PRODUCTION OF *CYDIA POMONELLA* GRANULOVIRUS (CpGV) IN A HETERALOGOUS HOST, *THAUMATOTIBIA LEUCOTRETA* (MEYRICK) (FALSE CODLING MOTH).

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ABSTRACT

Cydia pomonella (Linnaeus) (Family: Tortricidae), the codling moth, is considered one of the most significant pests of apples and pears worldwide, causing up to 80% crop loss in orchards if no control measures are applied. *Cydia pomonella* is oligophagous feeding on a number of alternate hosts including quince, walnuts, apricots, peaches, plums and nectarines. Historically the control of this pest has been achieved with the use of various chemical control strategies which have maintained pest levels below the economic threshold at a relatively low cost to the grower. However, there are serious concerns surrounding the use of chemical insecticides including the development of resistance in insect populations, the banning of various insecticides, regulations for lowering of the maximum residue level and employee and consumer safety. For this reason, alternate measures of control are slowly being adopted by growers such as mating disruption, cultural methods and the use of baculovirus biopesticides as part of integrated pest management programmes. The reluctance of growers to accept baculovirus or other biological control products in the past has been due to questionable product quality and inconsistencies in their field performance. Moreover, the development and application of biological control products is more costly than the use of chemical alternatives. Baculoviruses are arthropod specific viruses that are highly virulent to a number of lepidopteran species. Due to the virulence and host specificity of baculoviruses, *Cydia pomonella* granulovirus has been extensively and successfully used as part of integrated pest management systems for the control of *C. pomonella* in Europe and around the world, including South Africa. Commercial formulations have been typically based on the Mexican strain of CpGV. However due to long-term multiple applications of CpGV and the reliance on CpGV in organic farming practices in Europe, resistance to the CpGV-M strain has developed in a number of field populations of *C. pomonella*.

This study aimed to identify and characterize novel isolates of CpGV in South Africa and compare their virulence with the commercial standard CpGV-M. Secondly, since *C. pomonella* is difficult to culture on a large scale, an alternate method of CpGV production

was investigated in order to determine if CpGV could be produced more efficiently and at a reduced cost without negatively impacting the quality of the product.

Several isolates of CpGV were recovered either from field collected larvae or from a laboratory-reared *C. pomonella* colony. Characterisation of DNA profiles using a variety of restriction enzymes revealed that only a single isolate, CpGV-SA, was genetically different from the Mexican strain of the virus used in the commercially available CpGV based products in South Africa. In dose-response bioassays using CpGV-SA, LC_{50} and LC_{90} values for neonate *C. pomonella* larvae were 3.18×10^3 OBs/ml and 7.33×10^4 respectively. A comparison of these values with those of CpGV-M indicated no significant difference in the virulence of the two isolates under laboratory conditions. This is a first report of a genetically distinct CpGV isolate in South Africa. The biological activity and novelty of CpGV-SA makes this isolate a potentially important tool for CpGV resistance management in South Africa.

In order to justify production of CpGV in an alternative host, studies on the comparative biological performance of *C. pomonella* and *T. leucotreta* based on oviposition, time to hatch, larval developmental times and rearing efficiency as well as production costs were performed. *Thaumatotibia leucotreta* was found to be more fecund and to have significantly shorter egg and larval developmental times. In addition, larval production per unit of artificial diet was significantly higher than for *C. pomonella*. This resulted in *T. leucotreta* being more cost effective to produce with implications for reduced insectary space, sanitation practices as well as the labour component of production. Virus yield data generated by inoculation both *C. pomonella* and *T. leucotreta* with nine concentrations of CpGV resulted in comparable virus yields, justifying the continuation of the research into production of CpGV in *T. leucotreta*.

It was important to determine the LC and LT values required for mass production of CpGV in late instar *T. leucotreta* larvae. Dose- and time-response bioassays with CpGV-M were conducted on artificial diet to determine these values. Fourth instar LC_{50} and LC_{90} values were 5.96×10^3 OBs/ml and 1.64×10^5 OBs/ml respectively. LT_{50} and LT_{90} values were

81.10 hours and 88.58 hours respectively. Fifth instar LC_{50} and LC_{90} values were 6.88 x 10^4 OBs/ml and 9.78 x 10^6 OBs/ml respectively. LT_{50} and LT_{90} values were 111.56 hours and 137.57 hours respectively. Virus produced in fourth instar *T. leucotreta* larvae was bioassayed against *C. pomonella* neonate larvae and compared to CpGV-M to establish if production in the heterologous host negatively affected the virulence of the isolate. No significant difference in virulence was observed between virus produced in *T. leucotreta* and that produced in *C. pomonella*. The data generated in the bioassays was used in CpGV mass production trials to evaluate production. All production methods tested produced acceptable virus yields. To examine the quality of the virus product, genomic DNA was extracted from larval cadavers and subjected to REN analysis with *Hin*dIII. The resulting DNA profiles indicated that the virus product was contaminated with the homologous virus, CrleGV.

Based on the above results, the use of *T. leucotreta* as an alternate host for the *in vivo* production of CpGV on a commercial basis is not at this stage viable and requires further investigation before this production methodology can be reliable used to produce CpGV. However, this study has shown that CpGV can be produced in a homologous host, *T. leucotreta* and significant strides have been made towards developing a set of quality control standards that are essential for further development of successful production methodology. Finally a novel isolate of CpGV has been identified with comparable virulence to the CpGV-M. This is an important finding as it has broad reaching implications for resistance management of CpGV products in South Africa.

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LIST OF ABBREVIATIONS

µl – micro liter

µm - micrometer

% - percent

ºC – degrees Celsius

AFLP – amplified fragment length polymorphism

Ave. – Average

Bt – Bacillus thuringiensis

BV – Baculovirus

bp – Base pairs

cm – Centimetre

CM – Codling moth

CpGV – *Cydia pomonella* granulovirus

CrleGV - *Cryptophlebia leucotreta* granulovirus

DAT – Days after treatment

DNA – deoxyribonucleic acid

ds – double standard

egt – Ecdysone glycosyltransferase

Ethbr – Ethidium bromide

FCM - False codling moth

g – grams

h – hour

ha - hectare

GV – Granulovirus

l – litre

LC_{50} – medial lethal concentration for 50% mortality

LC_{90} – medial lethal concentration for 90% mortality

LT_{50} – medial lethal exposure time for 50% mortality

LT_{90} – medial lethal exposure time for 90% mortality

M – molar

m – metre

min - minute

ml - millilitre

mm – millimetre

n – number of replicates

nm – nanometre

nt - nucleotide

NPV – Nucleopolyhedrovirus

OB – occlusion body

PCR – polymerase chain reaction

PhopGV – *Phthorimaea operculella* granulovirus

PE – Production efficiency

PR – Production ratio

qPCR – quantitative PCR

RE – restriction enzymes

REN – restriction endonuclease

rpm – revolutions per minute

SD – standard deviation

SE – standard error

SDS – Sodium Dodecyl Sulphate

SIT – Sterile insect technique

SNP

ss – single standard

TEM – transmission electron microscope

USA – United States of America

USD - United States dollar

UV – ultraviolet

ZAR – South African Rand

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1

GENERAL INTRODUCTION

1.1 INTRODUCTION

Cydia pomonella (Linnaeus) (Family: Tortricidae), the codling moth, is considered one of the most significant pests of apples and pears worldwide, including the Western Cape, South Africa (Nel, 1983; Pringle *et al*., 2003; Addison, 2005; Timm *et al*., 2006; Reyes *et al*., 2007). Alternate hosts of *C. pomonella* include quince, walnuts, apricots, peaches, plums, nectarines and other *Juglans* species (Annecke & Moran, 1982; Riedl, 1983; Barnes, 1991; Ciglar, 1998; Reyes *et al*., 2007). Although the moth is oligophagous, most of the host plants belong to the family Rosaceae (Wearing *et al*., 2001). The apple originated from Eurasia and as *C. pomonella* is closely associated with this fruit, it is believed to have originated from the same area (Annecke & Moran, 1982; Mills, 2005). *Cydia pomonella* was first recorded in South Africa in 1885 (Lounsbury, 1898), however the first records of fruit infestation were only reported in 1898 (Annecke & Moran, 1982). The current infestation potential of *C. pomonella* in South Africa is considered to be exceptionally high, as the population is active from August to April during the warmer months of the year. During this active period, three to four generations may occur whereas in cooler regions of the world only one or two generations occur per season (Geier, 1964; Blomefield & Giliomee, 2014). Total infestation rates can be as high as 80% in apple orchards if no control measures are implemented (Myburg, 1980; Pringle *et al*., 2003).

The focus of this study was firstly to isolate and test a South African strain of *Cydia pomonella* granulovirus (CpGV), characterize it genetically and compare it in terms of virulence against laboratory-reared *C. pomonella* larvae to the CpGV-M stain found in

both the commercially available CpGV products. Secondly, this study's main focus was to assess the potential of producing CpGV in an alternate host, *Thaumatotibia leucotreta* (Meyrick) (Family: Tortricidae), the false codling moth (previously known as *Cryptophlebia leucotreta*).

1.2 THE HOST: *CYDIA POMONELLA*

1.2.1 Taxonomy and distribution

Cydia pomonella has a long and complex taxonomic history. It was first described from Europe by Linnaeus as *Phalaena tinea pomonella*, but in the economic and taxonomic literature from 1830 to 1960, *C. pomonella* was referred to as *Carpocapsa pomonella* (L.). During this time period a few authors used *pomonella* in combination with the genus *Cydia*, however this combination did not receive wide usage. In 1959, Obraztosc considered *Cydia* and *Carpocapsa* to be synonyms of *Laspeyresia*. As a result, *C. pomonella* was then referred to as *Laspryresia pomonella.* It was later realized that the genus *Laspryresia* was a misspelling of the genus *Laspryria* Germar and therefore could not be considered. The next available name for *pomonella* was *Cydia*, which is now considered the correct generic name (Wearing *et al*., 2001). Currently, *Cydia* and related genera are included in the tribe Grapholitini of the sub-family Olethreutinae (Tortricidae) (Wearing *et al*., 2001; Pajać *et al*., 2011).

Order:	Lepidoptera Linnaeus, 1758
Suborder:	Microlepidoptera
Family:	Tortricidae Latreille, 1803
Sub-family:	Olethreutinae
Tribe:	Grapholitini
Genus:	*Cydia* Hübner, 1825
Species:	*Cydia pomonella* (Linnaeus, 1758)

Cydia pomonella occurs in the temperate regions of all major continents of the world and has shown the ability to colonize apple and pear trees wherever the climate is suitable for production (Figure 1.1) (Barnes, 1991; Wearing *et al*., 2001; Franck *et al*., 2007; Thaler *et al*., 2008). A major historical contributor to the spread of *C. pomonella* was human migration and the movement of fruit along trade routes of the world. Infested apple trees have been transported by unsuspecting colonists from Europe to many countries around the world, particularly during the 19th century (Slingerland, 1898). Currently *C. pomonella* is a pest in North and South America as well as in Mexico. It is also widespread throughout Europe, from southern Scandinavia and eastward to Siberia, to the north of India and to Xinjiang and Gansu in China. It is also established in the fruit growing regions of Western Australia and New Zealand (Croft & Penman, 1989). Extensive eradication programmes have prevented the permanent establishment of the pest in Western Australia to date. Its distribution does not exclude Africa, with South Africa, Egypt, Libya, Tunisia, Algeria, Morocco, Madeira, Canary Islands and Mauritius all having established populations of the pest (CABI, 2011) (Figure 1.1).

