1.1. Bioassays were incubated at 26°C with a relative humidity of 60 – 70% and 18:6 photoperiod. After 24 hours, larval mortality was recorded and these larvae were excluded from the experiments. Mortality was then recorded on the seventh day to determine the virulence of the OBs collected from the cells. This was replicated twice.

6.2.2.6 Multiplicity of Infection (MOI) time course and expression of genes

Two six-well plates were seeded with 1×10^5 cells/well in 2 ml of complete medium. After cell attachment, the medium was removed and cells were infected with an MOI of 5 pfu/cell. After each time point (6, 12, 24, 48, 72 and 96 hours) a well containing infected cells and uninfected cells (control) were collected and placed into separate polyethylene centrifuge tubes. The tubes were centrifuged at 1000 rpm for 10 minutes and stored at -80°C.

The expression of six genes over the 96 hour time course (polh, IE-1, lef-8, Fusion, V-39 and DNApol) was determined by RT-qPCR analysis using a CFX90™ real-time system (Bio-Rad laboratories, Inc, California). RNA was extracted from the cells collected at each time point using a ReliaPrep™ RNA tissue miniprep system kit (Promega©, Madison USA). RNA was then transformed into cDNA using iScript™ Reverse transcription supermix for RT-qPCR kit (Bio-Rad laboratories, Inc, California). RT-qPCR was then performed in a 25 µl reaction containing 0.3pM of each primer (polh, IE-1, Lef-8, Vp39, fusion, DNApol and Actin as a standard), 12.5 µl of Maxima® SYBR green qPCR master mix (Thermo Fisher Scientific©, Inc, California) and 500 ng of cDNA. RT-qPCR was started with an initial denaturation step at 95°C for 10 minutes, followed by 40 cycles of denaturation at 95°C for 15 seconds, annealing at 58°C for 30 seconds and extension at 72°C for 30 seconds. Melting curve analysis was performed from 45 to 95°C with an increment of 0.5°C every 5 seconds. Data were analysed using Bio-Rad CFX Manager 2.0 (Bio-Rad laboratories, Inc, California) and the $2^{-\Delta\Delta CT}$ method (Livak & Schmittgen 2001).

6.3 RESULTS

6.3.1 Biological activity of CrpeNPV against CpS, CpRR1 and CpR5M

6.3.1.1 Infectivity bioassays with CpS, CpRR1 and CpR5M neonate larvae

The infectivity of CrpeNPV was tested against the three *C. pomonella* cultures: CpS, CpRR1 and CpR5M, at two discriminating dosages, 5.8×10^4 and 5.8×10^5 OBs/ml. From the results obtained, it was observed that the virus caused 100% mortality for all three cultures at both of the discriminating concentrations (Figure 6.2). Therefore, the virulence (dosage response) of CrpeNPV could be tested against the three insect cultures.

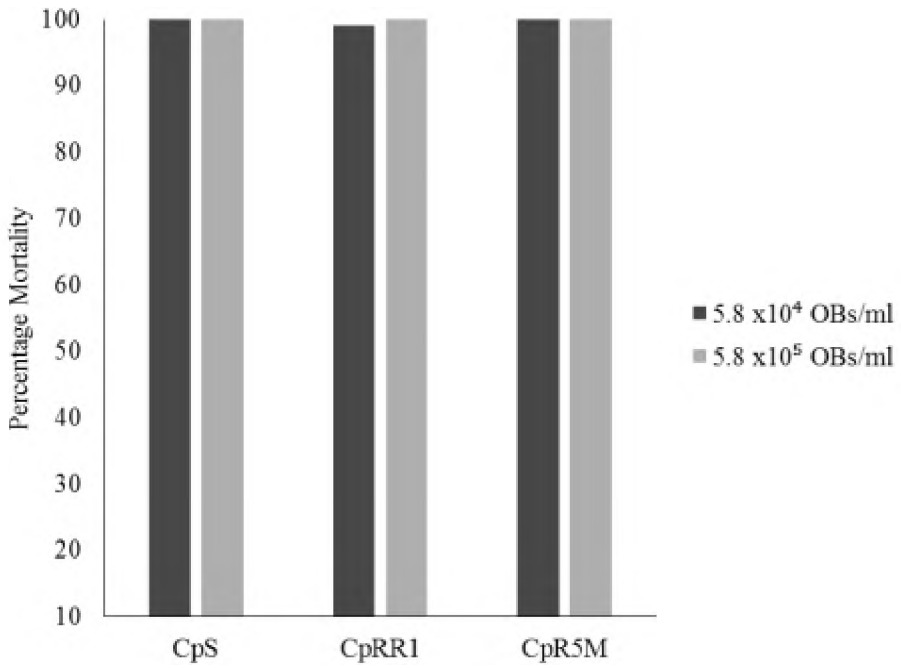


Figure 6.2: The infectivity of CrpeNPV against three European codling moth cultures: CpS, CpRR1 and CpR5M

6.3.1.2 Dosage response bioassays with CpS, CpRR1 and CpR5M neonate larvae

The regression line fitted to the data for seven days was y = 1.25122 (variance of slope = 0.00577) x – 4.29379 and for 14 days, y = 1.30998 (variance of slope = 0.00756) x – 3.83014 (Figure 6.3).

The regression lines were compared for all three replicates at seven days and were found to have a chi-squared value of 20.847 with three degrees of freedom and p-value < 0.05. Slopes were significantly different. The results obtained for the three replicates were used to calculate the mean LC_{50} and LC_{90} values (Table 6.2). The regression lines were compared for all three replicates at 14 days and were found to have a chi-squared value of 8.359 with three degrees of freedom and p-value < 0.05. Slopes were significantly different. The results obtained for the three replicates were used to calculate the mean LC_{50} and LC_{90} values (Table 6.3).

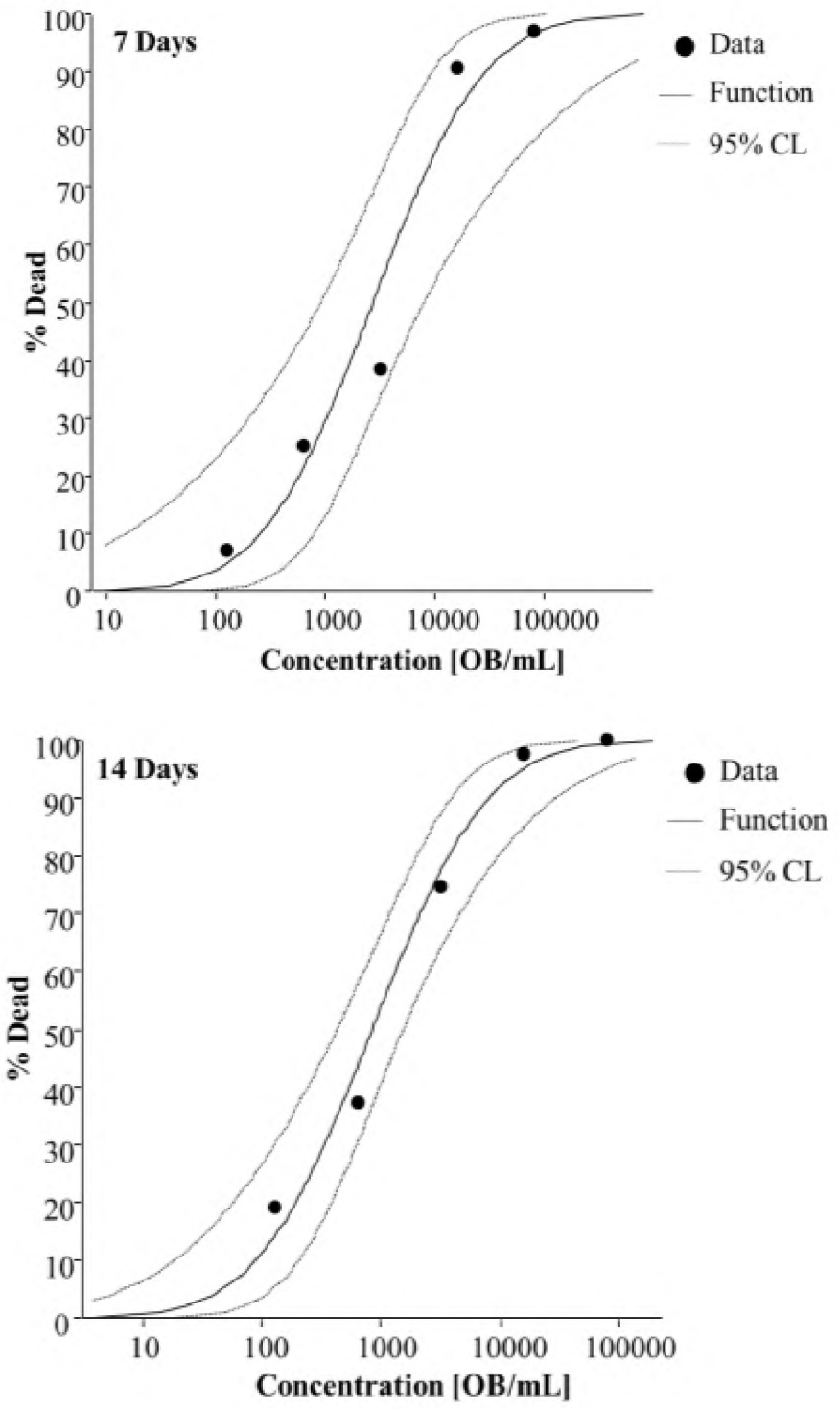


Figure 6.3: Dosage-response probit analysis for CrpeNPV against neonate larvae of CpS after seven and fourteen days.

The regression line fitted to the data for seven days was y = 1.35070 (variance of slope = 0.00652) x – 4.80997 and for 14 days, y = 1.18181 (variance of slope = 0.00549) x – 3.71487 (Figure 6.4).

The regression lines were compared for all three replicates at seven days and were found to have a chi-squared value of 8.343 with three degrees of freedom and p-value < 0.05. Slopes were significantly different. The results obtained for the three replicates were used to calculate the mean LC_{50} and LC_{90} values (Table 6.2).

The regression lines were compared for all three replicates at 14 days and were found to have a chi-squared value of 2.934 with three degrees of freedom and p-value < 0.05. Slopes were significantly different. The results obtained for the three replicates were used to calculate the mean LC_{50} and LC_{90} values (Table 6.3).

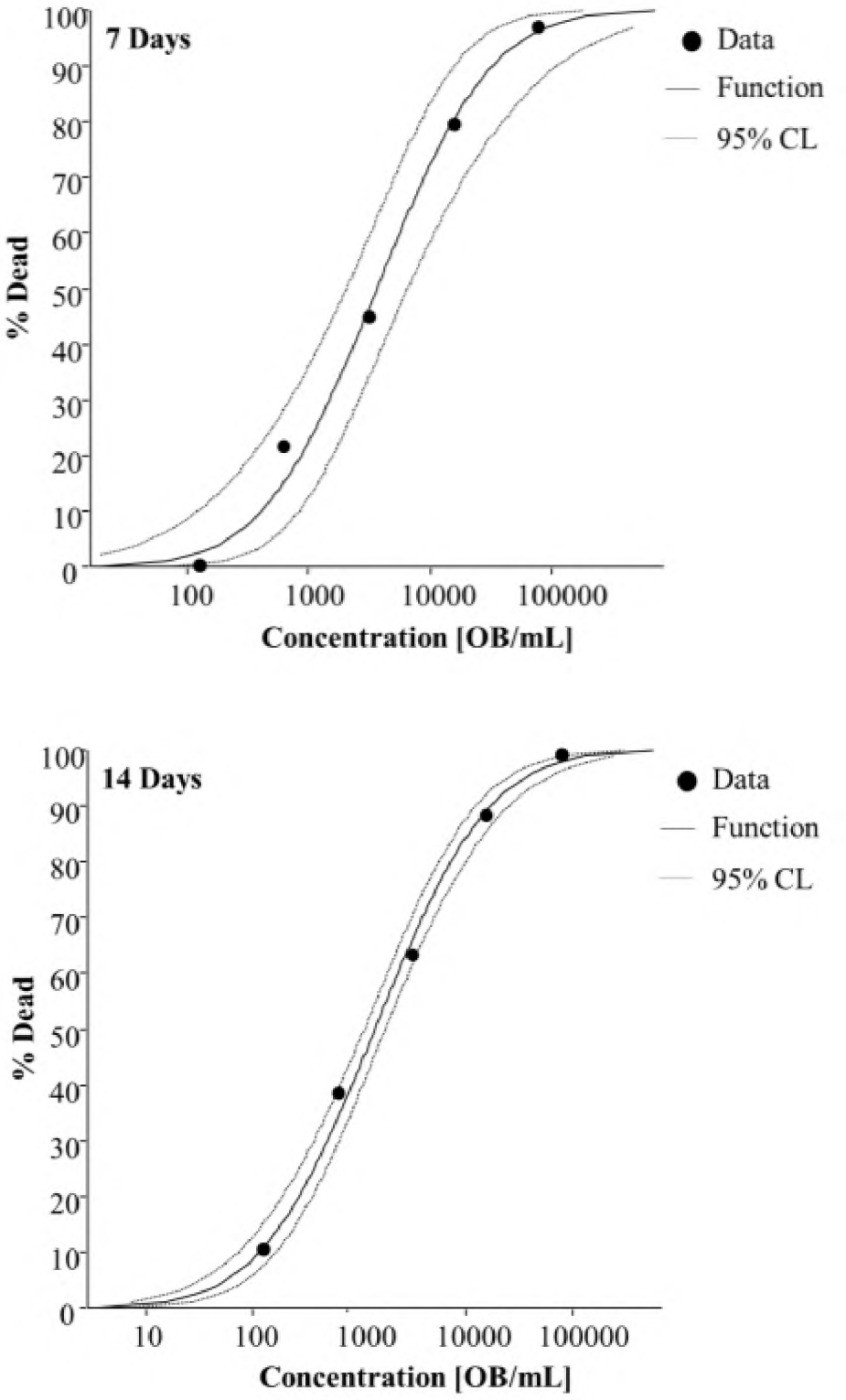


Figure 6.4: Dosage-response probit analysis for CrpeNPV against neonate larvae of CpRR1 after seven and fourteen days.

The regression line fitted to the data for seven days was y = 1.24940 (variance of slope = 0.00595) x – 4.17286 and for 14 days, y = 1.55490 (variance of slope = 0.01167) x – 4.42552 (Figure 6.5).

The regression lines were compared for all three replicates at seven days and were found to have a chi-squared value of 5.133 with three degrees of freedom and p-value < 0.05. Slopes were significantly different. The results obtained for the three replicates were used to calculate the mean LC_{50} and LC_{90} values (Table 6.2).

The regression lines were compared for all three replicates at 14 days and were found to have a chi-squared value of 13.567 with three degrees of freedom and p-value < 0.05. Slopes were significantly different. The results obtained for the three replicates were used to calculate the mean LC_{50} and LC_{90} values (Table 6.3).

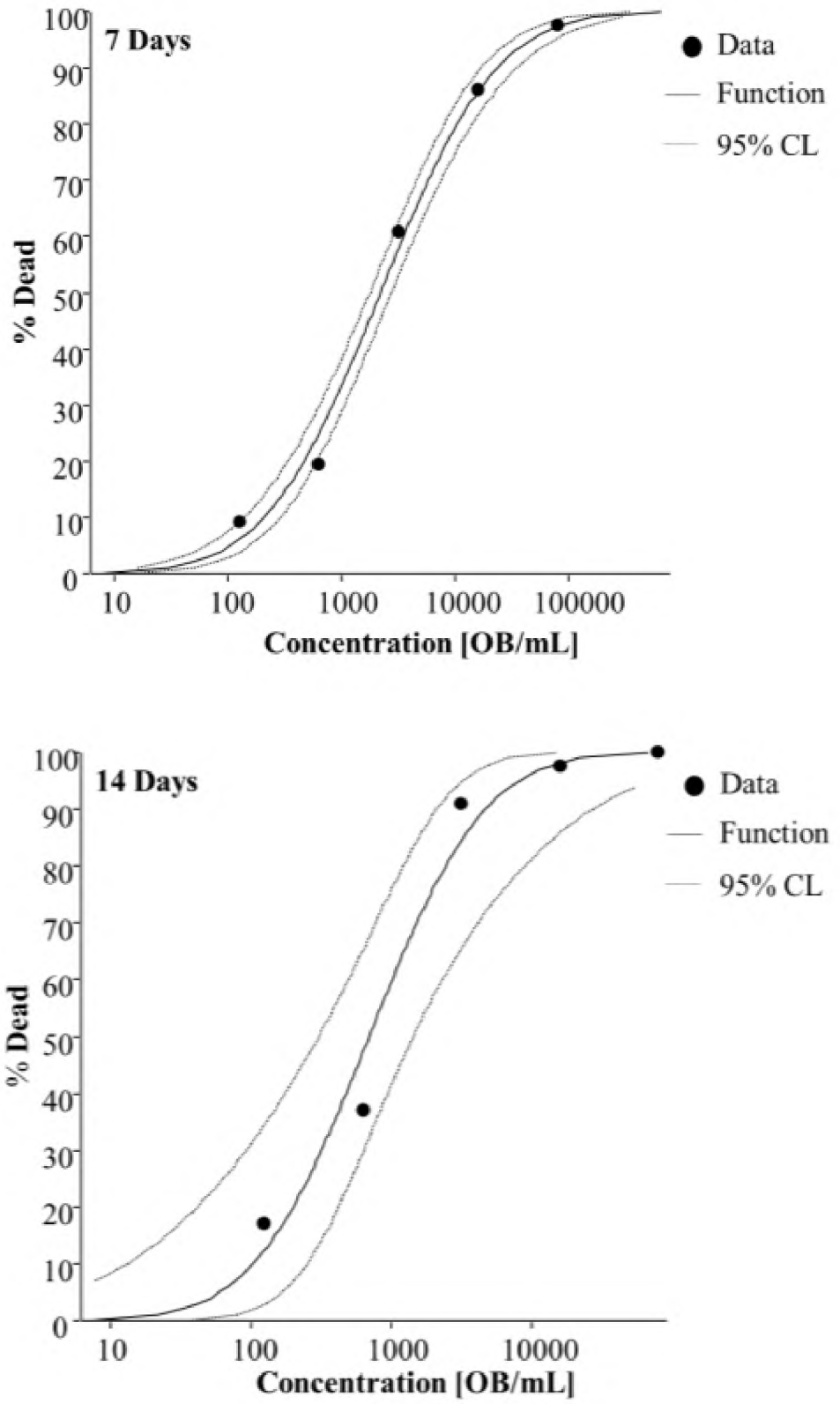


Figure 6.5: Dosage response probit analysis for CrpeNPV against neonate larvae of CpR5M after seven and fourteen days.

Table 6.2: Comparison of the LC_{50} and LC_{90} values for the three European codling moth cultures: CpS, CpRR1 and CpR5M after seven days

		Dosage (OBs/ml)	Lower 95%-CI	Upper 95%-CI
CpS	LC_{50}	2.70×10^3	9.06×10^2	7.85×10^3
	LC_{90}	2.86×10^4	9.38×10^3	4.41×10^5
CpRR1	LC_{50}	3.64×10^3	2.05×10^3	1.57×10^4
	LC_{90}	3.24×10^4	6.53×10^3	1.10×10^5
CpR5M	LC_{50}	2.19×10^3	1.76×10^3	2.72×10^3
	LC_{90}	2.32×10^4	1.68×10^4	3.43×10^4

Table 6.3: Comparison of the LC_{50} and LC_{90} values for the three European codling moth cultures; CpS, CpRR1 and CpR5M after fourteen days

		Dosage (OBs/ml)	Lower 95%-CI	Upper 95%-CI
CpS	LC_{50}	8.39×10^2	4.26×10^2	1.52×10^3
	LC_{90}	7.98×10^3	3.85×10^3	2.89×10^4
CpRR1	LC_{50}	1.39×10^3	1.11×10^3	1.74×10^3
	LC_{90}	1.69×10^4	1.22×10^4	2.51×10^4
CpR5M	LC_{50}	7.02×10^2	3.07×10^2	1.44×10^3
	LC_{90}	4.68×10^3	2.11×10^3	2.73×10^4

6.3.1.3 Time response bioassays against CpS and CpR5M neonate larvae using CrpeNPV and three CpGV isolates (CpGV-S, CpGV-E2 and CpGV-M)

Larval mortality was observed 56 hours after treatment and continued until mortality had stabilised for each of the four viral isolates. Mortality was recorded for a small number of larvae after 24 hours. This mortality was not symptomatic of viral infection and was recorded as non-responsive and omitted from the results. This is an acceptable practice, known as biological truncation (Moore *et al.* 2011). The time-mortality relationship for all four viral isolates (see Table 6.1) tested against neonate larvae of the susceptible *C. pomonella* culture, CpS were analysed using a logit regression analysis (Table 6.4). From the logit regression, the LT_{50} and LT_{90} for each viral isolate was determined (Table 6.6).

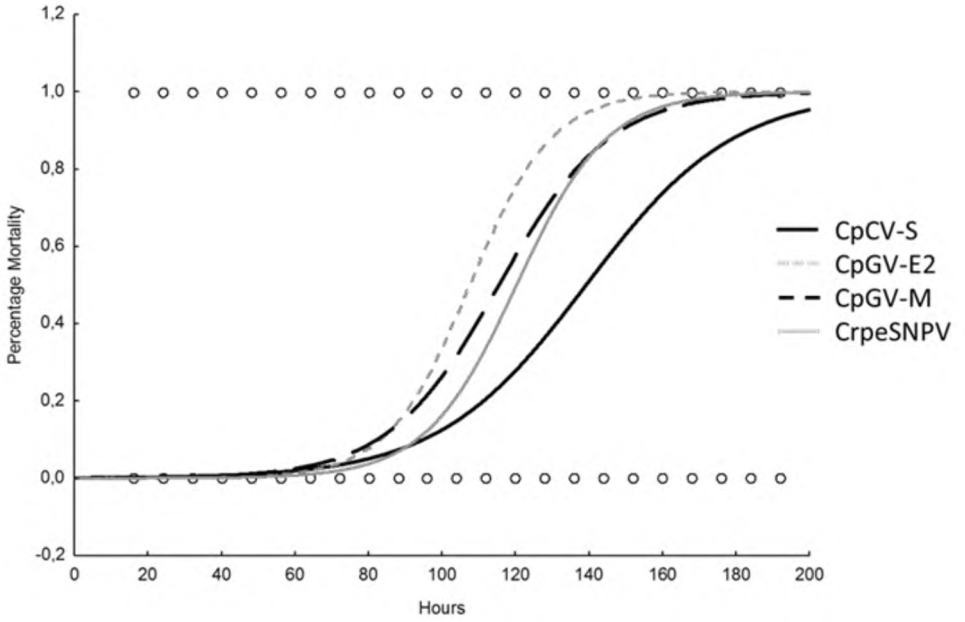


Figure 6.6: Time-mortality response between three CpGV isolates and CrpeNPV against the susceptible *Cydia pomonella* (CpS) culture.

Table 6.4: Logit regression data for four viral isolates tested against the susceptible *Cydia pomonella* culture, CpS.

Viral Isolate	Logit regression data for CpS							
	Regression Coefficient	SE	P-value	Chi-square	P-value	X-intercept	SE	P-Value
CpGV-S	-6.92	0.29	<0.001	1355.9	<0.001	0.05	0.002	<0.001
CpGV-E2	-9.70	0.47	<0.001	2273.3	<0.001	0.09	0.004	<0.001
CpGV-M	-7.66	0.33	<0.001	1927.8	<0.001	0.06	0.003	<0.001
CrpeNPV	-9.74	0.44	<0.001	2105.6	<0.001	0.08	0.004	<0.001

Larval mortality was observed 56 to 80 hours after treatment and continued until mortality had stabilised for each of the four viral isolates. Mortality observed after 24 hours was not symptomatic of viral infection and was recorded as non-responsive and omitted from the results. As in the previous trial with CpS, the time-mortality relationship for all four viral isolates tested against neonate larvae of the resistant *C. pomonella* culture, CpR5M were analysed using a logit regression analysis (Table 6.5). From the logit regression, the LT_{50} and LT_{90} values for each viral isolate were determined (Table 6.6). LT_{50} and LT_{90} values could not be obtained for CpGV-S and the LT_{90} value could not be obtained for CpGV-M due to the resistance to the isolates.

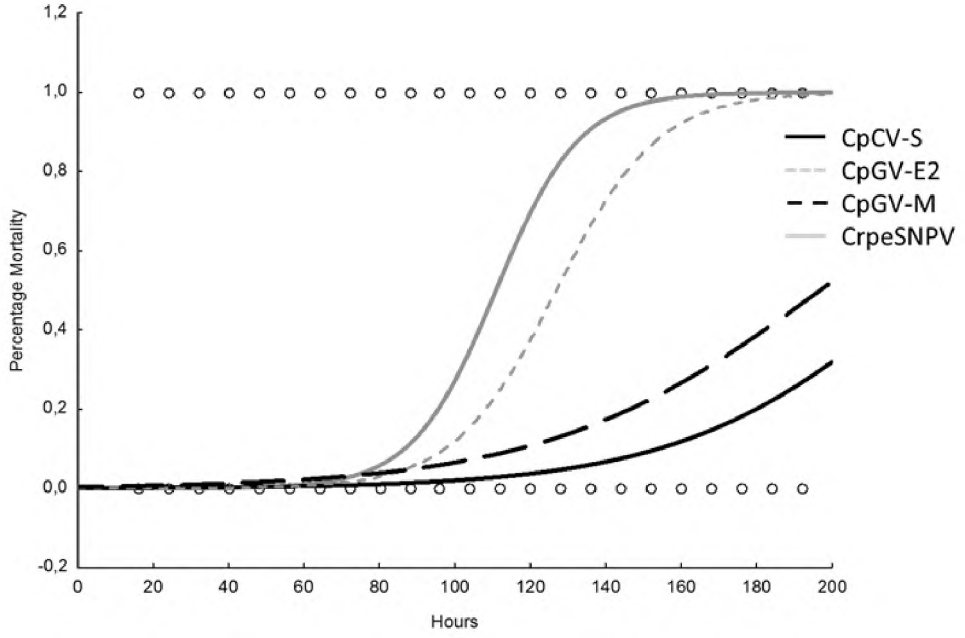


Figure 6.7: Time-mortality response between three CpGV isolates and CrpeNPV against the resistant *Cydia pomonella* (CpR5M) culture.

Table 6.5: Logit regression data for four viral isolates tested against the resistant *Cydia pomonella* culture, CpR5M

Viral Isolate	Logit regression data for CpR5M							
	Regression Coefficient	SE	P-value	Chi-square	P-value	X-intercept	SE	P-Value
CpGV-S	-6.99	0.46	<0.001	203.8	<0.001	0.03	0.003	<0.001
CpGV-E2	-9.42	0.44	<0.001	1556.4	<0.001	0.07	0.003	<0.001
CpGV-M	-5.41	0.27	<0.001	365.5	<0.001	0.03	0.002	<0.001
CrpeNPV	-10.04	0.49	<0.001	1760.3	<0.001	0.09	0.004	<0.001

Table 6.6: Comparison of the LT_{50} and LT_{90} values for two of the *Cydia pomonella* cultures; CpS (susceptible) and CpR5M (resistant) against four viral isolates; CpGV-S, CpGV-E2, CpGV-M and CrpeNPV.

	CpS		CpR5M	
	LT_{50}	LT_{90}	LT_{50}	LT_{90}
CpGV-S	139.28 hours	183.55 hours	-	-
CpGV-E2	107.68 hours	132.07 hours	126.89 hours	156.48 hours
CpGV-M	115.58 hours	148.74 hours	200.00 hours	-
CrpeNPV	120.34 hours	147.49 hours	110.92 hours	135.18 hours

6.3.2 Susceptibility of CrpeNPV to the Cp14R cell line

6.3.2.1 Transfection of Cp14R cells with CrpeNPV DNA

Cryptophlebia peltastica NPV DNA was used to complete a transfection to determine whether CrpeNPV could infect the Cp14R cell line. Three weeks after the transfection, OBs could be observed within the nuclei of the Cp14R cells (Figure 6.8).

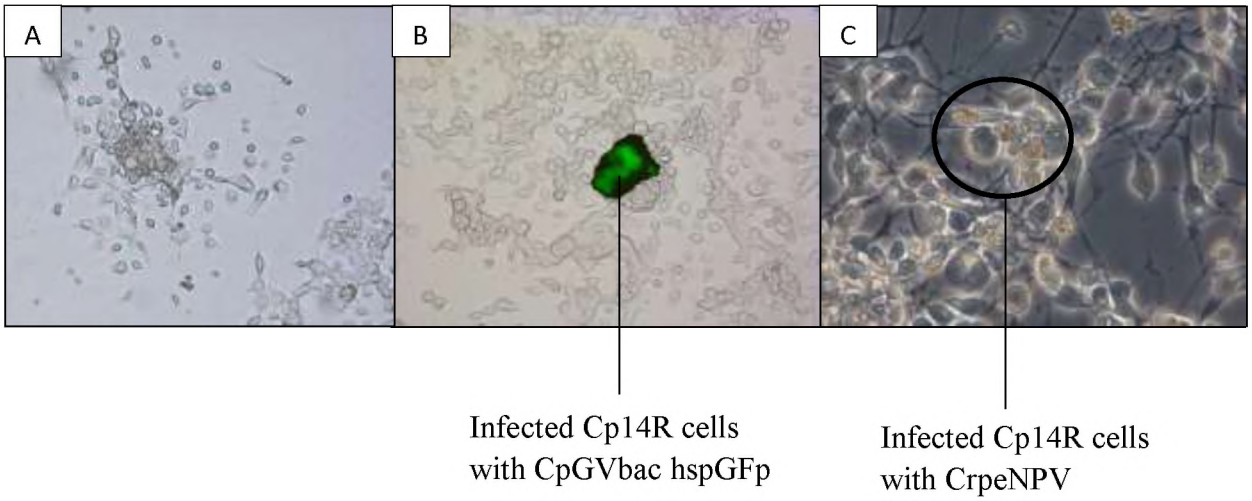


Figure 6.8: Transfection of Cp14R cells with CpGVbachspGFp and CrpeNPV DNA. (A) Uninfected cells (control) (100x magnification), (B) CpGVbachspGFp infected cells fluoresced under a green light and (100x magnification) (C) CrpeNPV infected cells (200x magnification).

6.3.2.2 Infection of Cp14R cells with budded virus

Budded virus collected from the haemolymph of 5th instar *C. pomonella* larvae was used to infect Cp14R cells. Three days after the infection, OBs could be observed within the nuclei of the Cp14R cells (Figure 6.9).