Cydia pomonella was first reported from South Africa in Graaf-Reinett around 1885. It is believed to have been introduced in infested apples transported by a traveller. Several unsuccessful attempts were made to eradicate the pest (Lounsbury, 1897). Since then *C. pomonella* has established itself in deciduous fruit orchards throughout South Africa (Giliomee & Riedl, 1998).

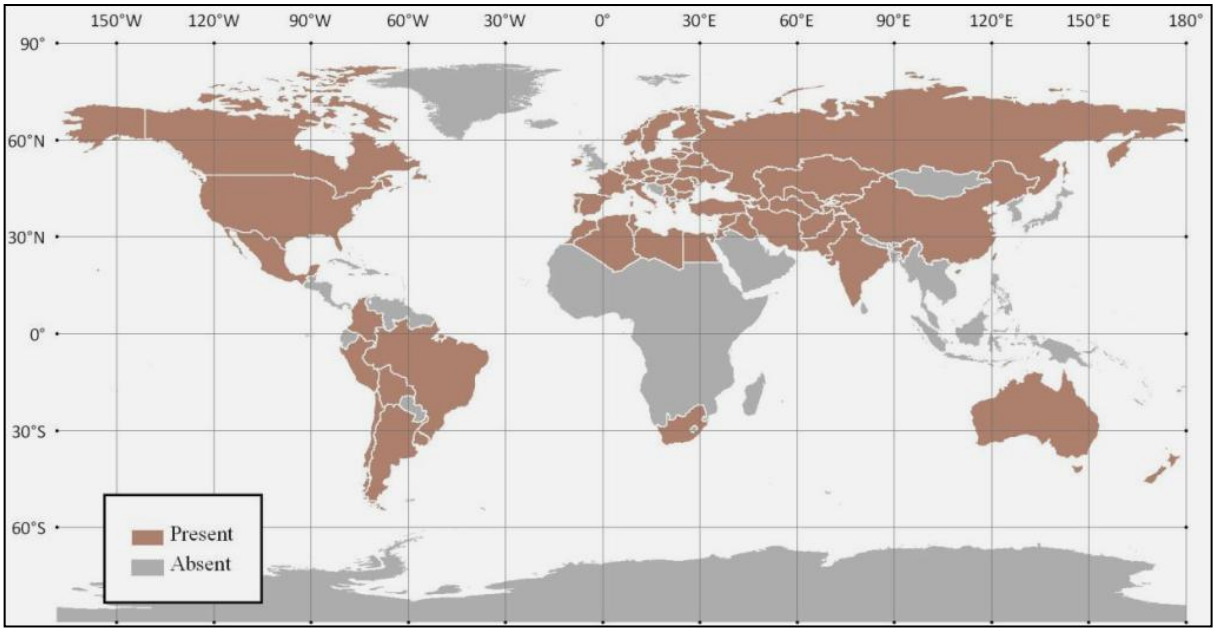


Figure 1.1 Global distribution of *Cydia pomonella* (L.) (Lepidoptera: Tortricidae) (CABI, 2011). Regions shaded in brown indicate areas where *C. pomonella* is present and regions shaded in grey indicate places where *C. pomonella* is absent.

1.2.2 Biology and life history

In South Africa *C. pomonella* is multivoltine with a facultative diapause, producing three to four generations per year (Blomefield, 2003; Pringle *et al*., 2003). Eggs are laid singly or in groups of two or three on the upper surface of leaves near fruit, on twigs or on the fruit itself and occasionally on the bark of the tree (Blomefield, 2003; Blomefield & Giliomee, 2012). The female can oviposit an average of 30 to 70 eggs depending on environmental conditions (Pajaĉ *et al*., 2011). Carter (1984) reported that a female may lay more than 100 or less than 12 eggs. Higher oviposition rates were recorded by Blomefield & Giliomee (2011) under South African conditions, with an average oviposition of 92.6 eggs per female for spring moths and 121.2 eggs for summer moths. The eggs are elliptical in shape and measure 1 to 1.2 mm in diameter, slightly flattened with a creamy white appearance when freshly oviposited (Figure 1.2 A). Midway through the egg development a distinct red ring becomes visible (Figure 1.2 B). Before hatching the dark head capsule of the neonate larva becomes clearly visible (Figure 1.2 C).

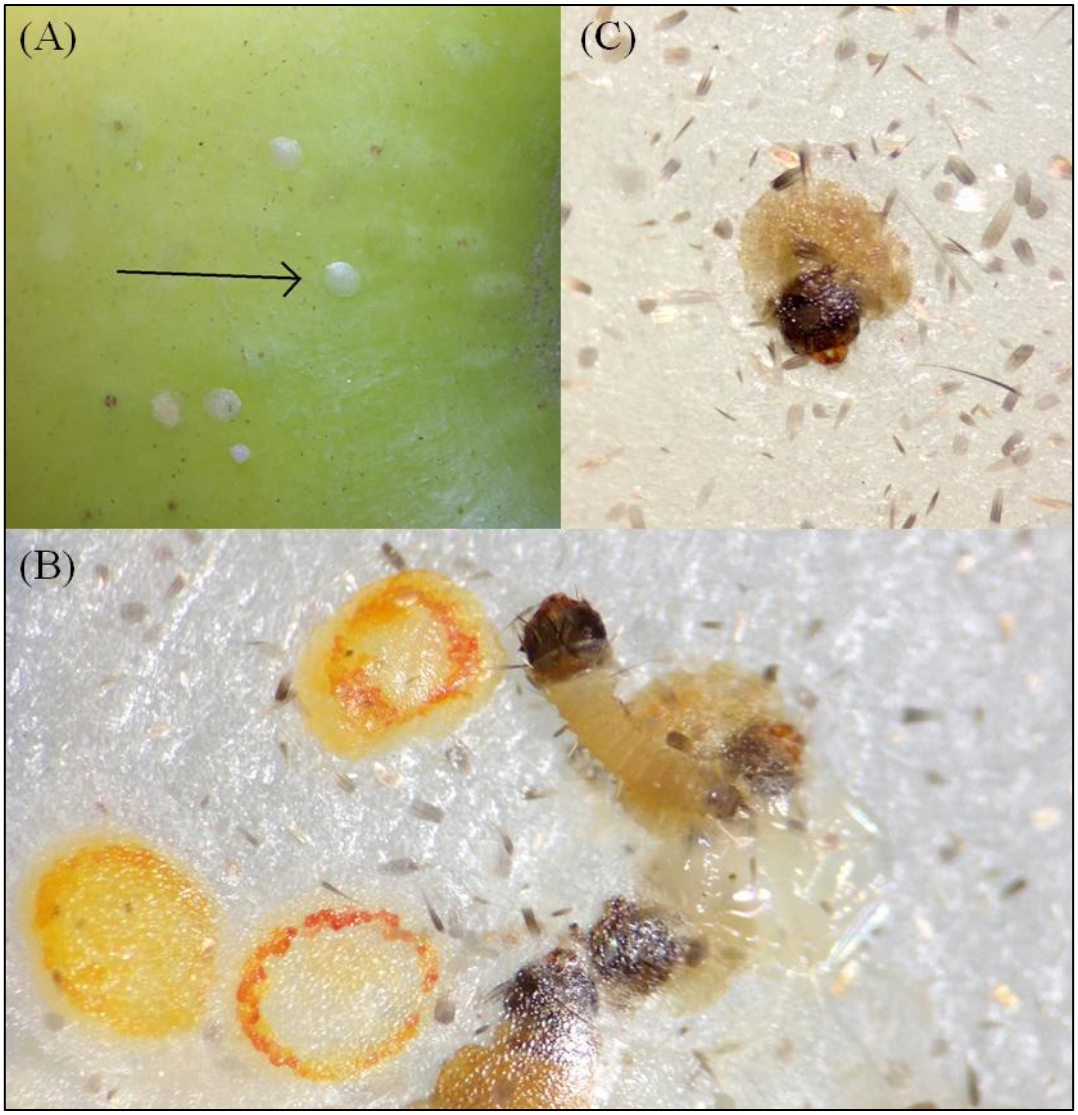


Figure 1.2 *Cydia pomonella* eggs. (A) Flattened appearance and creamy white colour, indicated by arrow, (B) egg showing distinct red ring during development, (C) larval head capsule visible through a transparent chorion.

The development threshold for the eggs is 10.0°C (Geier, 1963). Agnello & Kain (1996) reported time to egg hatch to be approximately 6 to 20 days with hatch being dependent on environmental conditions within the orchard. A study by Jackson (1979) revealed that 57% of all eggs were found on upper leaf surfaces, 35% on lower leaf surfaces and 8% on apples, but these percentages were not constant over the summer. His research also showed that 57% of all eggs laid in the field were within 7.5 cm and 91% within 20 cm of the fruit. Neonate larvae are pale yellow to white and measure approximately 2 mm in

length and 0.5 mm in diameter (Figure 1.3). The neonate larvae move in search of fruit and begin penetrating and feeding. Occasionally, the larvae have been observed to feed upon foliage if the search for fruit is prolonged (Blomefield, 2003). The time from egg hatch to successful entry into the fruit is a critically important period in the field biology of *C. pomonella*.

Figure 1.3 Neonate *Cydia pomonella* larva and eggs at various stages of development.

Once the larva has penetrated the fruit, feeding occurs just below the surface of the rind, after which the second instar larva moves towards the core of the fruit, where it feeds on the developing seeds (Figure 1.4). *Cydia pomonella* larvae undergo five larval stages, the first four of which are spent within the fruit. The fifth or pre-pupal stage exits the fruit in

search of a cocooning or pupation site. These sites include under loose bark, in pruning woods, leaf litter under the canopy or fruit bins (Cossentine & Jensen, 2004).

Figure 1.4 Fifth instar *Cydia pomonella* larva feeding on the core on an apple, indicated by arrow.

The development time of the larval stage is approximately 18 to 40 days and is a function of both ambient temperature and food quality (Wearing, 1979; Agnello & Kain, 1996; Welter, 2008; Davis *et al*., 2013). Rock & Shaffer (1983) found a positive correlation between temperature and survival rate of *C. pomonella*, with 27°C found to be the optimum temperature for development. *Cydia pomonella* larvae develop from 2 mm (neonate) to approximately 20 mm (fifth instar). Neonate larvae are white with black

heads. As the larvae mature to reach fifth instar, their colour changes to a dark pink and the head capsule is brown. Table 1.1 lists the degree-day requirements for the development of the various larval stages as well as larvae to pupae and larvae to adult developmental times. *Cydia pomonella* larvae have no anal comb which is a feature that distinguishes them from other larval pests attacking apples and pears (Wearing *et al*., 2001; Pajać *et al*., 2011)

Table 1.1 Thermal requirements for the development of *Cydia pomonella* larvae (Setyobudi, 1989).