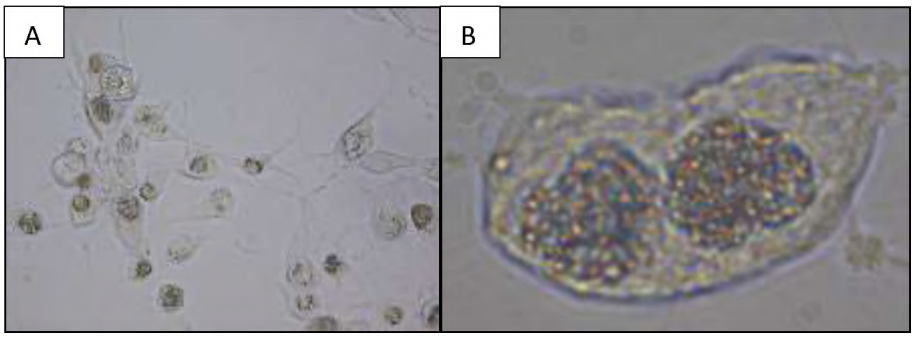


Figure 6.9: Infected Cp14R with CrpeNPV from an infection with budded virus after three days. (A) Infected cells (200 x magnification), (B) OBs in the nuclei of the Cp14R cells (400x magnification).

6.3.2.3 Virulence of OBs collected from Cp14R cells

The virulence of CrpeNPV OBs isolated from Cp14R cells were tested against the two *C. pomonella* cultures; CpS and CpR5M using the LC_{50} values obtained from section 7.3.1.2. From the results obtained, it was observed that the OBs produced from the cells (*in vitro*) caused over 50% mortality for both CpS and CpR5M larvae at the LC_{50} concentration (Figure 6.10). OBs produced *in vitro* have similar virulence to OBs produced *in vivo*.

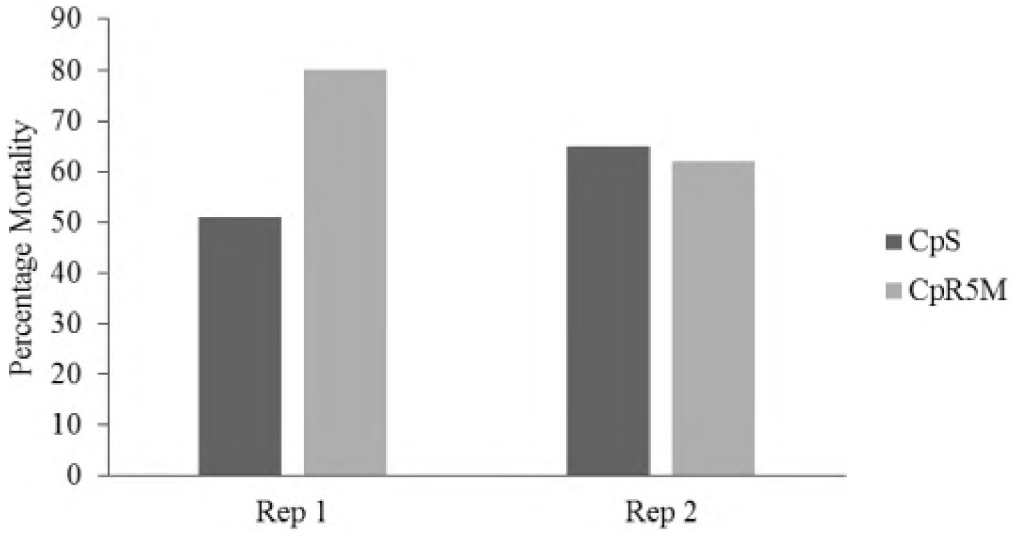


Figure 6.10: The virulence of CrpeNPV occlusion bodies collected from Cp14R cells against CpS and CpR5M *Cydia pomonella* cultures.

6.3.2.4 End point dilution assay and Multiplicity of Infection (MOI) time course

The $TCID_{50}$ was calculated after 9 days of infection. The budded virus supernatant was found to have a titre of 1.23 x 10^7 pfu/ml. The titre was then used to inoculate a six-well plate of Cp14R cells with an MOI of 5.

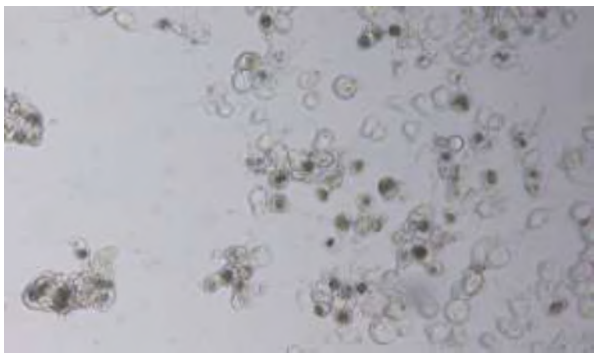


Figure 6.11: An infected well of a sixty well plate after nine days used to calculate the $TCID_{50}$

RNA samples were extracted from Cp14R cells infected with CrpeNPV budded virus (5 PFU/cell) at 6, 12, 24, 48, 72 and 96 hours post infection to determine when the polh gene was expressed (Figure 6.12). The RT-qPCR data for each sample was analysed using the $2^{-\Delta\Delta CT}$ method and β-actin as a standard (Livak & Schmittgen 2001). The polh gene was expressed at 6 hours, with peak expression at 48 hours after which expression decreased. Only the polh gene was amplified and no unspecified genes amplified as shown by the melt curve (Figure 6.13).

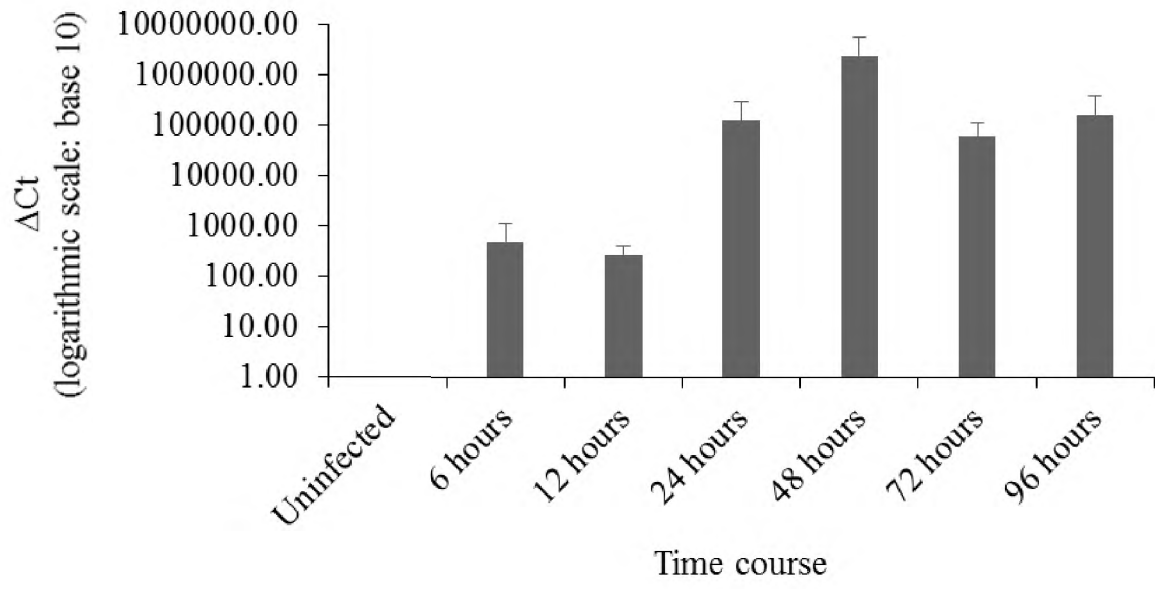


Figure 6.12: The RT-qPCR calibration of Cq using actin as a standard to determine the expression of the polyhedrin gene of CrpeNPV in Cp14R cells over 96 hours (y-axis is a logarithmic scale with a base of 10).

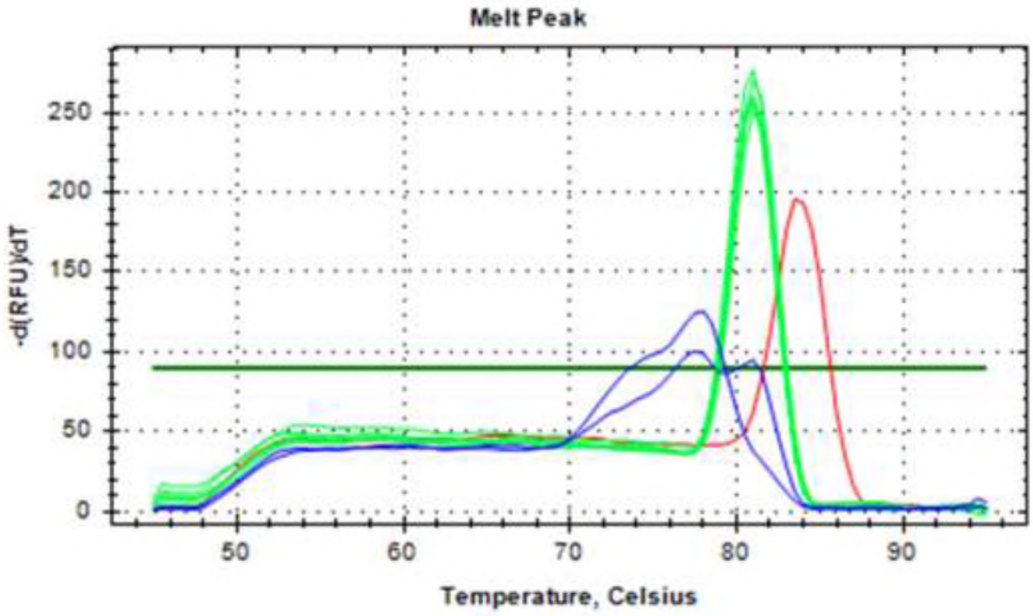


Figure 6.13: Melt curve of the RT-qPCR for polyhedrin gene expression of CrpeNPV in Cp14R cells. Samples at different time points (green), negative control and uninfected larvae (blue) and actin control (red).

To determine the expression of the IE-1 gene in CrpeNPV in the Cp14R cell line, RNA samples were extracted from Cp14R cells infected with CrpeNPV budded virus (5 PFU/cell) at 6, 12, 24, 48, 72 and 96 hours post infection (Figure 6.14). The RT-qPCR data for each sample were analysed using the $2^{-\Delta\Delta CT}$ method and β-actin as a standard. The IE-1 gene was expressed at 6 hours and increased until 72 hours after which expression decreased. Only the IE-1 gene was amplified and no unspecified genes amplified as shown by the melt curve (Figure 6.15).

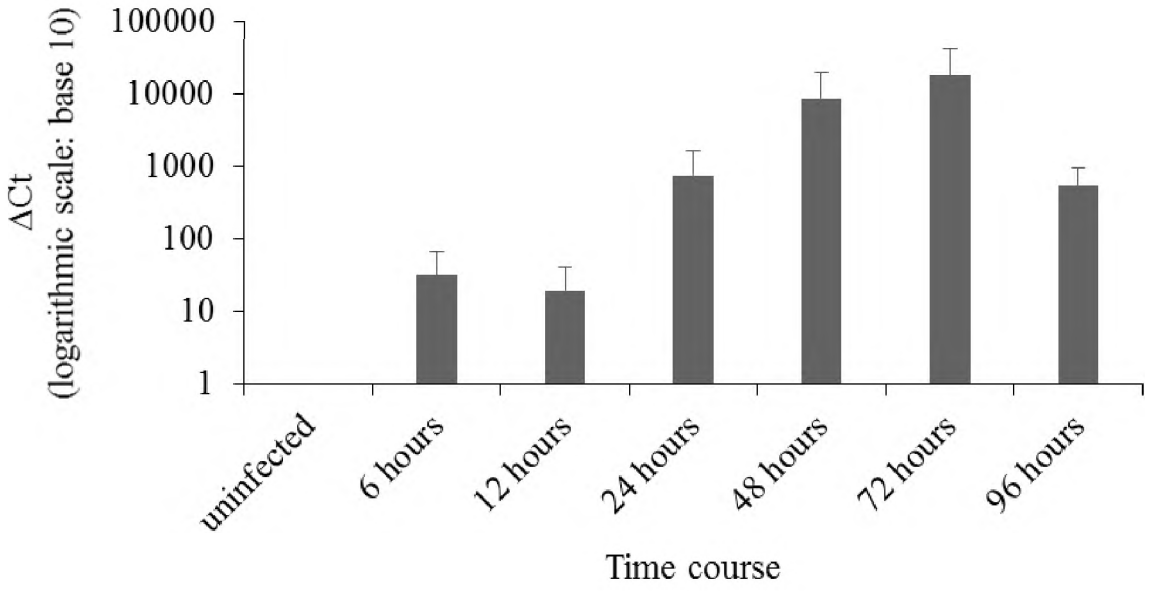


Figure 6.14: The RT-qPCR calibration of Cq using actin as a standard to determine the expression of IE-1 gene of CrpeNPV in Cp14R cells over 96 hours (y-axis is a logarithmic scale with a base of 10).

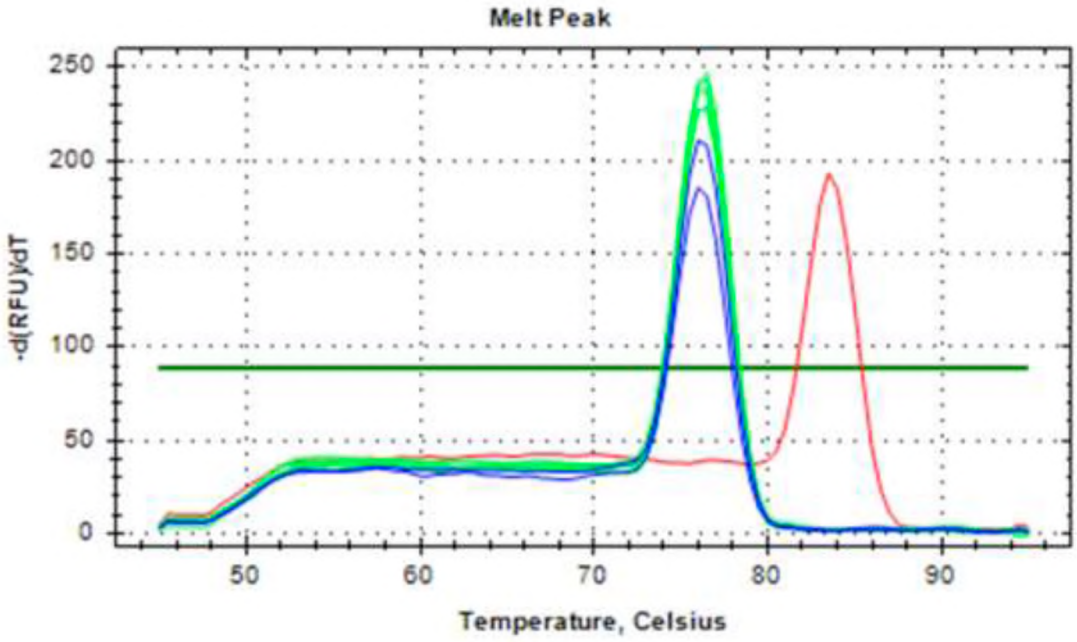


Figure 6.15: Melt curve of the RT-qPCR for IE-1 gene expression of CrpeNPV in Cp14R cells. Samples at different time points (green), negative control and uninfected larvae (blue) and actin control (red).

To determine the expression of the lef-8 gene of CrpeNPV in the Cp14R cell line, RNA samples were extracted from Cp14R cells infected with CrpeNPV budded virus (5 PFU/cell) at 6, 12, 24, 48, 72 and 96 hours post infection (Figure 6.16). The RT-qPCR data for each sample were analysed using the $2^{-\Delta\Delta CT}$ method and β-actin as a standard. The lef-8 gene was expressed at 6 hours and peaked at 72 hours after which expression decreased. Only the lef-8 gene was amplified and no unspecified genes amplified as shown by the melt curve (Figure 6.17).

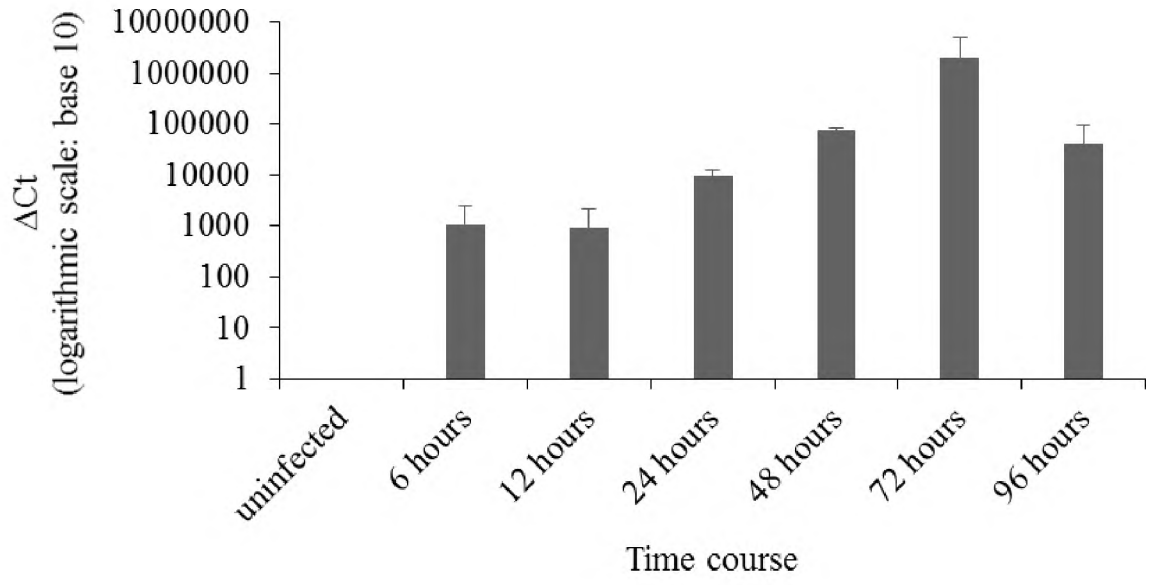


Figure 6.16: The RT-qPCR calibration of Cq using actin as a standard to determine the expression of lef-8 gene of CrpeNPV in Cp14R cells over 96 hours (y-axis is a logarithmic scale with a base of 10).

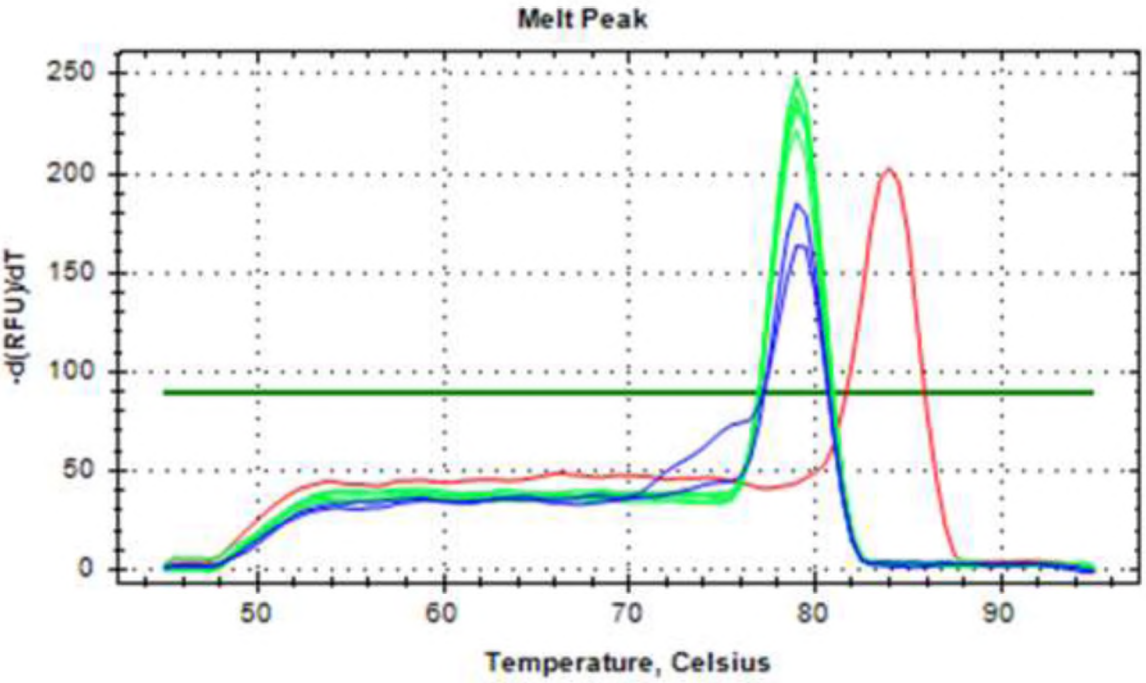


Figure 6.17: Melt curve of the RT-qPCR for the lef-8 gene expression of CrpeNPV in Cp14R cells. Samples at different time points (green), negative control and uninfected larvae (blue) and actin control (red).

To determine the expression of the fusion protein gene of CrpeNPV in the Cp14R cell line, RNA samples were extracted from Cp14R cells infected with CrpeNPV budded virus (5 PFU/cell) at 6, 12, 24, 48, 72 and 96 hours post infection (Figure 6.18). The RT-qPCR data for each sample were analysed using the $2^{-\Delta\Delta CT}$ method and β-actin as a standard. The fusion protein gene was expressed at 6 hours and increased until 72 hours after which expression decreased. Only the fusion protein gene was amplified and no unspecified genes amplified as shown by the melt curve (Figure 6.19).

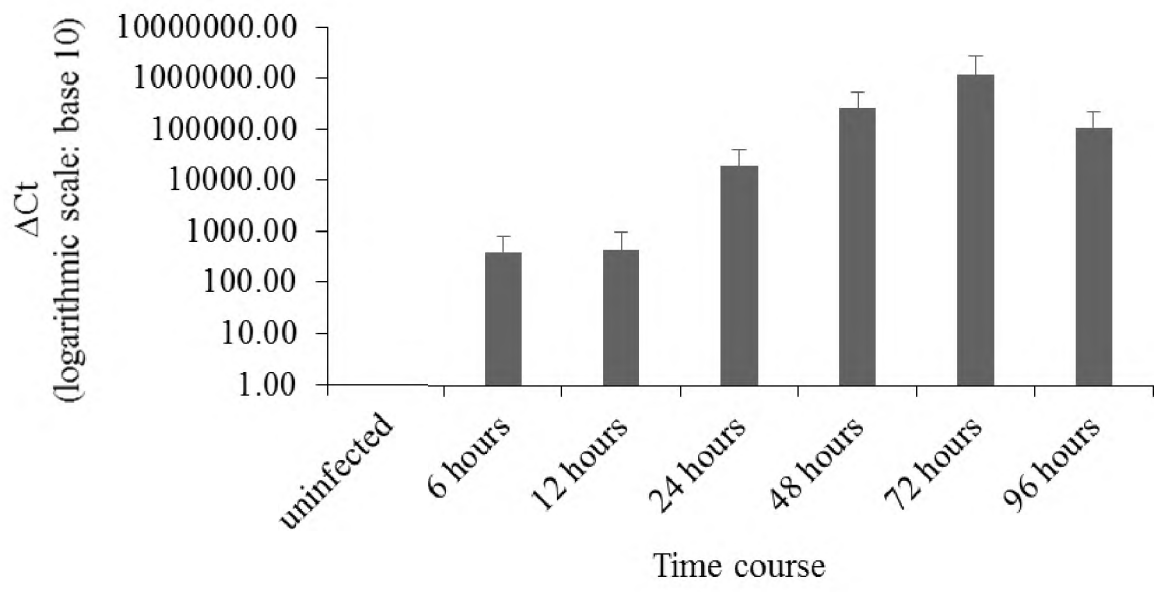


Figure 6.18: The RT-qPCR calibration of Cq using actin as a standard to determine the expression of fusion protein gene of CrpeNPV in Cp14R cells over 96 hours (y-axis is a logarithmic scale with a base of 10).

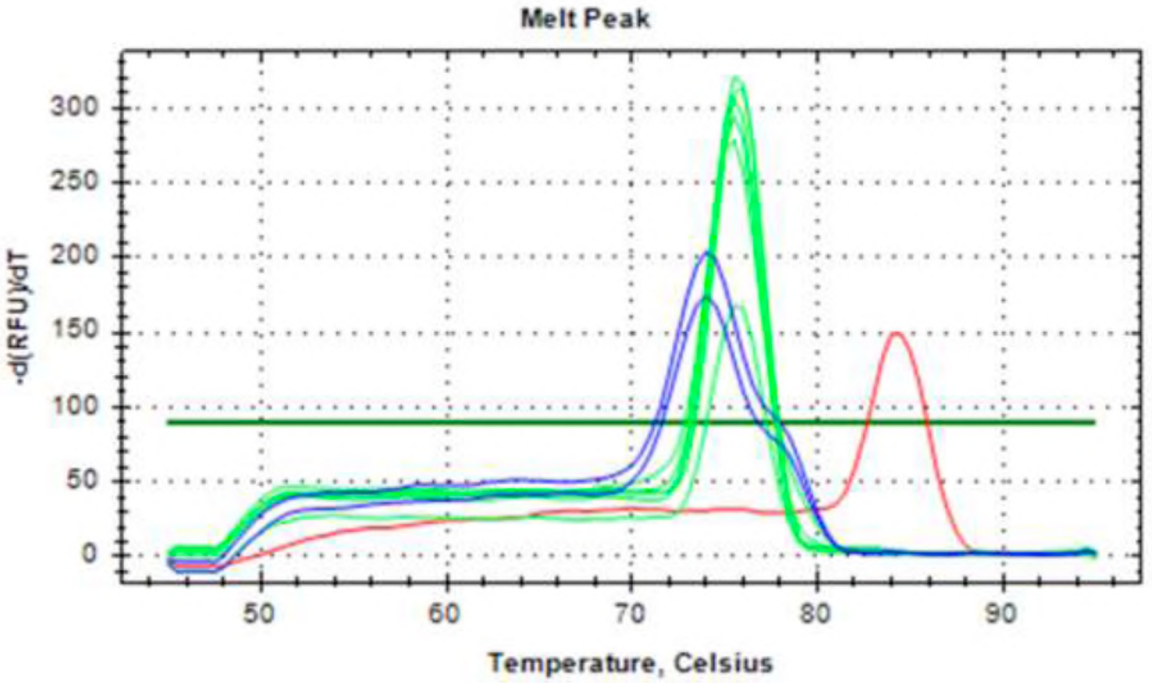


Figure 6.19: Melt curve of the RT-qPCR for the fusion protein gene expression of CrpeNPV in Cp14R cells. Samples (green) at different time points, negative control and uninfected larvae (blue) and actin control (red).

To determine the expression of the Vp39 gene of CrpeNPV in the Cp14R cell line, RNA samples were extracted from Cp14R cells infected with CrpeNPV budded virus (5 PFU/cell) at 6, 12, 24, 48, 72 and 96 hours post infection (Figure 6.20). The RT-qPCR data for each sample were analysed using the $2^{-\Delta\Delta CT}$ method and β-actin as a standard. The Vp39 gene was expressed at 6 hours and peaked at 48 hours after which expression decreased. Only the Vp39 gene was amplified and no unspecified genes amplified as shown by the melt curve (Figure 6.21).

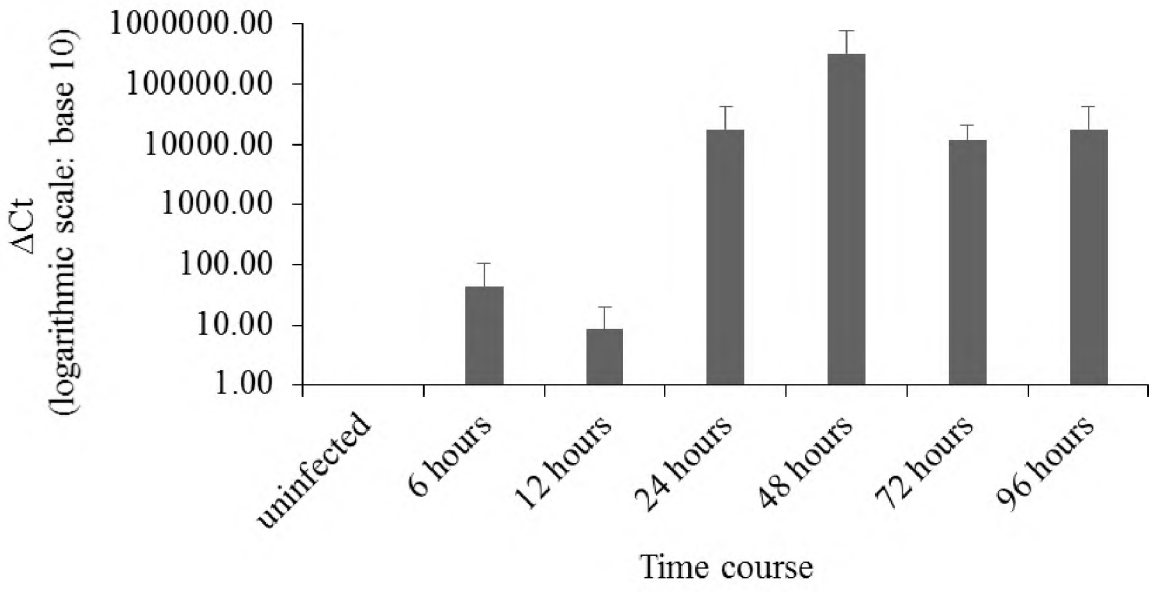


Figure 6.20: The RT-qPCR calibration of Cq using actin as a standard to determine the expression of Vp39 gene of CrpeNPV in Cp14R cells over 96 hours (y-axis is a logarithmic scale with a base of 10).