Stage of Life Cycle	**Sex**	**Degree-Days required**	
		Mean	**95% Confidence Index**
LARVAE			
First Instar	Combined	54.59	(53.10-56.08)
Second Instar	Combined	55.75	(53.09-48.41)
Third Instar	Combined	33.18	(28.32-38.04)
Fourth Instar	Combined	21.20	(17.50-24.80)
Fifth Instar	Combined	124.24	(115.2-132.96)
First to Fifth instar	Female	279.18	(243.99-314.37)
	Male	303.80	(287.07-320.53)
	Combined	299.96	(214.43-310.30)
PUPAE	Female	154.59	(146.93-162.25)
	Male	162.88	(154.76-171.00)
	Combined	152.30	(147.43-157.17)
LARVAE TO ADULTS	Female	433.77	(390.92-476.62)
	Male	466.68	(441.83-491.53)
	Combined	441.26	(261.86-467.56)

In early summer, the cocooned larvae develop into pupae and mature in approximately 7 to 30 days depending on environmental temperatures (Pajaĉ *et al*., 2011). The pupae vary in length from 12 to 14 mm. Male pupae can be identified by the presence of two clearly distinguishable circles on the ventral surface of the sixth abdominal segment and are usually smaller than the females. During the colder months of the year larvae may enter a diapause phase, surviving through winter as mature larvae and pre-pupae in cocoons. A study by Dickson (1949) was the first to show that decreasing photoperiod induced diapause in the larvae of *C. pomonella*. Riedl (1983) indicated that although the diapause appears to be facultative and influenced by the prevailing photoperiod and temperature, a natural tendency for univoltinism appears to be present in *C. pomonella* as some individuals do not respond to photoperiod changes and enter diapause.

Male moths tend to eclose slightly before females, and mating typically occurs within the first few days of the female's existence. The adult moths are speckled grey-brown in colour and approximately 8 mm in length. Their wings have a distinctive crisscrossed pattern with light gray lines and there is a bronze-coloured patch near the outer margins of the forewings (Figure 1.5).

Figure 1.5 Adult *Cydia pomonella* showing wing pattern and colouration.

1.2.3 Pest status

The status of a pest on a particular crop determines the extent to which the pest needs to be controlled (Pringle, 2006). Moran (1983) developed a mathematical formula to measure the pest status of an insect. His results ranked *C. pomonella* as the fifth most important plant feeding pest species in South African. Bell & McGeoch (1996) modified this formula to obtain a more objective assessment of each pest's relative importance. The pest-status values obtained from their study ranked *C. pomonella* as the third on the list, with *Helicoverpa armigera* (Hübner) and *Agrotis segetum* (Schiffermüller) ranked as first and second respectively. Currently, *C. pomonella* is considered to be a key pest and of major concern in most deciduous growing areas around South Africa (Pringle *et al*., 2003; Pringle, 2006). With the development of resistance to both synthetic insecticides as well as certain isolates of the *Cydia pomonella* granulovirus (albeit not yet in South Africa), the pest status of *C. pomonella* continues to increase (Barnes & Blomefield, 1997).

1.2.4 Economic importance

In South Africa, *C. pomonella* has one of the highest damage potentials in the world and infestation of up to 80% of an apple crop is possible if left untreated. Crop losses are difficult to assess, as most of the methods used are not comparable (CABI, 2011). Damage ranges from shallow wounds resulting in scarring of the fruit, to direct feeding damage to the fruit pulp. Secondary infections and indirect contamination by larval frass also result in crop loss (Welter, 2008). Deciduous fruit export accounts for approximately 15% of the South Africa's total agricultural export earnings. The Hortgro tree census (2013) indicated that there is a total of 22501 ha of apple and 12034 ha of pear orchards in South Africa, with apples making up 29% and pears 15% of the total area of deciduous fruit planted (Hortgo, 2013). The value of apples and pears to the South African industry was R9.912 billion (USD 1 = ZAR 11.18). Under good pest suppression, losses of 0.5% have been reported (Addison, 2005) which would equate to nearly a R10 million loss to the industry and with no control measures, losses may be in the billions of Rands (Table 1.2).

Table 1.2 Value of the deciduous fruit industry in South Africa in 2013 (Anonymous, 2013).

	Apples	**Pears**	**Plums**	**Peaches**	**Nectarines**	**Apricots**
Total sales (R millions)	6759.3	3153.6	1289.4	98.0	240.3	110.9
Export Volume (tons)	434663	197912	5959	3457	8102	4635

1.2.5 Control of *Cydia pomonella*

Many control measures have been used against the *C. pomonella*. Traditionally these have relied heavily on the use of broad spectrum insecticides. Concerns over employee safety, environmental impact and the sustainability of the synthetic pesticides have resulted in the development of softer control measures for implementation into an integrated pest management (IPM) strategy (Blomefield, 2003; Charleston *et al*., 2003; Lazarovits *et al*., 2007). Integrated pest management is a broad based ecological approach to agricultural pest control that integrates pesticides into a management strategy. The management strategy may incorporate a range of practices for economic control of a pest. Integrated pest management programmes use current, comprehensive information on the life cycles of pests and their interaction with the environment. This information, in combination with available pest control methods is used to control the pest with the least possible impact to people, property, and the environment. The various control measures are described below:

1.2.5.1 Chemical control

Historically, *C. pomonella* control measures have been based almost solely on the use of broad spectrum insecticides, particularly organophosphates (OPs) (Riedl *et al*., 1998). Over 70% of the insecticide treatments in apple orchards are currently applied to control *C. pomonella* populations (Pajaĉ *et al*., 2011). These sprays were usually applied on a

calendar basis. During the 1970s use of synthetic pheromone traps became the standard for monitoring *C. pomonella* activity within orchards. This enabled the application of insecticide sprays to be applied according to both pest pressure and activity, resulting in a general reduction in applications during times of low *C. pomonella* pressure. The correct timing of the insecticide application is critical if it is to be effective against *C. pomonella* due to the cryptic nature of the pest. The chemical application should be applied just before or during egg hatch. Currently, in South Africa, there are over 30 insecticides registered for *C. pomonella* control (Quinn *et al*., 2011), of which flufenoxuron (Cascade), azinphos-methyl (Azinphos, Azinphos Flo) and tebufenozide (Mimic) are regularly used by growers in the Western Cape. This is similar to the controls used in Israel and the United States with extensive use of organophosphorus compounds (OPs), most notably azinphos-methyl (Dunley & Welter, 2000; Reuveny & Cohen, 2004). Frequent use of broad spectrum insecticides can have a negative effect on the environment, resulting in a reduction of beneficial organisms, and more importantly, it has also resulted in *C. pomonella* developing resistance and cross-resistance to many of the chemical insecticide (Knight *et al*., 1994; Fuentes-Contreras *et al*., 2007; Mota-Sanchez *et al*., 2008; Rodríguez *et al*., 2011). This has forced growers to consider alternate control measures and the possible use of IPM based programmes for the control of *C. pomonella*. Not only is resistance reducing the suite of products available for pest control but due to human and food safety concerns the European Commission has in the past decade reviewed and removed over two thirds of pesticides that have been used to control pests and plant diseases (European Commission, 2013)

1.2.5.2 Monitoring *Cydia pomonella* in orchards

Pheromone traps have been used worldwide for the monitoring of *C. pomonella* for the past two decades (Witzgall *et al*., 2008). In South Africa, monitoring of *C. pomonella* populations is achieved using a single pheromone trap every 2 ha. Twenty five evenly spaced trees are selected throughout the block and used as data trees each time *C. pomonella* infestation is monitored (Pringle *et al*., 2003). Pheromone trapping provides an indication of moth activity within an orchard, however a number of factors may influence

trap counts thereby decreasing the reliability of the data obtained. Scouting data can be used to ascertain the level of *C. pomonella* prevalence in an orchard. Both the trap counts and scouting data are important in determining the level of *C. pomonella* activity within an orchard (Pringle *et al*., 2003). Blomefield & Giliomee (2014) reported that pheromone traps are dependent on weather conditions and the density of the insect population. An understanding of the pest population within the orchard allows one to time control measures relative to peak pest pressure. This can reduce unnecessary spray applications and improve control measures (Myburgh *et al*., 1974; Barnes, 1990).

1.2.5.3 Cultural control measures

The use of insecticides generally maintains *C. pomonella* populations at very low levels within an orchard. However, cultural control methods still play a vital role. Important cultural practices are the removal of abandoned apple, pear, and walnut trees in close proximity to productive orchards; and orchard sanitation and banding of the trees, which play a vital role in maintaining the population of *C. pomonella*. Abandoned orchards may act as reservoirs for the pest allowing continual re-infestation of nearby productive areas. Orchard sanitation is a critical tool for the control of *C. pomonella*. Moore & Kirkman (2008) suggested that strict sanitation practices are required when using either sterile insect releases, mating disruption and or a granulovirus application in a *T. leucotreta* control programme on citrus. This concept is equally important in the control of *C. pomonella* as the three control approaches work best under low pest pressure (Judd *et al*., 1997; Witzgall *et al*., 2008). Therefore infested fruit should be removed from the tree and fallen fruit should be collected and removed from the orchard, mulched or buried to prevent any larvae from exiting the fruit to cocoon or pupate. Banding of the tree trunks and branches to catch cocooning larvae, or grease banding the tree trunk, is not considered an effective stand-alone control method but may be used as part of an IPM strategy to reduce pest pressure within an orchard. A study by Judd *et al*. (1997) reported damage of 43.5% to 56.7% in an orchard with no control measures other than tree banding.

1.2.5.4 Mating disruption

Mating disruption is used worldwide for the control of *C. pomonella* and has long proven successful (Riedl *et al*., 1998; Pringle *et al*., 2003; Witzgall *et al*., 2008; Knight *et al*., 2012). It has been found to be particularly effective if applied in areas with low pest pressure or in combination with other control measures such as the sterile insect technology (SIT). Several factors impede the effectiveness of this method and these include high pest pressure, geographic layout of an area, wind and open spaces in the treatment area. It must be noted that the most important disadvantage of mating disruption is that the female moth's behaviour is not affected (Yan *et al*., 1999). Usually this control approach involves placing 500 to 1000 dispensers evenly per hectare. The presence of the pheromone impedes the male's ability to find females and reduces the frequency of mating (Pringle *et al*., 2003). Judd *et al*. (1997) concluded that an integrated control programme of mating disruption, orchard sanitation and tree banding could control *C. pomonella* effectively enough to make organic apple production viable in British Columbia. Over a three year trial period, damage at harvest averaged < 0.7% in four organic orchards. This result was comparable to five conventional orchards which received sprays of azinphosmethyl in which 0.5% damage at harvest was recorded (Judd *et al*., 1997).

New mechanisms of pheromone dispensing such as puffers, emulsified wax dispensers and sprays are also being considered as alternate means of mating disruption for *C. pomonella* (Shorey & Gerber, 1996; Stelinski *et al*., 2009; Knight *et al*., 2012). Codlemone, (*E,E*)–8,1O–dodecadienol, the female sex pheromone of *C. pomonella* has been reported to cause up to 98% disruption in pheromone communication as measured by a reduction in the numbers of males that oriented to codlemone lure–baited or female–baited traps, when release at 44 minute intervals from puffers (machines designed for the dispensing of aerosols from pressurized canisters) (Shorey & Gerber, 1996). A novel emulsified wax dispenser (SPLAT-OFM, ISCA, Riverside, United States of America) has been tested against the oriental fruit moth, *Grapholita molesta* (Busck) (Family: Tortricidae) with 98% disruption of the males ability orientate to optimally attractive pheromone traps relative to

the untreated control. This same technology (SPLAT-CYDIA ISCA, Riverside, United States of America) is being used to control *C. pomonella*.