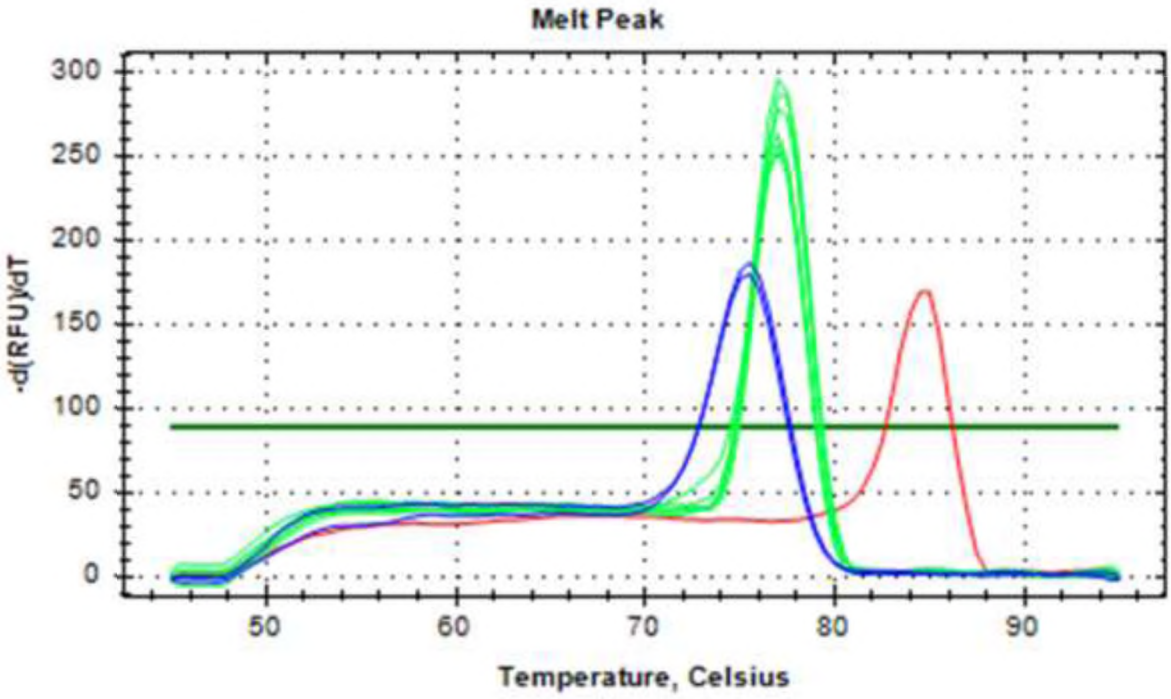


Figure 6.21: Melt curve of the RT-qPCR for the Vp39 gene expression of CrpeNPV in Cp14R cells. Samples at different time points (green), negative control and uninfected larvae (blue) and actin control (red).

To determine the expression of the DNA polymerase gene of CrpeNPV in the Cp14R cell line, RNA samples were extracted from Cp14R cells infected with CrpeNPV budded virus (5 PFU/cell) at 6, 12, 24, 48, 72 and 96 hours post infection (Figure 6.22). The RT-qPCR data for each sample were analysed using the $2^{-\Delta\Delta CT}$ method and β-actin as a standard. The DNA polymerase gene was expressed at 6 hours and peaked at 48 hours after which expression decreased. Only the DNA polymerase gene was amplified and no unspecified genes amplified as shown by the melt curve (Figure 6.23).

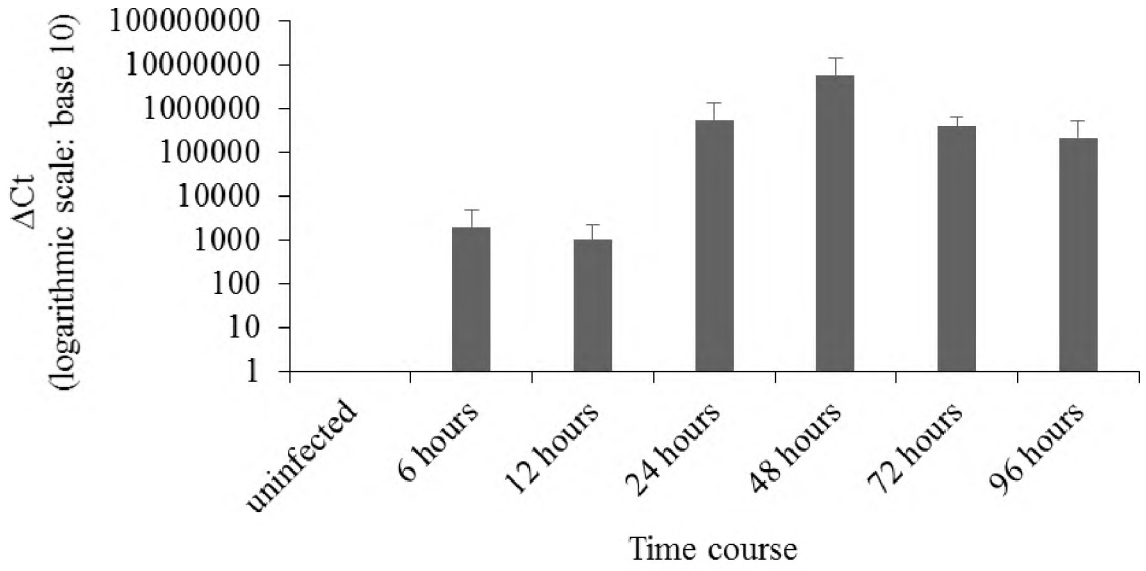


Figure 6.22: The RT-qPCR calibration of Cq using actin as a standard to determine the expression of DNA polymerase gene of CrpeNPV in Cp14R cells over 96 hours (y-axis is a logarithmic scale with a base of 10).

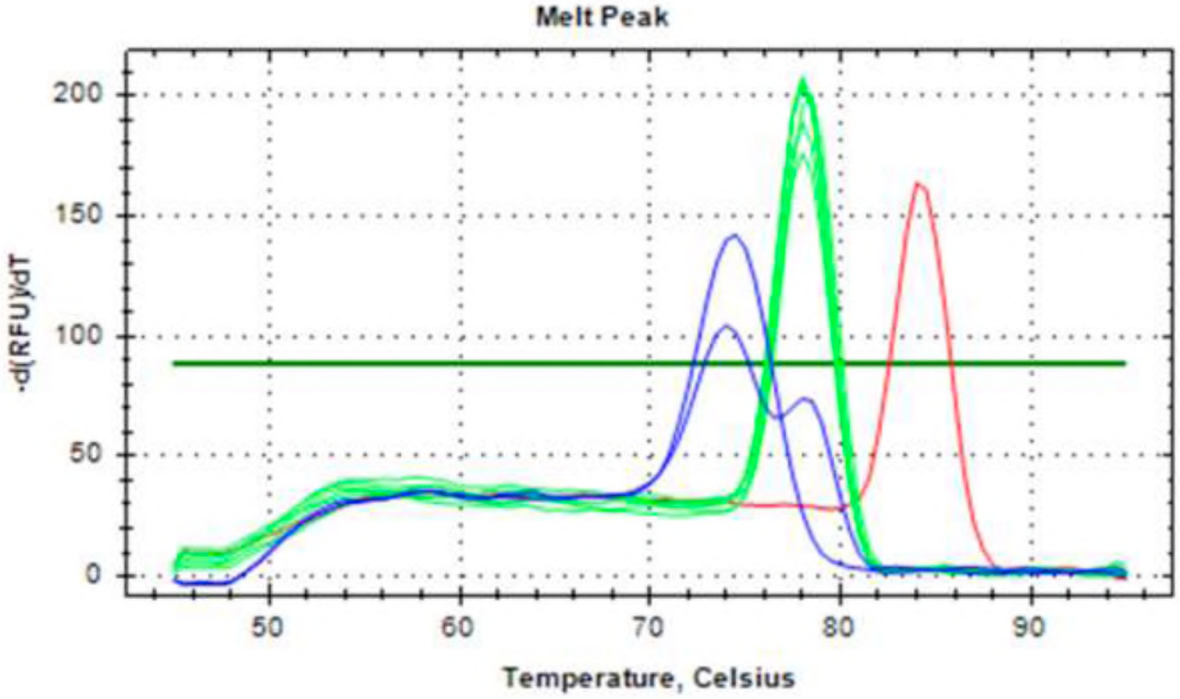


Figure 6.23: Melt curve of the RT-qPCR for the DNA polymerase gene expression of CrpeNPV in Cp14R cells. Samples at different time points (green), negative control and uninfected larvae (blue) and actin control (red).

6.4 DISCUSSION

Cryptophlebia peltastica NPV has the potential to be used as a microbial control agent against *C. pomonella*, as shown in the previous chapter. For this reason, the biological activity was also examined against CpGV resistant *C. pomonella* as an additional option in the resistance management programme in Europe. To determine the virulence of CrpeNPV against susceptible and resistant European *C. pomonella*, the dosage response relationship was determined using the diet incorporation method. The diet incorporation method was used as it is the standard method at the Julius Kühn Institute, where the original bioassays for various CpGV isolates against resistant *C. pomonella* were conducted.

Dosage response mortality curves were calculated and lethal concentrations (LC_{50} and LC_{90}) were determined for the susceptible (CpS) and resistant (CpRR1 and CpR5M) *C. pomonella* cultures. The LC_{50} of CrpeNPV against CpS was observed to be 2.70×10^3 OBs/ml at 7 days and 8.39×10^2 OBs/ml at 14 days. The LC_{50} values obtained for CrpeNPV against CpS were slightly higher than various other isolates of CpGV meaning that it is less virulent. For example, CpGV-M ("Mexican strain") had an LC_{50} of 1.90×10^3 OBs/ml, 8.97×10^2 OBs/ml for the CpGV-I12 ("Iran strain") isolate and 9.5×10^2 OBs/ml for the CpGV-E2 isolate after 7 days (Eberle & Jehle 2006; Eberle *et al.* 2008; Eberle 2010). The only isolate that CrpeNPV was more virulent against for CpS was the CpGV-S isolate, which had an LC_{50} of 9.3×10^3 OBs/ml after 7 days (Eberle 2010). The LC_{50} of CrpeNPV against CpRR1 was calculated to be 3.64×10^3 OBs/ml at 7 days and 1.39×10^3 OBs/ml at 14 days. The LC_{50} values obtained for CrpeNPV against CpRR1 were only less virulent than the CpGV-E2 isolate, which had an LC_{50} value of 1.89×10^3 OBs/ml. CrpeNPV was more virulent against CpRR1 than CpGV-M (LC_{50} could not be calculated), CpGV-I12 (1.27×10^4 OBs/ml) and CpGV-S (LC_{50} of 1.44×10^4 OBs/ml) (Eberle & Jehle 2006; Eberle *et al.* 2008; Eberle 2010). The LC_{50} of CrpeNPV against CpR5M was calculated to be 2.19×10^3 OBs/ml at 7 days and 7.02×10^2 OBs/ml at 14 days. No comparative results are available for CpR5M, as studies are currently be conducted by another student at Julius Kühn Institute (Jehle, personal communication). Various populations of European *C. pomonella* are resistant to the CpGV-M isolate, therefore alternative isolates of CpGV have been identified to help overcome the resistance in *C. pomonella* (Eberle *et al.* 2008).

An initial time response relationship was determined for the CpS and CpR5M cultures using three CpGV isolates: CpGV-S, CpGV-E2, CpGV-M and CrpeNPV. From the results obtained

for CpS, it was observed that CrpeNPV had a faster speed of kill than CpGV-S and CpGV-M. For the CpR5M culture, CrpeNPV had a faster speed of kill than the three CpGV isolates. Therefore, as CrpeNPV has the ability to overcome resistance in both CpRR1 and CpR5M cultures and has a faster speed of kill than certain CpGV isolates there is a great possibility that CrpeNPV can be used as an alternative control option in the resistance management of CpGV for *C. pomonella*, however, field trials still need to be completed.

To further develop CrpeNPV into a biopesticide it is important to have a good understanding of its molecular biology and have the possibility to genetically manipulate the virus in the future. In order to get a better understanding of this, a cell line permissive to CrpeNPV would be a great advantage (Winstanley & Crook 1993). From the above results, Cp14R cells were transfected with CrpeNPV DNA and infected with CrpeNPV budded virus. Both methods were successful in producing OBs within the Cp14R cells. This is the first report of an NPV infecting the Cp14R cell line. However, this is not the first time a NPV has been reported to infect a cell line of a different host insect. For example,the *Spodoptera frugiperda* cell line has been used to propagate *S. frugiperda* NPV, *Autographa californica* NPV, *S. exempta* NPV, *S. exigua* NPV and *Trichoplusia ni* NPV (Tsuda *et al.* 1984).

Occlusion bodies of CrpeNPV could be observed within the nuclei of the Cp14R using a light microscope, unlike OBs of CpGV which require electron microscopy, as GVs are much smaller than NPVs (Winstanley & Crook 1993). The initial cytopathic effect observed from the Cp14R cells infected with CrpeNPV was the expansion of the nucleus of the cells. This was then followed by numerous OBs of CrpeNPV being observed within the nucleus of the cells. This was also observed when *S. frugiperda* cells (SF2) were infected with SfNPV (Knudson & Harrap 1976). OBs collected from the infection were used in bioassays against CpS and CpR5M. From the results obtained, the OBs produced *in vitro* had a similar virulence against the two *C. pomonella* cultures as OBs produced *in vivo*. Thus, there is an opportunity to produce CrpeNPV in cell culture in the future when the method is economically feasible.

During this study Cp14R cells were infected with an MOI of 5 $TCID_{50}$ per cell in order to determine when the following randomly selected early and late expression genes were been expressed using real time qPCR: polh, ie-1, lef-8, fusion protein, vp39 and DNA polymerase. From the results, it was observed that the initial expression of polh was at 24 hours post infection (p.i) and reached maximum infection at 48 hours p.i, after which the expression of the gene decreased. This was also observed for vp39 and DNA polymerase. The lef-8 and

fusion protein genes showed initial expression at 48 hours p.i and peaked at 72 hours p.i after which the expression of lef-8 and fusion protein decreased. The ie-1 gene started to be expressed at 24 hours and increased until 72 hours p.i after which expression decreased. From this analysis it was shown that budded virus of CrpeNPV takes three days (72 hours) to reach maximum infection of Cp14R cells. Similar results were obtained for *S. frugiperda* cells infected with *A. californica* NPV, where virions were observed 72 hours p.i (Vlak 1979).

In conclusion, CrpeNPV has the ability to overcome CpGV resistance in *C. pomonella* and therefore can potentially be used as an alternative control option in the resistance management of *C. pomonella* in Europe. Not only can CrpeNPV be used as an alternative control option but it also has the potential to be mass produced in vitro in Cp14R cells in the future. The ability of CrpeNPV to infect Cp14R cell lines also allows for the opportunity to further genetically characterise and manipulate the virus to achieve a better understanding of the virus, improve its virulence and prevent hosts from developing resistance.

Chapter 7

A PRELMINARY INVESTIGATION OF THE POTENTIAL PRODUCTION OF CrpeNPV IN A HETEROLOGOUS HOST, *THAUMATOTIBIA LEUCOTRETA*

7.1 INTRODUCTION

The previous chapters dealt with the biological activity of CrpeNPV against three closely related host species, *Cryptophlebia peltastica, Thaumatotibia leucotreta* and *Cydia pomonella*. From the results it was shown that CrpeNPV was more virulent against the heterologous hosts (*T. leucotreta* and *C. pomonella*) than against its homologous host (*C. peltastica*) (Chapter 2). *Thaumatotibia leucotreta* would be an ideal candidate as a heterologous host for the production of CrpeNPV due to the established culture of *T. leucotreta* (River Bioscience (Pty) Ltd), the similar biology and close relationship with *C. peltastica* (Timm *et al.* 2007). Literature on the use of a heterologous host for baculovirus mass production is limited, however, a study by Chambers (2014) has covered various aspects. The ideal characteristics for a host for production of a heterologous virus are a close genetic relationship with the original host, the ability to produce a virus of good quality, increased volume of virus produced, efficient and cost effective to rear (Chambers 2014). *Thaumatotibia leucotreta* is potentially a suitable heterologous host for CrpeNPV as it conforms to the above criteria. Not only is the biology of *T. leucotreta* and *C. peltastica* similar (chapter 2), but as shown in the study by Chambers (2014), *T. leucotreta* is easy to rear and has a short developmental time. Thus, more larvae can be reared per unit diet, which allows for increased production of virus. A successful mass rearing facility for *T. leucotreta* has already been established at River Bioscience® (Pty) Ltd.

One important characteristic of a host for production of a heterologous virus, that is yet to be demonstrated, is the ability to produce the virus. Previously, bioassays were conducted against neonate larvae. However, in order to produce virus in a heterologous host, infection with later instars, preferably 5th instars, is required (Ravensberg 2011). Producing virus in these later instars could prove to be challenging and possibly not economically feasible, as stated by Payne (1986) "it is now well established that larval susceptibility to many baculoviruses decreases sharply as larvae age." Therefore it is important to conduct bioassays against later instars of both the homologous and heterologous host to determine and compare the virulence and dosage response. This will allow for a conclusion to be made on whether the heterologous host can economically feasible produce CrpeNPV.

Thus, the aim of this study was to determine whether CrpeNPV can be successfully produced in a heterologous host, *T. leucotreta.* Ten-fold dosage response bioassays were conducted against 5th instar *C. peltastica* and *T. leucotreta* larvae in order to compare virus production.

7.2 MATERIALS AND METHODS

7.2.1 Surface dosage-response bioassays with Cryptophlebia peltastica *fifth instar larvae*

Surface dosage bioassays were conducted in glass vials. Each glass vial was filled with 4 g of FCM artificial diet (Moore *et al.* 2014) and 3.5 ml of distilled water and autoclaved at 121°C for 13 minutes. Eight 10-fold dilutions (10^2 to 10^9 OBs/ml) of purified OBs of CrpeNPV (described in section 3.2.3) in sterilised distilled water were used for dosages. Sterile distilled water was used as a control (Figure 7.1). Thirty larvae were treated per dosage and assays were replicated three times. A volume of 150 µl of each viral dilution and a distilled water control were pipetted onto the centre of the diet surface. The fluid was spread evenly over the diet surface by rotating the vial at an angle. Inoculated glass vials were left for ± 30 mins, until the diet had dried. One 5th instar larva was placed into each vial. Vials were plugged with cotton wool and kept in a CE room at 27°C and a relative humidity of 60 – 80%.

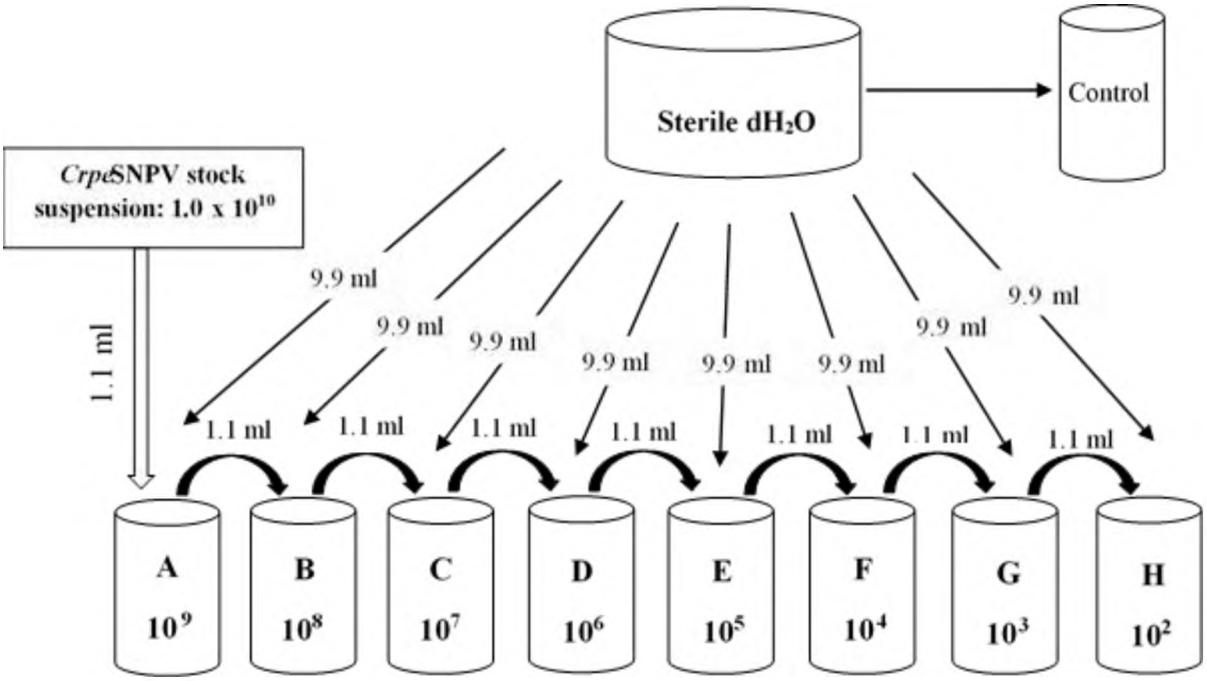


Figure 7.1: Ten-fold dilution series of CrpeNPV for surface dosage-response bioassays with neonate larvae of *Cryptophlebia peltastica* and *Thaumatotibia leucotreta*.

Vials were inspected for dead larvae or pupae every second day. Ten dead larvae were removed from each dosage and placed into individual vials and weighed. These larvae will then be used to determine the yield of virus produced per larva. The dosage-response curve was calculated using ToxRat® Solutions GmbH 2001-2015, standard version 3.2.1 software. Mortality of control larvae was taken into consideration by ToxRat® and corrected using Abbott's formula. From the analysis the average LC_{50} and LC_{90} for all three replicates were calculated.

7.2.2 Surface dosage-response bioassays with Thaumatotibia leucotreta *fifth instar larvae*

Surface dosage bioassays were conducted in glass vials. Each glass vial was filled with 4 g of FCM artificial diet (Moore *et al.* 2014) and 3.5 ml of distilled water and autoclaved at 121°C for 13 minutes. Eight 10-fold dilutions (10^2 to 10^9 OBs/ml) of purified OBs of CrpeNPV (described in section 3.2.3) in sterilised distilled water were used for dosages. Sterile distilled water was used as a control (Figure 7.1). Thirty larvae were treated per dosage and assays were

replicated three times. A volume of 150 µl of each viral dilution and control was pipetted onto the centre of the diet surface. The fluid was spread evenly over the diet surface by rotating the vial at an angle. Inoculated glass vials were left for ± 30 mins, until the diet had dried. One 5th instar larva was placed into each vial. Vials were plugged with cotton wool and kept in a CE room at 27°C.

Vials were inspected for dead larvae or pupae every second day. Ten dead larvae were removed from each dosage and placed into individual vials and weighed. The dosage-response curve was calculated using ToxRat® Solutions GmbH 2001-2015, standard version 3.2.1 software. Mortality of control larvae were taken into consideration by ToxRat® and corrected using Abbott's formula. From the analysis the average LC_{50} and LC_{90} for all three replicates were calculated.

7.2.3 Purification and Enumeration of OBs

Occlusion bodies were purified and enumerated from five of the ten larvae infected 5th instar larvae collected and weighed from section 7.2.1 and 7.2.1 for all dosage treatments and for each replicate using the same methodology as in chapter 3, section 3.2.2 and 3.2.7.

7.3 RESULTS

7.3.1 Surface dosage-response bioassays with Cryptophlebia peltastica *fifth instar larvae*

The regression line fitted to the data was y = 0.36510 (variance of slope = 0.00080) x – 1.29053 (Figure 7.2). The regression lines were compared for all three replicates and were found to have a chi-squared value of 17.43266 with six degrees of freedom and p-value < 0.001. Slopes were significantly different. The results obtained for the three replicates were used to calculate the mean LC_{50} and LC_{90} values (Table 7.1).

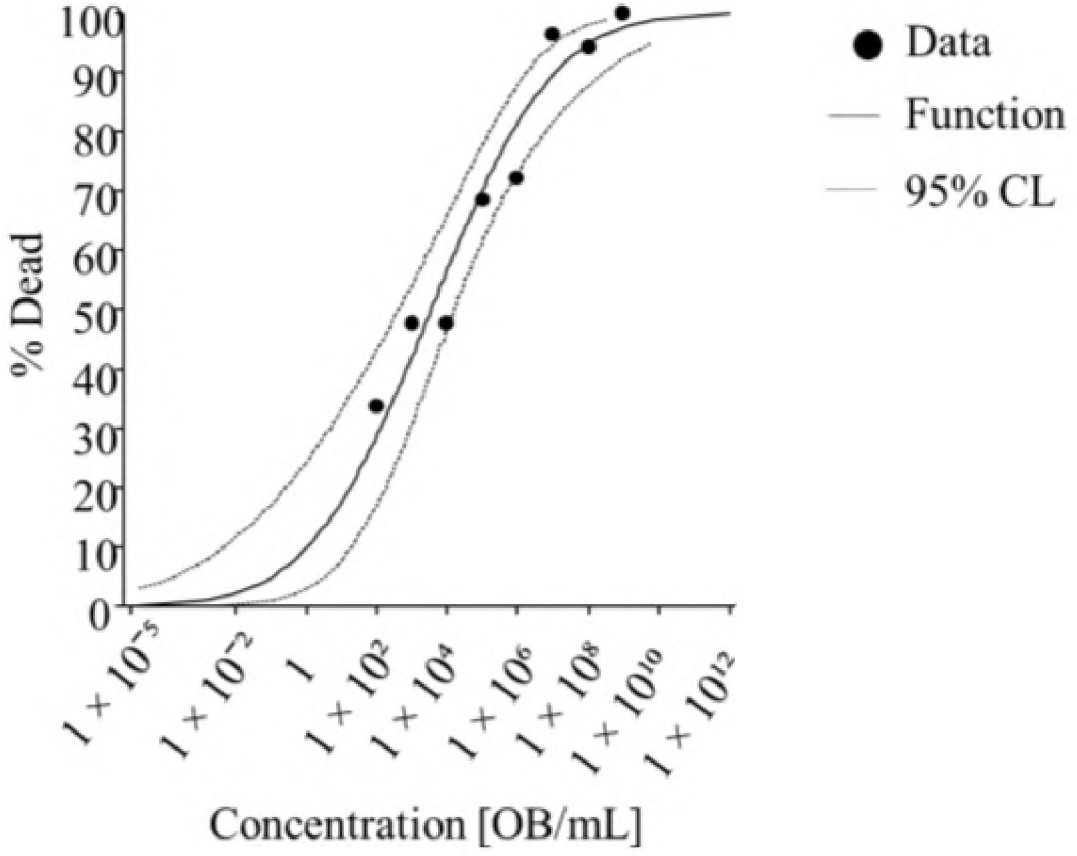


Figure 7.2: Dosage response probit analysis for CrpeNPV against fifth instar larvae of *Cryptophlebia peltastica.*

7.3.2 Surface dosage-response bioassays with Thaumatotibia leucotreta *fifth instar larvae*

The regression line fitted to the data was y = 0.36172 (variance of slope = 0.00081) x – 1.23079 (Figure 7.3). The regression lines were compared for all three replicates and were found to have a chi-squared value of 17.43866 with six degrees of freedom and p-value < 0.001. Slopes were significantly different. The results obtained for the three replicates were used to calculate the mean LC_{50} and LC_{90} values (Table 7.1).

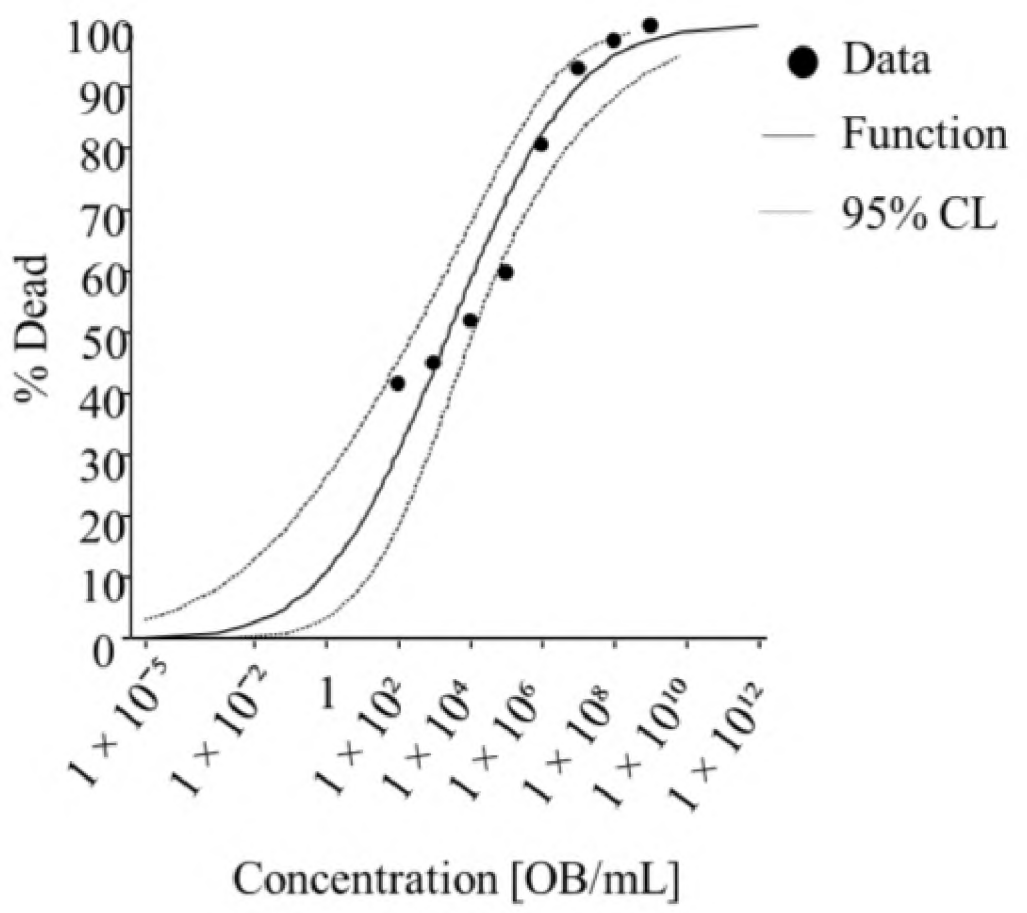


Figure 7.3: Dosage response probit analysis for CrpeNPV against fifth instar larvae of *Thaumatotibia leucotreta.*

Table 7.1: Comparison of the LC_{50} and LC_{90} values for fifth instar larvae of *Cryptophlebia peltastica* and *Thaumatotibia leucotreta*.

		Dosage (OBs/ml)	Lower 95%-CI	Upper 95%-CI
C. peltastica	LC_{50}	3.43×10^3	4.14×10^2	1.55×10^4
	LC_{90}	1.11×10^7	1.70×10^6	2.60×10^8
T. leucotreta	LC_{50}	2.53×10^3	2.73×10^2	1.18×10^4
	LC_{90}	8.82×10^6	1.35×10^6	2.11×10^8

7.3.3 Purification and Enumeration of OBs

The average yield of CrpeNPV produced per *C. peltastica* larva was determined for all eight dosages for each replicate (Table 7.2). The number of OBs per larvae ranged from 2.69×10^{10} to 6.43×10^{10} OBs/larva for replicate one, 2.52×10^{10} to 5.19×10^{10} OBs/larva and 3.05×10^{10} to 5.77×10^{10} OBs/larva for replicate two and three respectively. The weight of the infected larvae ranged from 0.04974 to 0.10972 g per larva for all replicates. The larva with the lowest weight of 0.04974 g produced 2.69×10^{10} OBs and the larva with the highest weight of 0.10972 g had a virus yield of 4.78×10^{10} OBs.

Table 7.2: The average yield of virus produced per fifth instar larva of *Cryptophlebia peltastica* at each concentration for all three replicates.