1.2.5.5 Sterile insect technique (SIT)

The sterile insect technique is a method of control which involves area-wide inundative release of sterile insects. Released sterile moths mate with the wild population reducing the number of progeny in the field (Knipling, 1955; Klassen, 2005). The success of SIT relies on an initial low pest pressure. Therefore in most instances, other forms of control are required to supplement SIT releases in the first few years of application. Riedl *et al*. (1998) suggested a pre-release damage of 0.05% to be acceptable. The aim of the technique is to suppress the wild population numbers below the economic threshold of the crop or to eradicate the pest.

SIT has been used around the world in many area-wide integrated pest management programmes. The following examples testify to the success of this technique: screw worm (*Cochliomyia hominivorax*) (Coquerel) (Family: Calliphoridae) has been eradicated from the United States, Mexico, and Libya; the Mexican fruit fly (*Anastrepha ludens*) (Loew) (Family: Tephritidae) has been eradicated from most of northern Mexico; the Tsetse fly (*Glossina* spp.) has been eradicated from Zanzibar; and the Medfly (*Ceratitis capitata*) (Wiedemann) (Family: Tephritidae) has been eradicated from parts of Chile, Peru and Mexico (Anonymous, 2011a). Sterile insect releases of *C. pomonella* were first started in the Similkameen Valley in British Columbia, Canada (Proverbs *et al*., 1982). The Okanagan-Kootenay SIR programme was the first commercial SIT programme to target this pest in Canada. The programme was officially started in 1992 with the first releases of sterile moths in 1994. This programme has proved a great success with the pest being reduced to almost undetectable levels. The main advantage of an SIT control programme is that there is no negative environmental impact and non-target organisms are therefore not affected (Myers *et al*., 2000). The disadvantage of such an approach is that it is usually expensive and relies on very good maintenance and release management (Addison, 2005).

In South Africa, a *C. pomonella* SIT pilot project was initiated in 2003 in the Western Cape (Addison, 2005). The original trial facility produced round 1500 moths per week and was used to supply a single hectare. In 2009, the construction of a 560 m² rearing facility at the University of Stellenbosch's Welgevallen Experimental Farm was commenced. The facility was commissioned in late 2010 and was geared to produce in excess of 2 million moths per week, with has been supplying moths to just over 3% of the local apple and pear industry, therefore still leaving ample room for expansion. Unfortunately, due to poor grower uptake of the technology the company shut down at the end of 2014.

1.2.5.6 Biological control

Biological control includes both pathogens (microbial control) and arthropods (parasitoids and predators). Pathogens infecting or attacking *C. pomonella* larvae include bacteria, fungi, viruses and entomopahogenic nematodes (EPNs). Of these, viruses and EPNs have been commercially produced for biological control of *C. pomonella* (Lacey & Unruh, 2005). There is little literature available on the specific interaction between entomopathogens and arthropod natural enemies of *C. pomonella* (Lacey & Unruh, 2005). Biological control will be favoured when there is compatibility between the two. It is also important to fully understand the positive and negative effects of a combined IPM approach for the control of *C. pomonella*.

1.2.5.6.1 Predators and parasitoids

Cydia pomonella predators are the least studied of all the biological control measures. Assessing predation is considered to be difficult because in most instances the predation goes unobserved or cannot be measured with confidence. In many instances no evidence of predation is visible because the prey is entirely consumed. Historically, studies have focused on the visible groups of predators, such as birds, and those that can easily be trapped, such as larger insects (Solomon & Glen, 1979; Riddick & Mills, 1994). Various authors consider birds to be important predators of *C. pomonella* larvae with a number of studies showing predation in unsprayed orchards ranging from 50% to 95% (LeRoux,

1961; MacLellan, 1971; Glen & Millsom, 1978; Wearing, 1979). Glen (1975) showed that predators inflicted heavy mortality on *C. pomonella* eggs. Predatory Heteroptera fed on 35% and 57% of the eggs during the two year study. A smaller number of eggs (16%) simply disappeared. These were presumed to have been consumed by earwigs, *Forficula auricularia* Linnaeus. Various authors have reported on the diversity of spiders found within deciduous orchards, with multiple species of these been observed feeding of *C. pomonella* larvae (Dondale, 1956; Monsour *et al*., 1980; Bogya & Mols, 1996). Mites are thought to be important predators of eggs and possible small larvae (Glen, 1977). The effect of spiders and mites as control agents of *C. pomonella* have been poorly studied due to their predominantly nocturnal habits as well as their small size. No significant quantitative studies of their predations have been conducted. Insects are the largest group of *C. pomonella* predators (Lacey & Unruh, 2005).

More than 100 species of parasitoids have been recovered from *C. pomonella*. Only a few of these species have been recognized as specialist parasitoids of *C. pomonella*, with the majority causing negligible levels of parasitism (Lacey & Unruh, 2005). *Trichogramma* species (Hymenoptera: Trichogrammatoidea) have been used as egg parasitoids and are considered to be the most important biological agents of *C. pomonella* (Mansfield & Mills, 2002; Makee, 2006). In South Africa *Trichogrammatoidea lutea* (Girault) has been investigated as a promising biological control agent of *C. pomonella* (Wahner, 2008; Wahner *et al*., 2008). Control of viable eggs with *T. platneri* Nagarkatti has been reported by Zhang & Cossentine (1995). Mills (2005) reported the release of three species of parasitoids *Bassus rufipes* Wesm., *Liotryphon caudatus* L. and *Mastrus ridibundas* Grav for the control of *C. pomonella*. *Bassus rufipes* was not recovered in the field, while *L. caudatus* had temporarily established in a few release areas. *Mastrus ridibundas* established itself in the field and good recoveries were obtained at numerous trial sites. The effective use of parasitoids is subject to several confounding factors which include the timing of releases, temperature and population density all of which can have an impact on *C. pomonella* control (Zhang & Cossentine, 1995).

1.2.5.6.2 Bacteria

A few strains of bacteria have been successfully tested against *C. pomonella*. The most extensive being *Bacillus thuringiensis* (*Bt*) Berliner, which has been successfully used against *C. pomonella* in conjunction with reduced rates of chemical applications (Lacey & Unruh, 2005). Due to the feeding behavior of the neonate larvae, they only have a short period in which to come into contact with the bacterium on the fruit surface and therefore its value as a control agent is limited by the improbability of a larva ingesting a lethal dosage of *Bt* toxin (Lacey & Unruh, 2005). Falcon & Berlowitz (1986) were unable to obtain acceptable levels of control using *Bt* as a standalone control measure.

1.2.5.6.3 Fungal entomopathogens

Several fungi have been reported to infect *C. pomonella* (Glen, 1982; Zimmerman & Weiser, 1991). *Beauvaria bassiana* has been shown to be one of the most abundant species infecting *C. pomonella* in nature (Jaques & MacLellan, 1965; Ferron & Vincent, 1978; Falcon & Huber, 1991). Garcia-Gutierrez *et al*. (2004) noted effective control of neonate larvae using a native strain (BbP1) and two commercial formulations, namely Mycotrol and Meta-Sin at a concentration of 1.2×10^{12} conidia/ha. The main factor limiting the efficiency of fungal pathogens in insect control is their requirement for high humidity and moderate temperatures for spore germination, growth and sporulation (Cross *et al*., 1999). Attempts to develop *B. bassiana* into a successful microbial control agent of several insects have been somewhat successful (Goettel *et al*., 2005), however it has not been commonly used for *C. pomonella* control (Lacey & Unruh, 2005).

1.2.5.6.4 Entomopathogenic nematodes (EPNs)

Steinernima feltiae (Filipjev) and *S. carpocapsae* (Weiser) have been found to be the most promising nematode species for the control of *C. pomonella* and have been recovered from natural populations of *C. pomonella* in various parts of the world (Kaya *et al.*, 1984; Lacey & Chauvin, 1999; Vega *et al.*, 2000; Lacey & Unruh, 2005; Lacey *et al.*, 2006). The

cocooned diapausing larvae, which occur in late autumn, winter and early spring in temperate areas, have been considered to be the most logical target for control with EPNs (De Waal, 2008). During this period, *C. pomonella* overwinters under loose bark, in litter at the base of trees and in nearby woodpiles and fruit bins. The above mentioned sites are documented as being relatively favourable for the survival of EPNs (Lacey & Unruh, 2005). A significant reduction in the *C. pomonella* population would have a marked suppression effect on the first generation for the following season. Vega *et al*. (2000) reported up to 81% control of *C. pomonella* using laboratory bioassay methodologies. Similar results were obtained by Lacey & Unruh (1998) who reported up to 90% mortality as a result of infection. Field trial results have also shown significant control of C. pomonella with up to 95% control being recorded in orchard trials (Lacey *et al*., 2006). Malan & Addison (2008) documented the first use of EPNs for the control of *C. pomonella* in South Africa as part of a preliminary laboratory bioassay study that was aimed at selecting a promising local nematode isolate for future investigation. *Heterorhabditis zealandica* (SF41) was selected from this study as the most promising South African isolate.

Malan *et al*. (2006) conducted a survey for EPN prevalence in orchard soils of the southwestern parts of South Africa. Entomopathogenic nematodes were isolated in 7% of 498 soil samples baited with wax moth *Galleria mellonella* L. (Family: Pyralidae) *Heterorhabditis* was the dominant genus isolated with *Steinernema* species being rare. Within the soil, EPNs are buffered from environmental extremes. Entomopathogenic nematodes, being soft bodied invertebrates that require surface moisture in which to move and survive, are negatively affected by various environmental conditions (Lacey & Unruh, 1998; Glazer, 2002; Wright *et al.*, 2005). For this reason, their suitability as biological control agents in the aerial parts of deciduous tree has been questioned.

EPNs have not only been used to target larvae in the orchards but have been effectively used to control larvae in fruit bins (Lacey & Chauvin, 1999; Cossentine *et al*., 2002). Infested fruit bins can act as an inoculum source and aid in re-infestation of an area previously under good control. Good levels of control have been reported by soaking bins

in a suspension containing infective juveniles (Lacey & Chauvin, 1999; Lacey *et al*., 2005b). Bins immersed in suspensions of *S. carpocapsae*, ranging from 5 to 100 IJ/ml, resulted in 68 to 100% mortality (Lacey & Chauvin, 1999).

1.2.5.6.5 Virus

Baculoviruses found naturally in pest populations are important biological control agents and have been utilized to control lepidopteran pests of economically important crops (Rodrigeuz *et al*., 2012; Knox *et al*., 2015). One of the most efficacious and highly selective pathogens of *C. pomonella* are granuloviruses (GVs) belonging to the family *Baculoviridae*. This group of DNA viruses is known to infect insects and has been successfully commercialized into bio-pesticides that are used worldwide (Szewczyk *et al*., 2006). The use of virus as a biological control measure is summarized in section 1.3.2.6.