Dosage	Replicate 1			Replicate 2			Replicate 3		
	Mean weight of larva (g)	Mean OBs/larva	SE	Mean weight of larva (g)	Mean OBs/larva	SE	Mean weight of larva (g)	Mean OBs/larva	SE
10^2	0.04974	2.69×10^{10}	7.06×10^{9}	0.08886	4.19×10^{10}	1.35×10^{9}	0.09920	4.72×10^{10}	8.89×10^{9}
10^3	0.10864	5.15×10^{10}	1.72×10^{10}	0.09316	5.19×10^{10}	5.35×10^{9}	0.09160	5.77×10^{10}	1.10×10^{10}
10^4	0.09724	6.11×10^{10}	2.63×10^{10}	0.09672	4.39×10^{10}	6.37×10^{9}	0.09582	5.03×10^{10}	3.64×10^{9}
10^5	0.10972	4.78×10^{10}	1.18×10^{10}	0.09032	3.91×10^{10}	2.67×10^{9}	0.09058	4.44×10^{10}	6.13×10^{9}
10^6	0.09212	5.05×10^{10}	1.31×10^{10}	0.09678	2.52×10^{10}	6.03×10^{9}	0.09114	3.19×10^{10}	2.95×10^{9}
10^7	0.09676	6.43×10^{10}	1.78×10^{10}	0.09046	3.28×10^{10}	4.81×10^{9}	0.09024	4.73×10^{10}	1.03×10^{10}
10^8	0.07896	4.87×10^{10}	1.08×10^{10}	0.08646	2.87×10^{10}	5.29×10^{9}	0.07458	3.05×10^{10}	2.90×10^{9}
10^9	0.06082	3.43×10^{10}	2.76×10^{9}	0.07220	3.34×10^{10}	5.53×10^{9}	0.08198	3.28×10^{10}	1.39×10^{9}

The average yield of CrpeNPV produced per larva of *T. leucotreta* was determined for all eight dosages for each replicate (Table 7.3). The number of OBs per larvae ranged from 1.49×10^{10} to 3.78×10^{10} OBs/larva for replicate one, 1.71×10^{10} to 3.20×10^{10} OBs/larva and 1.53×10^{10} to 3.07×10^{10} OBs/larva for replicate two and three respectively. The weight of the larvae ranged from 0.01806 to 0.08540 g per larva for all replicates. The larva with the lowest weight of 0.01806 g produced 1.95×10^{10} OBs and the larva with the highest weight of 0.08540 g had a virus yield of 3.07×10^{10} OBs.

Table 7.3: The average yield of virus produce per fifth instar larva of *Thaumatotibia leucotreta* at each concentration for all three replicates.

Dosage	Replicate 1			Replicate 2			Replicate 3		
	Mean weight of larva (g)	Mean OBs/larva	SE	Mean weight of larva (g)	Mean OBs/larva	SE	Mean weight of larva (g)	Mean OBs/larva	SE
10^2	0.03084	3.78×10^{10}	1.85×10^{9}	0.01806	1.95×10^{10}	1.85×10^{9}	0.08540	3.07×10^{10}	4.50×10^{9}
10^3	0.02348	2.36×10^{10}	9.81×10^{9}	0.02874	2.41×10^{10}	9.81×10^{9}	0.03334	2.68×10^{10}	2.34×10^{9}
10^4	0.02846	2.98×10^{10}	7.41×10^{9}	0.03108	2.73×10^{10}	7.41×10^{9}	0.03340	2.37×10^{10}	1.36×10^{9}
10^5	0.03254	2.56×10^{10}	1.52×10^{10}	0.02806	2.28×10^{10}	1.52×10^{10}	0.03000	2.46×10^{10}	2.59×10^{9}
10^6	0.03544	2.71×10^{10}	1.00×10^{10}	0.02790	2.75×10^{10}	1.00×10^{10}	0.03676	1.53×10^{10}	3.78×10^{9}
10^7	0.03542	2.00×10^{10}	1.30×10^{9}	0.03174	1.71×10^{10}	1.30×10^{9}	0.03688	1.77×10^{10}	3.52×10^{9}
10^8	0.02520	1.49×10^{10}	5.19×10^{9}	0.03746	3.20×10^{10}	5.19×10^{9}	0.03198	1.92×10^{10}	4.50×10^{9}
10^9	0.02728	1.98×10^{10}	3.11×10^{9}	0.02770	2.22×10^{10}	3.11×10^{9}	0.03674	2.12×10^{10}	7.11×10^{9}

The average yield of CrpeNPV produced per larva was compared between the homologous host, *C. peltastica* and the heterologous host, *T. leucotreta* (Table 7.4). The highest average yield per larva of virus produced by *C. peltastica* was 5.37×10^{10} OBs/ml with a larval weight of 0.0978 g at the concentration of 1×10^3 OBs/ml. The highest average yield per larva of virus produced by *T. leucotreta* was 2.93×10^{10} OBs/larva with the average weight of the larva being 0.0448 g at a dosage of 1×10^2 OBs/ml. From the results it was observed that *C. peltastica* produced a higher yield of virus per larva compared to *T. leucotreta* (Table 7.4).

Table 7.4: Comparison of the mean yield of virus produced per fifth instar larva of *Cryptophlebia peltastica* and *Thaumatotibia leucotreta* at each concentration.

Dosage	*Cryptophlebia peltastica*				*Thaumatotibia leucotreta*			
	Mean Mortality	Mean Weight (g)	Mean OBs/ml	SE	Mean Mortality	Mean Weight (g)	Mean OBs/ml	SE
10^2	36.67	0.0793	3.87×10^{10}	1.05×10^{10}	43.44	0.0448	2.93×10^{10}	9.23×10^{9}
10^3	50.00	0.0978	5.37×10^{10}	3.47×10^{9}	46.56	0.0285	2.48×10^{10}	1.72×10^{9}
10^4	50.00	0.0966	5.18×10^{10}	8.69×10^{9}	53.33	0.0310	2.69×10^{10}	3.07×10^{9}
10^5	70.00	0.0969	4.38×10^{10}	4.38×10^{9}	61.22	0.0302	2.43×10^{10}	1.42×10^{9}
10^6	73.67	0.0933	3.59×10^{10}	1.31×10^{10}	81.22	0.0334	2.33×10^{10}	6.93×10^{9}
10^7	96.67	0.0925	4.81×10^{10}	1.58×10^{10}	93.44	0.0347	1.83×10^{10}	1.53×10^{9}
10^8	94.33	0.0800	3.60×10^{10}	1.11×10^{10}	97.89	0.0315	2.20×10^{10}	8.90×10^{9}
10^9	100.00	0.0717	3.35×10^{10}	7.55×10^{8}	100.00	0.0306	2.11×10^{10}	1.21×10^{9}

The production of CrpeNPV was compared for both 5th instar larvae of *C. peltastica* and *T. leucotreta* using the productivity ratio and production efficiency equations (Moore 2002; Chambers 2014). The productivity ratio (PR) was calculated as:

PR = yield/larva (OBs) ÷ No. of infecting OBs (OBs/mm^2 for surface inoculation)).

The production efficiency (PE) was calculated as:

PE = OBs per larva ÷ weight of larva (mg).

The mean productivity ratio for *C. peltastica* was 2.12×10^{10} and 1.53×10^{10} for *T. leucotreta*. The mean production efficiency for *C. peltastica* was 4.81×10^{8}, which was lower than that of *T. leucotreta,* which had a production efficiency of 7.27×10^{8}.

Table 7.5: Comparison of the production ratio and efficiency between fifth instar larva of *Cryptophlebia peltastica* and *Thaumatotibia leucotreta* at each concentration.

Dosage	*C. peltastica*		*T. leucotreta*	
	Production Ratio	**Production efficiency**	**Production Ratio**	**Production efficiency**
10^2	1.47×10^{11}	4.88×10^8	1.11×10^{11}	6.55×10^8
10^3	2.04×10^{10}	5.49×10^8	9.44×10^9	8.71×10^8
10^4	1.97×10^9	5.36×10^8	1.02×10^9	8.69×10^8
10^5	1.66×10^8	4.52×10^8	9.25×10^7	8.06×10^8
10^6	1.36×10^7	3.84×10^8	8.85×10^6	6.98×10^8
10^7	1.83×10^6	5.20×10^8	6.94×10^5	5.27×10^8
10^8	1.37×10^5	4.50×10^8	8.37×10^4	6.98×10^8
10^9	1.27×10^4	4.67×10^8	8.01×10^3	6.89×10^8
Mean	2.12×10^{10}	4.81×10^8	1.53×10^{10}	7.27×10^8

7.4 DISCUSSION

Dosage response bioassays were conducted against fifth instar larvae of *C. peltastica* and *T. leucotreta* to determine which species produced a higher yield of CrpeNPV. Bioassay results were also used to determine whether or not *T. leucotreta* could be used as a heterologous host for the production of CrpeNPV. The first objective in order to determine the above was to obtain the LC_{50} and LC_{90} values for each of the species. From the results of *C. peltastica* the LC_{50} was found to be 3.43×10^3 OBs/ml and the LC_{90} was 1.11×10^7 OBs/ml. This was compared to *T. leucotreta* where the LC_{50} was 2.53×10^3 OBs/ml and the LC_{90} was 8.82×10^6 OBs/ml. Once again, as mentioned in chapter 5, CrpeNPV was more virulent against *T. leucotreta* than *C. peltastica*. This is unusual as baculoviruses are generally more virulent against their homologous host, with decreased virulence against the heterologous host (Payne 1986). This was observed with bioassays of AgNPV against its homologous host, *Anticarsia gemmatalis* and the heterologous hosts, *Bombyx mori*, *Chlosyne lacinia saundersii*, *Spodoptera*

latifascia and *Trichoplusia ni*, where high virulence was recorded against the homologous host and decreased susceptibility with the other species (Moscardi 1989).

Not only was CrpeSNPV more virulent against *T. leucotreta* than *C. peltatstica*, but it was also more virulent against *T. leucotreta* than its homologous virus, CrleGV. The LC_{50} of CrleGV against fifth instar larvae of *T. leucotreta* was 2.69×10^7 OBs/ml and a LC_{90} of 9.12×10^9 OBs/ml (Moore 2002). As previously explained, this is because NPVs contain more than one virion per OB compared to a GV with a single virion per OB (Payne 1986; Abdul Kadir *et al.* 1999; Lacey *et al.* 2002). The virulence of CrpeNPV against *C. peltastica* and *T. leucotreta* is highly virulent, as a study by Escribano *et al.* (1999) found the LC_{50} of SfNPV against *S. frugiperda* to be 1.84×10^8 OBs/ml. This LC_{50} value of *S. frugiferda* lies within the LC_{90} of CrpeNPV against the two hosts. Therefore, CrpeNPV is highly virulent against the two host species.

The next objective was to determine the yield of virus produced by *C. peltastica* and *T. leucotreta*. From the results, the highest yield of virus produced by *C. peltastica* was 5.37×10^{10} OBs/larva and 2.93×10^{10} OBs/larva for *T. leucotreta*. From this analysis it shows that *C. peltastica* produces a higher yield of virus as compared to *T. leucotreta*. When comparing the production efficiency of CrpeNPV in *C. peltastica* and *T. leucotreta* to other NPVs, the production efficiency is of CrpeNPV is lower. The general range of NPV production was found to be 1×10^9 to 5×10^9 OBs per larva (Grzywacz & Moore 2017). An example of the NPV production efficiency is SeNPV against *Spodoptera exigua* which produced 2×10^9 OBs per larva. Though, when comparing the productivity ratio of CrpeNPV to other NPV, it is much higher. The productivity ratio of CrpeNPV against *C. peltastica* was 2.12×10^{10} and *T. leucotreta* was 1.53×10^{10}. Whereas, the highest productivity ratio for an NPV was 1.2×10^6 for SeNPV against *S. exigua* (Grzywacz & Moore 2017).

In conclusion, the lethal concentrations of CrpeNPV against fifth instar *C. peltastica* and *T. leucotreta* larvae was determined and it was found that CrpeNPV was more virulent against *T. leucotreta* than *C. peltastica*. The viral yields were also compared between each species and taking various factors into consideration, the virus yields for each species can be considered to be similar.

The results from this chapter are only a preliminary study into determining the yield of CrpeNPV produced by both host species and the potential production of CrpeNPV in a heterologous host. Further research is required to focus on whether the CrpeNPV sample

produced from *T. leucotreta* is contaminated with CrleGV, which can be determined using restriction analyses or multiplex PCR to determine the presence of CrpeNPV and CrleGV. The CrpeNPV sample produced from *T. leucotreta* also needs to be tested to determine if the virus is still highly virulent against all three tested hosts. This can be accomplished by conducting bioassays against all three host. Lastly, it needs to be determined if the 5th instars are the most suited for the production of CrpeNPV at a commercial scale.

Chapter 8

GENERAL DISCUSSION

8.1 THESIS OVERVIEW

This study investigated the isolation and characterisation of a baculovirus that has potential to be commercially developed into a microbial agent for the control of *Cryptophlebia peltastica*. In order to identify a baculovirus, a laboratory culture of *C. peltastica* was required (Chapter 2). Once a laboratory culture was successfully established, larvae showing symptoms of a baculovirus infection were collected and screened by TEM (Chapter 3). The isolation and characterisation of the baculovirus involved genetic analyses such as PCR, whole genome sequencing and REN analysis (Chapter 3 & 4). The biological activity of the isolated virus was then determined against *C. peltastica*, as well as *Thaumatotibia leucotreta* and *Cydia pomonella* (Chapter 5 & 6). Lastly, surface dosage bioassays against 5th instar larvae of *C. peltastica* and *T. leucotreta* were completed to compare the virus yield in both species and to determine whether the virus could be produced *in vivo* in the homologous and heterologous hosts. The study also made use of the Cp14R cell lines to investigate whether CrpeNPV could potentially be mass produced *in vitro*, also allowing for the further characterisation of the virus and genetic manipulation in the future (Chapter 6 & 7).

8.2 ISOLATION OF AN EFFECTIVE VIRUS

The use of microbial agents against various pests worldwide is gaining acceptance as an effective mode of controlling insect pests. This is due to the sustainable and ecological suitability of microbial control agents. However, while the hope is that microbial control agents and other biological and biorational products will one day replace chemical insecticides, they still have a long way to go, as between 2004 and 2007 the microbial control market increased by 47%, but this only comprised of 1 to 2% of the pesticide market (Ravensberg 2011; Lapointe

et al. 2012; Lacey *et al.* 2015). Many microbial control agents do not make it onto the market, due to various issues such as inconsistent quality, poor efficacy, cost of production, competition with chemical insecticides, host specificity and reduced persistence as compared to chemical pesticides, registration of the product and the time taken to get the product to the market (Ravensberg 2011; Lacey 2017). However, with the systematic model that has been proposed by Ravensberg (2011) and a project team that contains biological, technical and marketing skills, the possibility of developing and commercialising a microbial control agent is promising (Ravensberg 2011). For this study, collaboration between Rhodes University, River Bioscience (Pty) Ltd and Citrus Research International has enabled use of these skills, ensuring the development and future commercialisation of CrpeNPV as a microbial control agent.

The host range of microbial control agents (particularly baculoviruses) is often considered unfavourable by the market, as they are generally highly host specific, often only infecting a single species. This results in a small market for the product and unless it is targeting a major pest, it may not be economically viable to develop and produce (Ravensberg 2011). The biological activity of CrpeNPV was tested against three closely related species, with overlapping host ranges (Timm *et al.* 2007). This was done as NPVs generally have a wider host range than GVs and sometimes even have the ability to infect several species in the same genus or family (Ishii *et al.* 2003; Szewczyk *et al.* 2006; Rao *et al.* 2015). The ability of CrpeNPV to infect more than one species would ultimately mean that there would be a larger market, therefore it will be more profitable to develop.

Another important aspect in developing a baculovirus is the question of whether the product will be able to reduce a high percentage of the pest population sufficiently rapidly (referred to as knock down or kill rate). In order to determine whether this was possible for CrpeNPV, surface dosage bioassays were conducted against *C. peltastica*, *T. leucotreta* and *C. pomonella*. The bioassays were used to calculate the lethal concentration and lethal time for each species using the surface dosage technique. If the lethal concentration is too high it will not be economically viable to produce and commercialise the virus. A long lethal time means that the moth remains alive for longer, resulting in the pest causing more damage to the crops (Payne 1986). Therefore, low lethal concentrations and times are ideal for a microbial control agent. From this study it was shown that CrpeNPV had low lethal concentrations indicating that the virus is highly virulent and has a fast speed of kill against all three species tested. Not only was CrpeNPV highly virulent against all three species, but it was also shown that CrpeNPV was more virulent against *T. leucotreta* and *C. pomonella*, than the pest's homologous viruses,

CrleGV and CpGV respectively. A reason for the higher virulence of the NPV compared to the GVs is that NPVs comprise of more than one virion per OB, whereas GVs have only one virion per OB. Therefore, NPVs have a greater number of infecting propagules at the start of infection as compared to GVs, resulting in the higher virulence and fast speed of kill of NPVs (Payne 1986; Adul Kadir *et al.* 1999; Lacey *et al.* 2002). Both CrleGV and CpGV are successful commercialised biopesticides, CrleGV had an LC_{50} of 4.095×10^3 OBs/ml against *T. leucotreta* and an LT_{50} of 118 hours (Moore *et al.* 2011). CpGV had an LC_{50} of 1.632×10^3 OBs/ml against *C. pomonella* and an LT_{50} of 135 hours (Motsoeneng 2016). The LC_{50} of CrpeNPV ranged from 1.43 to 8.19×10^3 OBs/ml for each of the three species and the LT_{50} ranged from 73 to 125 hours. Thus, with the similar LC_{50} and LT_{50} values between CrpeNPV, CrleGV and CpGV, the development of CrpeNPV into a commercially available microbial control agent is a possibility. The low LC and LT values are favourable for the development of the virus, as the grower requires a control agent that significantly controls the majority of the pest population within a short period of time (Ravensberg 2011). Further support for the development of CrpeNPV into a microbial control agent, is the commercialisation of a successful baculovirus biopesticide, HearNPV against *Helicoverpa armigera*, which was found to have a higher LC_{50} and LT_{50} than CrpeNPV. The LC_{50} of 3.5×10^4 OBs/ml and an LT_{50} of 168 hours was obtained for HearNPV against *H. armigera* (Whitlock 1974; Whitlock 1978; Moore *et al.* 2010). The HearNPV has been successfully developed into various commercially available biopesticides such as Helicovir™ (River Bioscience) and Bolldex® (Andermatt-Biocontrol AG, Switzerland) and has been greatly successful against first instar larvae of the pest and is IPM-compatible in the citrus industry (Knox *et al.* 2015).

With the potential to control these species and the promising results obtained, CrpeNPV was also tested against the two cultures of CpGV resistant European *C. pomonella* cultures, CpRR1: type 1 resistance which is sex-linked found on the Z-chromosome and CpR5M: type 2 resistance which is dominant autosomal inheritance (Asser-Kaiser *et al.* 2007; Jehle *et al.* 2016). This was necessary as *C. pomonella* is a major pests of pome fruits and has developed resistance to various isolates of CpGV in Europe (Asser-Kaiser *et al.* 2007; Lacey *et al.* 2008; Jehle 2008). Various attempts have been made to find alternative options for the control of resistant *C. pomonella* in Europe. However, these still do not provide adequate control against resistant *C. pomonella* in the field. From the results obtained, CrpeNPV has the ability to overcome resistance in *C. pomonella* under laboratory conditions and thus has the potential to be incorporated into the resistance management programme in Europe. This will have a

positive affect for the control of *C. pomonella*, as well as *T. leucotreta* if in the future the pest developed resistance to CrleGV. Thus, the novel NPV has the potential to be developed into a new microbial control agent against *C. peltastica, T. leucotreta* and *C. pomonella*.

Another issue with the development and commercialisation of baculovirus biopesticides, is the application against endophagous insects. The application of biopesticides is focused on the first instar larvae due to baculoviruses been highly infective against the early instars as compared to the late instars and to reduce the amount of damaged caused, the probability of the larvae coming into contact and ingesting the virus before burrowing into the crop and late instars have a lower chance of coming into contact with the virus as they are inside the fruit (Moscardi 1999; Grzywacz 2017). For this reason, there is a short window of opportunity for the larvae to ingest the virus. Thus, the application of the biopesticide needs to focus on direct and full cover application of the virus at the correct lethal dosage, ensuring that the larvae ingest the virus before burrowing into the fruit (Moscardi 1999). This has been possible for various commercially available biopesticides used against endophagous insects. For example the application of CrleGV against *T. leucotreta* on citrus, avocadoes and grapes and CpGV against *C. pomonella* on apples and pears (Moscardi 1999; Moore *et al.* 2014). Thus, with the correct dosage and application of CrpeNPV and monitoring of the adult population, it will ensure the success of the biopesticide.

8.3 PRODUCTION OF CrpeNPV

Further development of a microbial agent requires a suitable mass production technique that is economically viable, generates a high yield of infective OBs and preserves the virulence and stability of the agent (Ravensberg 2011). There are two methods that can be used for the mass production of baculoviruses, *in vivo* and *in vitro*. *In vivo* production involves the mass production in the homologous host, or if possible in a heterologous host. The *in vivo* method is currently the only technique used for the production of baculovirus biopesticides. However, there are a few disadvantages of using the *in vivo* technique including contamination of the product with microorganisms, specifically bacteria, degeneration of the virus, rearing is labour intensive and time consuming (Ravensberg 2011; Knox *et al.* 2015; Grzywacz & Moore 2017). Nonetheless, *in vivo* mass production is still advantageous as it allows for an easy start-up and the interaction between virus and host, an interaction which will hopefully promote the evolution of the virus with the host (Ravensberg 2011).

Initial investigations into the use of *T. leucotreta* as a heterologous host were conducted in this study. The use of a heterologous host is advantageous when it is more cost effective to rear than the homologous host and when there is an increase in production of the control agent relative to the homologous host (Beas-Catena *et al.* 2014). The mass rearing of *T. leucotreta* is already successfully established at River Bioscience (Pty) Ltd and this species is more cost effective to rear than *C. peltastica*, as discussed in Chapter 2. Therefore *T. leucotreta* would be an ideal heterologous host for the production of CrpeNPV. However, it was important to further justify the use of *T. leucotreta* as a heterologous host by determining the virus yield in 5th instar larvae and comparing it to *C. peltastica*. *Thaumatotibia leucotreta* can be considered for use as a heterologous host for the production of CrpeNPV as the yield of virus, when taking the weight of the larvae into account, was similar for both the homologous and heterologous host. A study completed by Chambers (2014) focused on mass producing CpGV in *T. leucotreta*, this study showed that *T. leucotreta* is more cost effective and efficient to rear than *C. pomonella*. The study concluded that *T. leucotreta* can be used as a heterologous host for the production of CpGV, due to the similar yield of virus produced in both late instar larvae (Chambers 2014). However, the study also identified various concerns with the quality of the produced virus and therefore stated that a quality control protocol needs to be established. These concerns include: reduced virus yields, contaminated virus samples with the heterologous host's virus and negative effects on the biological activity of CrpeNPV (Chambers 2014). For this study the yield of virus produced in the heterologous host is not an issue, the virus yield in both the homologous and heterologous host was similar. However, an important concern is the contamination of the harvested CrpeNPV with the heterologous host's virus, CrleGV. Future work will need to determine if the sample of CrpeNPV is contaminated with CrleGV. Contamination of the samples may be reduced by incorporating quality control protocols, as well as using restriction analyses or multiplex PCR's to determine if CrleGV is present in the sample. Quality control protocols need to focus on reducing the risk of CrleGV contamination by using virus free laboratory cultures of *T. leucotreta* and ensuring optimal environmental conditions; ideal temperatures, adequate food supply and no overcrowding of larvae in order to prevent the larvae from becoming stressed and expressing CrleGV. Future research is required to ensure the virus produced from *T. leucotreta* is not contaminated with CrleGV and to test the biological activity of the produced virus against all three test species to ensure that the high virulence of this virus is maintained.

In vitro technique involves the production of baculoviruses in an established cell line. Production in cell lines can be an advantage when it comes to the need to manipulate the genetic makeup of the virus in order to make it more favourable, for example increasing the speed of kill of the virus which is one of the main disadvantages of a baculovirus biopesticide and to study any occurrence of resistance in the host (Smagghe *et al.* 2009; Ravensberg 2011). Baculovirus genomes can be altered through genetic modification to improve insecticidal activity, such as the speed of kill or to alter the feeding activity of the larvae. This can be done by creating a recombinant virus that has genes inserted or deleted in the genome of the specific virus. However, genetically modified organisms (GMOs) are not accepted by the public and prevent the commercialisation of these products. No genetically modified baculoviruses have been commercially developed (Claus *et al.* 2012; Knox *et al.* 2015), thus a baculovirus should preferably not be modified and should have an acceptable natural speed of kill, which is the case for CrpeNPV, nonetheless future field trials are required to confirm whether this is the case.

The *in vitro* method of mass production is not used as it can be costly to grow and maintain the cell cultures. The cells are very sensitive, easily contaminated and are not susceptible to infection by all baculoviruses (Beas-Catena *et al.* 2014; Knox *et al.* 2015). The methods of *in vitro* production need to be improved on and with continued research, cell cultures could potentially be used for the mass production of viral biopesticides. The main concerns that need to be focused on in the future are to develop a cost effective medium to produce the cells in, the development of suitable bioreactors and the quality of the virus produced (Grzywacz & Moore 2017). The ability for CrpeNPV to infect the Cp14R cell lines allows for the genetic make-up of the virus to be further characterised and for *in vitro* production of the virus to possibly take place, if necessary. Thus, CrpeNPV can potentially be mass produced *in vivo* in both the homologous and heterologous host, as well as *in vitro* in the Cp14R cells.

8.4 FUTURE WORK

The virus has the potential to be mass produced in both *C. peltastica* and *T. leucotreta.* However, further research in using *T. leucotreta* as a heterologous host for mass production of the virus is required. This research should focus on determining the quality of virus produced from *T. leucotreta* and whether the virulence of CrpeNPV is the same standard. Another factor that needs to be considered is the purity of the virus sample obtained from *T. leucotreta*,

ensuring that CrpeNPV is not contaminated with CrleGV. In some cases, when the heterologous host is exposed to the different virus or a contaminated sample, the larva may become stressed and express the homologous virus, contaminating the virus being produced, and a mixed infection may occur (Chambers 2014). Thus, quality control protocols need to be put in place and samples need to be tested regularly to ensure the purity of the sample.

The next step in the development of CrpeNPV into a biopesticide will be to determine the optimal formulation of the virus. The formulation of a biopesticide is important in order to allow the virus to remain at optimal efficiency, increased shelf life and protection from various environmental factors such as UV. Formulation of the product is dependent on the active ingredient, in this case the virus, and on the mode of application. The mode of application relies on the most conventional equipment available to the grower, which in most cases will be by spray application. When considering the formulation of the biopesticide it is essential to consider several factors such as, the stabilization of the virus, prevention of contamination, economic viability, user friendliness, protection of the virus against environmental factors and the ability to persist on the crop ensuring enough time for the pest to come into contact with the virus (Ravensberg 2011; Grzywacz & Moore 2017). The stability of the product is important to guarantee that it has a long shelf life. To ensure the stability of the product, it is important to standardise the formulation protocol and select the optimal formulation that keeps the physical, chemical and biological stability of the product. For virus products, the shelf life is a minor problem, as virus biopesticides have the ability to be stored for several years at -20°C or a year at 4°C (Ravensberg 2011). Two important issues with viral biopesticides is contamination with micro-organisms, specifically bacteria and protecting the virus from harmful environmental factors. To ensure the product is not contaminated, it is imperative to perform quality tests and bioassays to determine if the potency of virus is still optimal (Ravensberg 2011). However, as the relationship between micro-organisms are commensal there will always be a certain level of contamination. This contamination is only acceptable at 1×10^8 CFU/ml for liquid formulations and 5×10^8 CFU/g for dry formulations (Grzywacz & Moore 2017). Environmental factors that need to be considered during formulation of a viral biopesticide are UV protection, feeding stimulants for the active ingredient and rain-fastness. The use of additives such as molasses and sorbitol have been shown to improve the infectivity, rain-fastness and persistence of the virus. Molasses has been shown to improve the efficacy of Cryptogran™ (CrleGV, River BioScience (Pty) Ltd, South Africa) and the rain-fastness by acting as a sticker and feeding attractant (Kirkman 2007). To protect the virus from UV

irradiation, additives such as optical brighteners can be added for protection (Ravensberg 2011). A study by Kirkman (2007) found that by adding lignin to Cryptogran as a UV protectant, breakdown of the virus was decreased and larval mortality and field persistence of Cryptogran were improved. Adjuvants may also be used either in the formulation or as a tank additive to improve the efficacy of the biopesticide. However, care should be taken when considering adjuvants as they may increase the cost of the product, not be compatible with the active ingredient and have non-target effects on beneficial insects. For example the use of oil in baculovirus biopesticides has been found to have a repellent effect against the pest (Ravensberg 2011).

Before CrpeNPV can be a commercially available biopesticide other aspects still need to be addressed, most notably field trials and registration of the product. The first aspect that needs to be determined is the persistence and efficacy of CrpeNPV in the field against all three species discussed in this study. One of the favourable characteristics of a baculovirus is that it persists in the field. When identifying a new baculovirus biopesticide, it is important for the virus to persist in the environment and somewhat establish within the pest population. The virus also needs to be able to persist outside its host and remain stable, deal with the various environmental factors such as ultra-violet light (sunlight), rain and changes in temperatures (Payne 1986). Not only is the persistence of the virus important but it also needs to provide effective control in the field by significantly reducing the pest population, preferably below the economic threshold. Lastly, the CrpeNPV product needs to be registered as a plant protection product before it can be placed on the market.

8.5 CONCLUSION

This study focused on isolating and characterising a baculovirus that could potentially be used as a microbial control agent against *C. peltastica*. From the results obtained from this study, a novel alphabaculovirus was isolated from the laboratory reared culture of *C. peltastica* at Rhodes University. The alphabaculovirus was characterised to confirm the novelty through whole genome sequencing. Bioassays were then conducted to determine the virulence of CrpeNPV and whether it could potentially be developed into a biopesticide. The bioassays showed great potential for the control of not only *C. peltastica*, but also *T. leucotreta* and *C. pomonella*. In addition to the opportunity for CrpeNPV to potentially control resistant *C. pomonella* in Europe. Even though additional research is needed before CrpeNPV can be

commercialised, it can one day be a very useful biopesticide that can complement IPM programmes for *C. peltastica*, *T. leucotreta* and *C. pomonella*, and with further research the virus could potentially control various other Tortricidae pests and possibly Lepidoptera occurring in a pest complex. Thus, the research completed in this study can be seen as the start of the development of a successful biopesticide.

Chapter 9

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