1.3 THE PATHOGEN

1.3.1 Baculoviruses

1.3.1.1 History of baculoviruses

Baculoviruses were discovered as far back as the sixteenth century, in reports of 'wilting disease' infecting silk worm larvae. Paillot (1926) was the first to describe a GV infection. From as early as the 1940s, rod shaped virions in polyhedral structures were observed by electron microscopy (Bergold, 1948). GVs have been isolated from about 150 insect species, all of which belong to the order Lepidoptera (Francki *et al*., 1991). Currently GVs are been used and studied widely as biological control agents.

1.3.1.2 Classification

More than 20 groups of insect viruses are known to be insect pathogens and have been placed into 12 virus families. Many remain unclassified. Viruses are separated into DNA or

RNA viruses. The DNA viruses associated with arthropods include the following families: *Ascoviridae*, *Baculoviridae*, *Birnaviridae*, *Iridoviridae*, *Parvoviridae*, *Polydnaviridae* and *Poxviridae* (Miller, 1996; Evans, 2000) (Table 1.3). The family *Baculoviridae* is taxonomically characterized by a large, double stranded, circular DNA genome which is packed into a rod-shaped capsid and further enveloped by a unit membrane (Miller, 1996). Only the *Baculoviridae* and *Poxviridae* are known to produce occlusion bodies from which virions, at a certain stage of their development, may be randomly occluded (Tanada & Kaya, 1992). These occlusion bodies (OBs) contribute to the stability and persistence of the virus in the environment. Generally OBs are about 0.5 to 20 µm in diameter, and are visible under a light microscope (Hunter-Fujita *et al*., 1998).

Table 1.3 The main characteristics of the DNA based viruses that influence their use as potential biological control agents against arthropods (Miller, 1996; Evans, 2000).

Family	Genus	Characteristics	Presence of occlusion bodies	Insect host	Site of viral replication	Large scale use
Ascoviridae	*Ascovirus*	dsDNA, enveloped	-	Lepidoptera Noctuidae only	Nucleic: fat body, hypodermis, tracheal.	No
Baculoviridae	*Alphbaculovirus* *Betabaculovirus* *Gammabaculovirus* *Deltabaculovirus*	dsDNA, enveloped dsDNA, enveloped	Polyhedral Granular	Lepidoptera, Diptera and Hymenoptera	Nuclei: gut and systemic	Yes Yes
Birnaviridae	*Birnavirus*	dsRNA nonenveloped	-	Diptera	No tissue symptoms, adults sensitive to CO_2	No
Iridoviridae	*Iridovirus*, *Chlorirdovirus*	dsDNA, nonenveloped	-	Wide range of insect hosts	Cytoplasm: fat body, haemocoel, systemic	No
Parvoviridae	*Densovirus*	ssDNA nonenveloped	-	Diptera: Blattoidae, Lepidoptera: Odonata, Orthoptera	Tissue	No
Polydnaviridae	*Ichnovirus*	dsDNA, enveloped	-	Hymenoptera: Ichneumonidae	No effects on parasitoids	No
Poxviridae	*Entomopoxvirus*	dsDNA, enveloped	Spheriod	Coleoptera, Diptera, Hymenoptera, Lepidoptera, Orthoptera	Cytoplasm: mainly fat bodies	No

Baculoviruses are the most common group of viruses known to infect insects. They are a large diverse group and have very species-specific tropisms among invertebrates with over 600 host species described. Advances in genome analysis have improved the accuracy of classification of baculoviruses (Jehle *et al*., 2006). Baculovirus genomes consist of a circular double-stranded DNA molecule approximately 80 to 180 kbp that encodes for 90 to 180 genes (Rohrmann, 2011). Previously this group had been divided into three subgroups: nucleopolyhedroviruses (NPVs); granuloviruses (GVs) and nonoccluded, rod-shaped nuclear viruses (NOVs) (Tanada & Kaya, 1993). Currently the family *Baculoviridae* is divided into four genera: *Alphbaculovirus* (lepidopteran-specific NPV), *Betabaculovirus* (lepidopteran-specific GV), *Gammabaculovirus* (hymenopteran-specific NPV) and *Deltabaculovirus* (dipteran-specific NPV) (Jehle *et al*., 2006).

Cydia pomonella granulovirus (CpGV) belongs to the *Betabaculovirus* genus (Miller, 1996; Theilmann *et al*., 2005). The occlusion bodies of the GVs are smaller (0.2 to 0.5 µm) than those of the NPVs (0.15 to 15 µm). The GVs usually contain a single enveloped nucleocapsid, while the OBs of the NPVs may contain several hundred virus particles (Hunter-Fujita *et al*., 1998) (Figure 1.6).

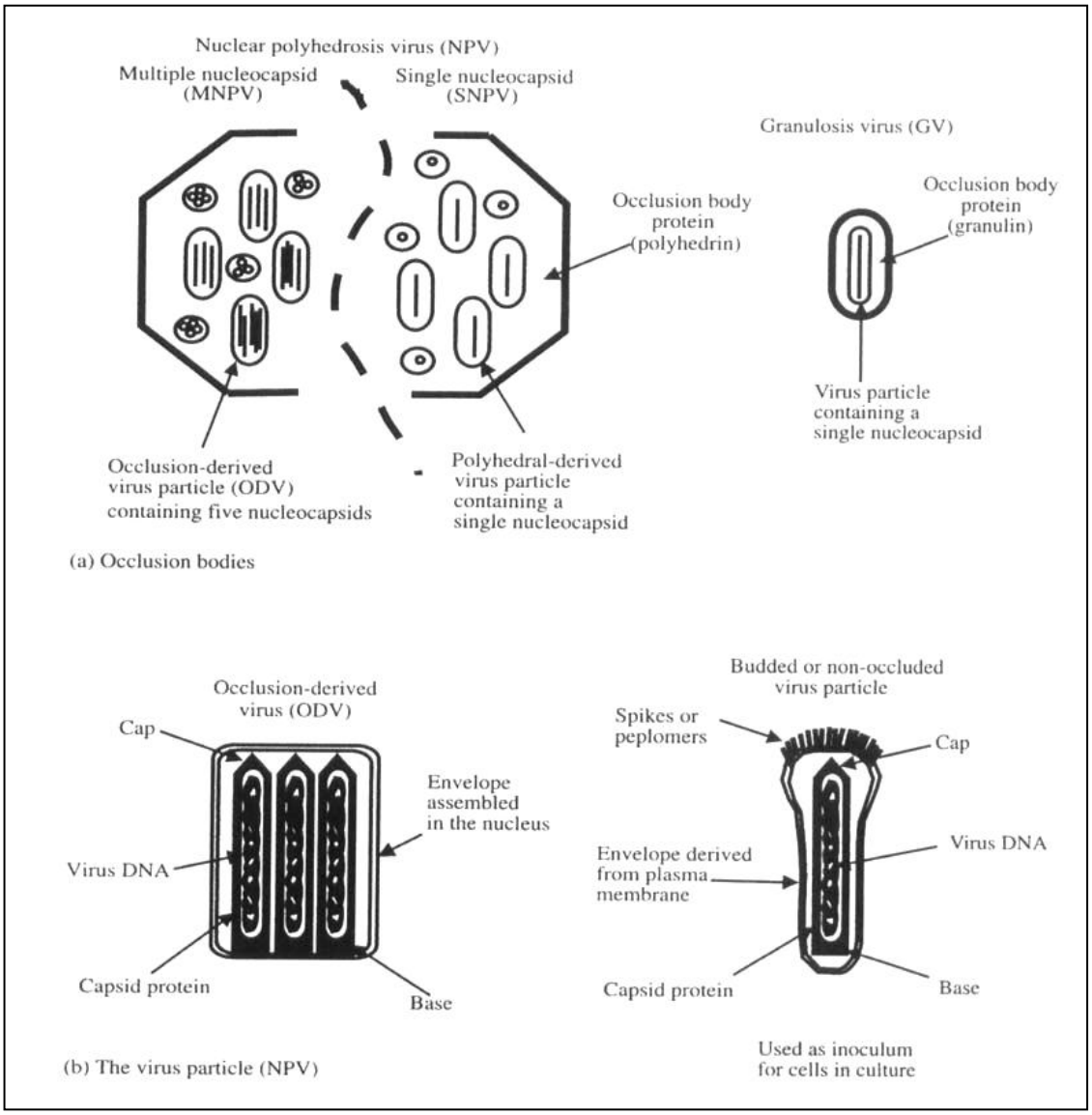


Figure 1.6 The morphology of members of the Baculoviridae family of insect pathogenic viruses (Hunter-Fujita *et al.*, 1998).

1.3.1.3 Pathology and pathogenesis of GVs

Initial symptoms of viral infection include a loss of appetite by the larvae and a progressive colour change to pale and milky white, especially on the ventral side (Huger, 1963). As the virus replicates and infection progresses, the larvae become progressively weaker, sluggish and flaccid (Tanada & Kaya, 1993). With GV infection there is a proliferation and

hypertrophy of infected fat cells which results in an enlarged larva during the latter part of the infection. With a systemic granulosis infection, larvae usually die more rapidly, than during an infection involving the fat body. A systemic infection therefore results in the larvae being smaller and wilted upon death (Tanada & Kaya, 1993).

The speed of kill of a GV depends on several parameters such as dose and susceptibility of the host. GVs have been described as "slow" or "fast" (Winstanley & O'Reilly, 1999). Type 1 GVs have high median lethal dose (LD_{50}) values, and a slow speed of kill. Type 2 GVs tend to be highly infectious with low LD_{50} values of < 5 OBs per neonate and a rapid speed of kill (Winstanley & O'Reilly, 1999). If neonate larvae ingest a lethal dose of the virus, death may occur in as little as 3 days. The LD_{50} has been estimated as low as 1.2 to 5 OBs per neonate larvae, with higher estimates being suggested for artificial diets (Sheppard & Stairs, 1977; Huber, 1986). Older instars require a higher dose of the virus in order to result in an infection.

1.3.1.4 Life cycle of a baculovirus

Viral infection is only possible if the virus is ingested by a larva. This usually occurs as the larvae penetrate the fruit, but may also occur during its scouting of the fruit for the penetration point. Shortly after ingestion of the virus particle, the granulin coating is dissolved in the alkaline midgut of the larvae which results in the liberation of the nucleocapsid (Summers, 1971). The nucleocapsids pass through the peritrophic membrane and fuse to the microvilli. They then enter a microvillus, migrate to the nucleus, and attach to the nuclear pores within 2 to 6 hours post infection (Tanada & Kaya, 1993; Ohkawa *et al.*, 2010). The viral DNA genome enters the nucleus through the nuclear pore.

Virus replication takes place in the stroma of the nuclei in the midgut cells and eventually in both the nuclei and cytoplasm when the nuclear membrane ruptures. Infection of these cells occurs without the production of OBs. Nucleocapsids are budded through the basal lamina membrane of the cell and into the haemocoel. The infective virus is then transported around the insect and results in secondary infections of various insect tissues.

Virus replication is followed by budding of nucleocapsids from infected cells to infect other cells within the insect body or by inclusion of the nucleocapsids in granulin to form the characteristic OBs (Tanada & Kaya, 1993). Fat body cells produce the highest numbers of OBs. The OBs are infectious only upon ingestion by other larvae (Figure 1.7).

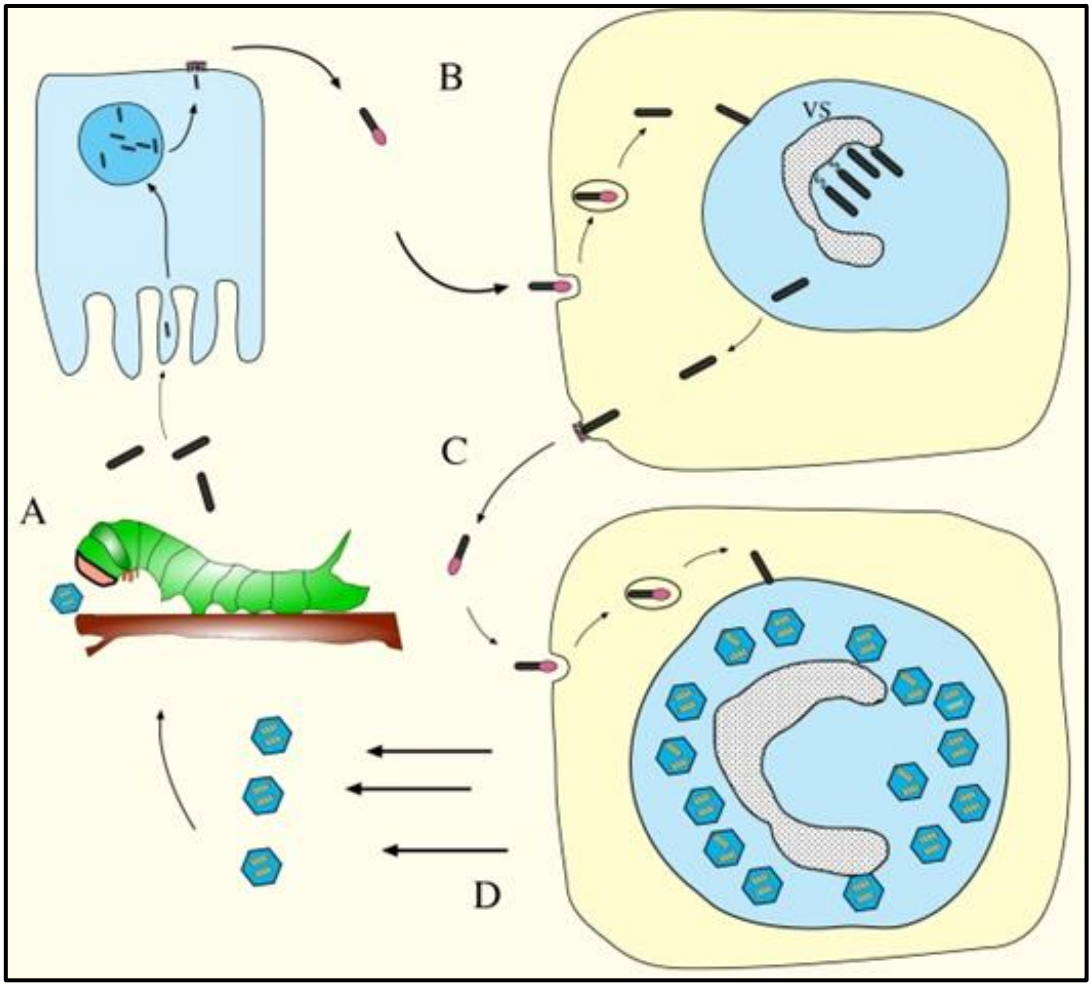


Figure 1.7 The main features of the biology of baculoviruses A: OB ingested by an insect; B: Occlusion derived virus is released in the midgut, bind to epithelial cells and replicate near the nucleus; C: Budded virus produced spread the infection throughout the insect; D: Occluded virions are produced, and the cell then dies releasing the occlusion bodies and the cycle is repeated (Rohrmann, 2013)

1.3.1.5 Natural occurrence

Baculoviruses are thought to occur in an enzootic state within a host population. This means that the virus is constantly present in the host population but at a very low prevalence (Tanada & Kaya, 1993). Observations of natural incidence of baculovirus disease are limited to relatively few isolates (Crook, 1991); however natural epizootics caused by baculoviruses are not uncommon. African examples of epizootics include the NPV of the African armyworm, *Spodoptera exempta*, in Kenya (Odindo, 1983) and the NPV of four species of semi-looper on soybean in Zimbabwe (Kunjeku *et al*., 1998). These two examples as well as many others worldwide involve surface feeding pests. *Cydia pomonella* larvae are cryptic pests, spending the majority of their life-cycle feeding within the fruit and therefore are considered to not be as susceptible to epizootics.

Eastwell *et al*. (1999) surveyed four sites within British Columbia, Canada and established that 23% of the wild population of *C. pomonella* larvae carried CpGV. They found that the frequency of positive PCR reactions revealed significant levels of CpGV in *C. pomonella* populations in all the commercial fruit production areas sampled. The data also suggested that, within *C. pomonella* populations of British Columbia, infection by CpGV was endemic but no epizootics were reported.

1.3.2 *Cydia pomonella* Granulovirus (CpGV)

The *Cydia pomonella* granulovirus was first isolated from infected larvae collected near Valle de Allende in Mexico (Tanada, 1964). Although CpGV is known to be one of the most virulent baculoviruses, its isolation from naturally occurring populations of *C. pomonella* is problematic and only a few isolates have been fully described worldwide. These include CpGV-M, CpGV-E2, CpGV-I12, CpGV-I07 and CpGV-S (Luque *et al*., 2001; Gebhardt *et al*., 2014). The main contributing factor to this problem is that diseased larvae usually rupture and disintegrate before being found.

1.3.2.1 History of CpGV based products

The first commercial formulation of CpGV, SAN 406, was developed by Sandoz Corporation (Switzerland). It was granted an experimental permit in 1981 and was tested worldwide till 1984, after which all work on the virus was terminated until fairly recently. In Europe the virus was tested extensively and in 1979 the Commission of European Communities (CEC) initiated the 'Biological Control in Apple Orchards' programme (Falcon & Huber, 1991). Work on the virus continued in Europe after the abandonment of the product by Sandoz. It was a collaborative effort between European government agencies scientists and European scientists working in conjunction with commercial companies that led to the development and commercialization of CpGV products. Three products were commercialized: Madex® which was registered in 1988 by Andermatt Biocontrol; Granupom™ which was registered in 1991 by Hoechst and Carpovirusine™ which was registered in 1993 by Calliope. All commercial products of CpGV produced prior to 2000 were based on the Mexican isolate (CpGV-M). With current research, more isolates have been identified and are currently being utilized in commercial formulations of the product (Table 1.4)

Table 1.4 Commercially available CpGV based products.

Producer	Country of origin	Product	Isolate used	OBs/litre
Andermatt Biocontrol AG	Switzerland	Madex, Madex *Plus*, Madex *Max*, Madex Top, Madex Twin and Madex I12	CpGV-M Laboratory selected and CpGV-I12	3×10^{13}
Arysta Lifescience Corporation	France	Carpovirusine, Caropvirusine Super SC	CpGV-M not kown	1×10^{13}
Probis GmbH	Germany	Granupom	CpGV-M	2.2×10^{13}
Certis USA	USA	Cyd-X	CpGV-M	3×10^{13}
BioTepp	Québec, Canada	Virosoft CP4	not known	4×10^{13}

1.3.2.2 Specificity

The specificity of CpGV to *C. pomonella* and its safety to non-target organisms has been well documented by several authors and reviewed by Gröner (1990). CpGV may infect other *Cydia* spp. as well as some species in closely related genera (Lacey *et al*., 2008a). However, the dose of virus required to other *Cydia* spp. is significantly higher. Due to the specificity and virulence of CpGV it should be considered as a key component of any pest management programme within pome fruit orchards (Lacey & Thomson, 2004). The use of CpGV will also contribute significantly to the conservation of other natural enemies in the orchard agroecosystem not only due to the specificity of the product but also the reduced reliance on 'hard' chemicals for the control of the pest (Lacey & Thomson, 2004). Reducing the requirements for harsh chemical applications further reduces the risk of

secondary pest repercussions as well as limiting the negative impact on natural enemies within an orchard complex.

1.3.2.3 Formulation and product shelf life

Currently all formulations of CpGV are manufactured and sold as suspension concentrates. All formulated products contain various additives, either in the product or that are required to be added before field application. The use of these additives and various formulation techniques are required to increase the shelf life of the product as well as to increase the ease and effectiveness of its application in the field (Burges & Jones, 1998). It is important for commercial purposes to consider both consistent product potency as well as the stability of the product. Specific product formulation is generally not public knowledge as most private concerns consider this to be confidential information. It is, however, a very important aspect of the commercial product and research into various additives is ongoing.
Wetting and sticking agents are generally recommended for use in conjunction with the virus. This improves mixing, reduces surface tension and increases deposition of the virus over the plant surface (Burges & Jones, 1998). A virus application is only effective if the larvae ingest a large enough dose of OBs, therefore the limited feeding of larvae before entry into the fruit is a major constraint to the effectiveness of a GV. Various phagostimulants have been tested in conjunction with CpGV applications of which a number have shown positive results (Pszczolkowski *et al*., 2002; Pszczolkowski & Brown, 2004). Molasses has been reported as one of the most effective feeding stimulants for Lepidoptera pests (Ballard *et al*., 2000). From 2004 to 2011, Citrus Research International, South Africa conducted nine field trials in which 11 comparisons were made between virus products (based on the *Cryptophlebia leucotreta* granulovirus) applied at the registered rate, with and without molasses. Eight of these comparisons were conducted with Cryptogran™ (River Bioscience PTY Ltd, South Africa) and the other three with Cryptex™ (Andermatt, Switzerland), both commercially available baculovirus products for the control of *T. leucotreta*. The results showed that in all cases, efficacy was superior where molasses was added with statistically significant differences shown in most instances (Moore & Kirkman, 2011). The addition of 15% cane molasses was found to

reduce the medium exposure time for *C. pomonella* neonates to acquire a lethal dose of CpGV from 269 to 5 min (Ballard *et al*., 2000). However it was noted that at a concentration of 1% (v/v) it had no effect.

A product shelf life of 12 to 18 months has been recommended for CpGV. Shelf life studies have concluded that CpGV had no loss of activity if stored at -20°C for 3 years (Geissler, 1994). Lacey *et al*. (2008b) showed no decline in the activity of Cyd-X and Virosoft formulations stored at 2°C for 3 years or at room temperature for 2 years. However prolonged exposure to high temperatures may have a detrimental effect on virus potency. The pathogenicity of the virus may be significantly reduced when exposed to 35°C for 20 to 40 weeks (Lacey *et al*., 2008b).

1.3.2.4 Application

Spraying is the most common method of applying baculoviruses for the control of insect pests (Hunter-Fujita *et al*., 1998). Virus applications are generally applied as full cover applications. This is an important consideration because baculoviruses need to be ingested in order to infect the target pest and therefore it is essential to achieve complete coverage with the application. Although a number of the commercial products available have ultra violet (UV) protectants within their formulation, the virus's sensitivity to solar radiation (UVB, 180 to 320 nm) is still the main contributing factor which limits the virus's persistence in the field (Jaques, 1985; Ignoffo, 1992). A recent report has shown that the addition of ZnO and TiO_2 to CpGV formulations hold significant promise as UV protectants at application rates which are considered safe and effective (Wu *et al*., 2015) It is essential to apply a virus product either at night or during a time period of low UV pressure. Due to limited persistence, virus should be reapplied throughout the peak egg hatch period. The recommended application intervals for multivoltine populations range from 7 to 14 days (Huber & Dicker, 1977; Stará & Kocourek, 2003; Arthurs *et al*., 2005). The number of applications will vary depending on the number of generations experienced in the control area.

Smith & Bouse (1981) emphasised the importance of accurately selecting, calibrating and recording the factors pertaining to spray application. The records should include the type of spray machinery used, spray pressure, and flow rates of spray fluids. This information, in combination with the details on the formulation of the product and on environmental factors should allow analysis of the appropriateness and success of the mode of application.

1.3.2.5 Resistance in field populations

Until the discovery of resistance to CpGV in a *C. pomonella* population, field observations of baculovirus resistance were considered extremely rare (Cory & Myers, 2003). Historically *C. pomonella* has developed resistance to a variety of chemical pesticides used for its control and currently continues to do so (Dunley & Welter, 2000; Reyes *et al*., 2007). With the extensive use of CpGV in organic orchards throughout Europe the threat of *C. pomonella* developing resistance was a cause for concern. Recently resistance has been reported in Germany and France in orchards treated with multiple applications of the virus (Jehle *et al*., 2008; Jehle *et al*., 2010; Schmitt *et al*., 2013). These populations were found in organic apple orchards, where intensive CpGV application failed in *C. pomonella* control. Jehle (2008) reported that more than 35 orchards in Germany, France, Italy, Switzerland and the Netherlands have been identified as having very low CpGV susceptibility. It was also noted that the resistance was geographically widely distributed but local due to the fact that single orchards and not large areas were affected. Therefore it was concluded that the genes for resistance were widely distributed but appear in very low frequencies in non-resistant populations of *C. pomonella* and it is possible to select for resistance under CpGV selection pressure. Resistance ratios in some populations have been found to exceed 1000 (Asser-Kaiser *et al*., 2007), and laboratory studies have shown it is possible for *C. pomonella* to develop extreme resistance due to sex-linked inheritance of a dominant-resistant gene (Asser-Kaiser *et al*., 2007). In order to predict or determine the potential for resistance to develop in a population, several factors need to be taken into account. These include; the frequency of genes responsible for resistance in *C. pomonella* populations; the number and frequency of CpGV applications per season and the size of

the treated areas and its proximity to untreated *C. pomonella* populations (Lacey *et al*., 2008a). An integrated approach that alternates CpGV with soft interventions (eg, mating disruption) along with a clear integrated management strategy should be considered if multiple virus applications are used extensively in an area. This area of research requires urgent attention and further study in the future.

The mode of inheritance of CpGV resistance was determined by mass crossing experiments (Eberle & Jehle, 2006) and single pair crossing experiments (Asser-Kaiser *et al*., 2007) between a susceptible and a resistant *C. pomonella* strain, followed by back-crossings and susceptibility tests of the offspring. These crossing experiments will determine whether the inheritance of resistance is recessive or dominant, linked to the sex chromosomes or to the autosomes, polygenic or monogenic. From both approaches it was concluded that resistance is incomplete dominant. Mass crossing performed with a non-homogenous resistant *C. pomonella* population initially suggested that the resistance gene or genes may be located on an autosome (Eberle & Jehle, 2006). Asser-Kaiser *et al*. (2007) unequivocally demonstrated that in single-pair crossing experiments performed with a genetically homogenous resistant *C. pomonella* strain, the allele responsible for CpGV resistance were sex-linked and located on the Z-chromosome of the moth. In *C. pomonella* as with most Lepidoptera, the sex chromosomes are ZW in females and ZZ in males and the dominance is concentration-dependent (Jehle, 2008). Recently published work has shown that resistance in *C. pomonella* to CpGV is isolate specific and related to a deletion in the *pe38* gene which is not only essential for the infectivity of CpGV, but also a key factor in managing CpGV resistance (Gebhardt *et al*., 2014).

1.3.2.6 The use of CpGV for the control of *Cydia pomonella*

As a result of the increased resistance to chemical control measures, the banning of various insecticides and lowering of acceptable maximum residue levels, research into the use of softer, safer control measures needs to be prioritized. A further factor driving this research is the growing public concern over the environmental and food safety aspects. Baculoviruses are considered low risk control agents which under suitable field conditions

can persist in the environment. Most baculovirus formulations are applied using standard spray methods (Moscadi *et al*., 2011; Rodrigeuz *et al*., 2012). Furthermore generally the efficacy of baculoviruses are not affected when mixed with other chemicals in a tank mix (Moscadi, 1999). However CpGV should not be mixed with sodium silicate, calcium polysulfide, *Bt* products and copper based fungicides as these products may adversely affect the viability of the OBs (Gut, 2005; Fritsch *et al*., 2012). The use of CpGV as a soft approach to *C. pomonella* control has received much attention over the last two decades. Extensive field trials have demonstrated promising activity of CpGV at orchard level.

Most commercial formulations of CpGV make use of the original Mexican strain (CpGV-M) of the virus. CpGV is one of the most virulent GVs known and is categorized as a type 2, which is faster acting then the type 1 GV (Lacey *et al*., 2008a). The OBs must be consumed in order to infect the larvae. Estimated LD_{50} values for neonate larvae range from 3 to 5 OBs (Sheppard & Stairs, 1976; Harvey & Volkman, 1983; Crook *et al*., 1985).
A number of authors have compared genetic variation within CpGV (Harvey & Volkman, 1983; Crook *et al*., 1985; Eberle *et al*., 2009; Eberle, 2010). Crook *et al*. (1985) compared different strains of CpGV using restriction enzyme analysis, most of which were indistinguishable from the Mexican isolate. However two isolates, one from Russia (CpGV-R) and another from England (CpGV-E) showed small genotypic differences. CpGV-E was found to be equally virulent when compared to the Mexican strain. Resulting infectivity of the CpGV-R isolate has varied considerably from being equal to CpGV-M (Crook *et al*., 1985) to up to 70 times less infectious to neonate larvae when compared to the Mexican strain (Harvey & Volkman, 1983). Recently, Eberle *et al*. (2009) identified eight new field isolates of CpGV. The isolates, originating from Iran, Georgia and England, were analysed using restriction fragment length polymorphisms and by partial genome amplification and sequencing. The results of the study indicate the predominant genotypes of these isolates could be assigned to those present in previously found isolates originating from Mexico (CpGV-M), England (CpGV-E) and Russia (CpGV-R). It was suggested that CpGV isolates can be described by four major genome types, which appeared to exist in different isolates as genotypic mixtures. Further studies have revealed that CpGV isolates that differed in their restriction profiles from CpGV-M were able to

overcome resistance. Isolates with similar profiles to CpGV-M showed a similar biological activity. It was noted that resistance to CpGV is not only isolate specific, but seemed to be specific to all CpGV-M like isolates (Eberle, 2010).

The first field tests with CpGV were conducted in California from 1966 to 1977 (Burgerjon, 1986). During the 1970s further testing was conducted in the USA, Australia and in New Zealand. SAN 406, an experimental-commercial formulation of CpGV was produced by Sandoz in the early 1980s. This allowed for more extensive field testing of CpGV against the *C. pomonella* on apple, pear and to a lesser extent walnuts (Burgerjon, 1986). Successful field trials have been conducted in North America, Europe, New Zealand, Argentina, South Africa and Australia (Jaques *et al*., 1981; Glen & Payne, 1984; Falcon & Huber, 1991; Cross *et al*., 1999; Suckling *et al*., 1999; Stará & Kocourek, 2003; Lacey *et al*., 2007; Simon *et al*., 2007). Lacey *et al*. (2008b) suggested that the level of population control depends on a number of variables including dosage, frequency and timing of the virus application. Early work on CpGV showed the virus to have promising signs as a potential biological control agent. Huber & Dickler (1977) tested CpGV in a commercial apple orchard for two years and compared it to organophosphorus insecticides. They found that the efficiency of the virus was influenced by the soil management in the orchard and were able to achieve an 88% reduction in fruit damage. The use of CpGV resulted in a 44 to 85% reduction in injury to apples as opposed to a 72 to 98% reduction with the use of chemical applications. They also indicted that the use of the virus had no effect on reducing other arthropod numbers in the orchards. Later studies by Jaques *et al*. (1994) reported that the use of CpGV could reduce the deep-entry damage to apples by 40 to 83% compared to the respective control plots. In some of their trial data the protection of fruit by CpGV exceeded that of the organophosphate insecticide, Phosmet. They concluded from their studies that CpGV may be substituted for some, if not all, chemical insecticides for the control of *C. pomonella* through the growing season.

Dosage rate studies using CpGV have been conducted by various authors (Sheppard & Stairs, 1976; Glen & Payne, 1984; Stará & Kocourek, 2003). Sheppard & Stairs (1976) tested a range of doses from 10^7 to 10^9 OBs/tree. All the doses tested had a similar effect

on the reduction of infestation but it was found that with the higher dosages there was a larger reduction in larval population as they entered the fruit. They also found the length of time for CpGV development to be inversely proportional to virus dosage. LT_{50} values range from 9.7 days for 3 OBs per larva to 3.7 days for 280 OBs per larva. Falcon *et al*. (1968) reported a 90% reduction in shallow entries, whereas shallow entries were only reduced by 40%. Their conclusion was that high doses of virus are no more effective than the lower doses at protecting fruit from feeding damage; however, higher doses were more effective at reducing the larval populations. Glen & Payne (1984) concluded that CpGV is an effective control agent particularly in reducing the number of larvae and the more severe forms of fruit damage such as deep entries, which confirms the conclusions drawn by Sheppard & Stairs (1976). In their field trials the applications of two high-volume CpGV sprays (7×10^{10} OBs/litre) reduced both the number of mature larvae and fruit damage to levels similar to those obtained from two applications of azinphos-methyl. Increasing the concentration of CpGV resulted in the damage to fruit diminishing slowly with increasing virus concentration.

Stará & Kocourek (2003) tested various concentrations of CpGV ranging from 0.5 to 1×10^{13} OB/ha, as well as varying numbers of applications per season. Their results indicated that the biological efficacy of CpGV in reducing *C. pomonella* population densities ranged from 75.5 to 96% compared to 90.8 to 97.5% achieved with the use of teflubenzuon. These results are comparable to those achieved by Arthurs *et al*. (2005) who tested three concentrations of CpGV against high *C. pomonella* populations, resulting in 81 to 99% larval mortality in fruit and a reduced number of mature larvae collected in tree bands by 54 to 98%. Both these studies showed that CpGV was more effective at reducing the *C. pomonella* population density than reducing fruit injury.

Glen and Clark (1985) found that different concentrations of CpGV did not significantly affect the survival of the neonate larvae before they entered the fruit. In the initial trials, 49% of the larvae survived long enough to cause recognizable damage to the fruit. In the second experiment 69% of the larvae produced damage to the fruit irrespective of the treatment applied. It was however noted that the neonate larvae usually died shortly after

entering treated fruit. This study highlights a potential shortcoming of CpGV namely its speed of kill, which has been highlighted in the literature.

1.4 PROJECT AIMS

1.4.1 Justification

Cydia pomonella is considered one of the most significant pests of apples and pears worldwide with infestation rates of up to 80% in apple orchards recorded when no control measures are implemented (Myburg, 1980; Pringle *et al*., 2003). CpGV-M was first isolated from infected larvae in Mexico and has been used in all commercial CpGV products until recently when resistance was discovered in *C. pomonella* populations (Jehle *et al*., 2008; Jehle *et al*., 2010; Schmitt *et al*., 2013). Resistance has been reported in 35 countries in Europe. This has led to a significant amount of research being conducted on the identification of novel isolates of CpGV in an attempt to manage resistant *C. pomonella* populations. In South Africa, both commercially available CpGV products make use of the Mexican isolate and the development of resistance in insect populations poses a potential problem if the use of CpGV is not managed correctly. The first overall aim of this study was to identify and generically characterise novel isolates of CpGV with comparable or superior biological activity that could be applied either in conjunction with or as an alternative to CpGV-M as a resistance management tool to reduce the selection pressure on CpGV-M.

CpGV is produced *in vivo* in *C. pomonella*. Rearing sufficient quantities of larvae for *in vivo* virus production is generally the limiting factor as production is in many instances labour intensive and thus costly. Reiser *et al*. (1993) considered *T. leucotreta* as a promising alternative host for the mass production of CpGV. Both *C. pomonella* and *T. leucotreta* were found to produce similar amounts of OBs per larva. However, it required a 10000 fold higher inoculum of CpGV to infect the fifth instar larvae of *T. leucotreta*. No lethal concentration (LC_{50}) or lethal time (LT_{50}) figures were reported in the study. The authors concluded that the ease of rearing *T. leucotreta* as an alternate host compensated

for the greater amount of inoculum required. Their final conclusion was that there is substantial scope to further the study and they suggested that it can be continued under large scale production conditions. The second overall aim of this study was thus to further evaluate CpGV production in *T. leucotreta* by determining biological activity against late instar *T. leucotreta* larvae and to develop a production technique.

1.4.2 Study objectives

1. To identify novel South African CpGV isolates from field populations of *C. pomonella* and to genetically characterise them using restriction endonuclease analysis of genomic DNA and sequencing of selected viral genes. CpGV-M will serve as the reference isolate for comparison with novel strains.
2. To test the virulence of any novel isolates recovered against *C. pomonella* neonate larvae and compare this to the Mexican isolate of CpGV.
3. To evaluate the rearing parameters of both *C. pomonella* and *T. leucotreta*, and compare them in terms of efficiency and cost.
4. To quantify the yield of virus obtained from fourth and fifth instar larvae of both *C. pomonella* and *T. leucotreta* when inoculated with various concentrations of CpGV.
5. To assess the viability of producing CpGV in *T. leucotreta* by determining dose- and time-response curves (LC and LT values) using surface dose bioassays in the laboratory.
6. To evaluate the production of CpGV in fourth and fifth instar *T. leucotreta* larvae using mass production techniques.
7. To evaluate the purity of CpGV produced in *T. leucotreta* using REN analysis of genomic DNA and quantitative PCR and then to determine whether the production of CpGV in a heterologous host has any negative effects on the virulence of the virus, using surface dose bioassays.

2

A COMPARISON OF THE REARING OF *CYDIA POMONELLA* AND *THAUMATOTIBIA LEUCOTRETA*, ON ARTIFICIAL DIET, UNDER LABORATORY CONDITIONS FOR POTENTIAL VIRUS PRODUCTION

2.1 INTRODUCTION

Insects are reared for a variety of reasons. These may include classical biocontrol, augmentative (inundative or inoculative) biocontrol, human and animal feeds, genetic studies and for *in vivo* production of entomopathogenic biocontrol agents, such as viruses. Effective rearing of insects requires a reliable source of natural host material or a high quality artificial diet. Initially *C. pomonella* was reared on thinning apples (Dickson *et al*., 1952; Hathaway *et al*., 1971; Howell, 1972b; Toba & Howell, 1991; Howell & Neven, 2000). However this method was sufficient for research purposes but not considered to be economically viable for mass production or commercial use. It was also labour intensive and involved collecting the chemically or manually thinned apples, washing off the chemical residues and placing the apples in cold storage until required for rearing (Dyck, 2010). Artificial diets have been developed for rearing insects where the host material is not readily available or mass production of insects is required. Most of the initial diets developed for *C. pomonella* were agar based and as such, relatively expensive therefore a number of studies have been undertaken to find a replacement for agar as a gelling agent (Navon & Moore, 1971; Honda *et al*., 1996; Stenekamp, 2011). Various authors have described diets for *C. pomonella* and while some have been novel others were

modifications of existing diets (Ashby & Singh, 1990; Hansen *et al*., 2004; Eberle & Jehle, 2006). Navon & Moore (1971) developed a diet in which the agar was replaced with calcium-alginate gel to reduce costs. Further developments in the *C. pomonella* diet were described by Brinton *et al*. (1969), Howell (1972a), Toba & Howell (1991), Bloem *et al*. (2000), Botto (2006), Hansen & Anderson (2006) and Stenekamp (2011). All of these diets have been used for the mass rearing of *C. pomonella* and support the development of large numbers of healthy insects over many generations. The Canadian sterile insect release programme (SIR) facility has used a modified version of the diet described by Brinton *et al*. (1969) and has successfully reared *C. pomonella* for twelve years for use in a sterile insect release programme in British Columbia, Canada. The diet used by the Canadian rearing facility contains the following ingredients: paper pulp, sawdust, canola meal, whole wheat flour, sucrose, fumaric acid, choline chloride, vitamins, Wesson salts, wheat germ, gluten, methyl paraben canola oil and formaldehyde (Bloem *et al*., 2007).

Unlike a number of other lepidopteran species, *C. pomonella* larvae are cannibalistic and intolerant of overcrowding which results in a new set of challenges. Increasing larval densities may lead to a decrease in production efficiency (Pristavko *et al*., 1978; Hansen & Anderson, 2006). This reduction may result from overcrowding stress, increased effect of cannibalism due to an increased interaction of larvae or a reduction in larval size due to competition for nutrient resources, all of which would have a negative impact on virus production. Therefore, a compromise needs to be made between optimizing larval numbers, and avoiding stress as a result of high larval densities that could lead to decreased larval efficiency as well as an increased risk of disease. Various authors have reported on the quantity of diet required per larval unit. Howell (1971) reported that *C. pomonella* larvae required 1.3 ml of diet to complete development. However, in order to obtain maximum adult yield per rearing tray, each larva should be allocated 4.8 ml of diet. Siegal *et al*. (2001) produced healthy, disease free larvae, on 1.8 ml of diet per larva in individual tubes, however larval production in individual tubes makes the rearing process expensive.

Both *C. pomonella* and *T. leucotreta* have been mass reared for biological control programmes such as sterile insect technique and baculovirus production. Due to their close

genetic relationship it is possible that *T. leucotreta* could serve as a heterologous host for CpGV production (Reiser *et al*., 1993). A number of rearing parameters could benefit the production of CpGV in *T. leucotreta* as opposed to *C. pomonella*. *Thaumatotibia leucotreta* is not cannibalistic and larvae can be reared in large numbers in a confined area. Rearing densities of greater than 2.2 larvae per gram of diet have been achieved at River Bioscience in South Africa without any detrimental effect on larval or adult development (Moore *et al.*, 2014). Increased production per unit diet results in a decrease in the cost of rearing. The cost benefit is not only confined to dietary ingredients but also the labour requirement in preparation time and sanitation practices. *Thaumatotibia leucotreta* completes its life cycle in a shorter period of time than *C. pomonella*, both under laboratory rearing as well as mass production (Reiser *et al*., 1993). Therefore the decision to use one rearing or production protocol over another should be based on a number of factors: the cost of the ingredients, the safety and quality of the ingredients, the availability of the ingredients (local suppliers if possible), staff requirements, the infrastructural requirements of the rearing facility and finally the potential quantity and quality of the insects reared. Considerable numbers of large healthy larvae must be reared for optimal *in vivo* production of CpGV, whether it is produced in the homologous or a heterologous host. The literature reveals that this can be achieved for both *C. pomonella* and *T. leucotreta* (Brinton *et al*., 1969; Howell, 1972b; Bloem *et al*., 2000; Botto, 2006; Hansen & Anderson, 2006; Stenekamp, 2011; Moore *et al*., 2014). However, the cost per larval unit is significantly higher for *C. pomonella*. If production of the CpGV in *T. leucotreta* could be shown to produce virus of a high quality and more efficiently, this production technique could result in a reduced cost, increased profits and the ability for a company to produce a product than can compete in world markets.

The initial aim of this chapter was to establish and maintain viable *C. pomonella* and *T. leucotreta* populations in the laboratory for life table studies to assess which of the two hosts would have the greatest economic potential for *in vivo* production of CpGV on a commercial scale. Assessing the fecundity, rate of development, developmental mortality as well as taking into consideration the cost of rearing, which in itself is influenced by a number of factors, were considered important in assessing the suitability of each host. The

second aim was to determine the developmental time of each insect species and quantify the cost of rearing. Production of a virus in a heterologous host may increase the risk of a contaminated solution in the final virus product by triggering the covert virus of the heterologous host. It was therefore important to determine whether virus production in *T. leucotreta* would provide sufficient benefit over the production of CpGV in *C. pomonella* to warrant further investigation. These benefits would include a short life cycle, low mortality, efficient utilisation of the diet by the larvae, a cost effective diet, as well as a reduced labour component. From a commercial perspective, the aim would be to produce the largest amount of larvae in the shortest possible time with a minimal labour cost. Achieving this would result in a viable commercial product that will produce the maximum profit for the manufacture.

2.2 MATERIALS AND METHODS

2.2.1 Laboratory maintenance of lepidopteran colonies

2.2.1.1 *Cydia pomonella* rearing

Egg sheets were obtained from ENTOMON Technologies Pty Ltd, Stellenbosch, South Africa, to initiate a laboratory culture of *C. pomonella* at River Bioscience. A modified version of the diet described by Geunnelon *et al*. (1891) was used (Table 2.1). A closed rearing system, involving a honey jar, with a lid and breathable membrane, containing a sterile dietary environment was used. This method was preferred to the standard open tray rearing method described by Stenekamp (2011) and used by ENTOMON in a commercial SIT programme. The closed rearing system reduced the risk of contamination as once the jar had been inoculated with sterilised egg sheets the lid was closed and the risk of introducing virus particles and fungal spores was minimal.

Table 2.1 Dietary ingredients used the preparation of *Cydia pomonella* diet.

Ingredients	Weight or volume per rearing jar
Whole wheat flour	28.41
Wheat germ	7.15
Brewers yeast	7.61
Methyl paraben	1.00
Ascorbic acid	0.46
Benzoic acid	0.36
Reverse osmosis or distilled water (ml)	45

45 g of dry diet was measured into a glass honey jar (Consol). 45 ml of dH_2O water was dispensed into the surface of the diet after which a lid containing a breathable membrane (PTFE 2203, BreathTech, South Africa) was tightly fastened. Jars were allowed to stand for 30 min to ensure even dispersal of the water throughout the diet, reducing the risk of dry areas. The jars were placed into autoclave bags and cooked at 121°C for 15 min, after which the cooked diet was left overnight to cool. This allowed the excess moisture within the jars to evaporate leaving no visible water droplets.

Egg sheets were cut up into squares containing approximately 450 to 600 eggs, and surface sterilized using an 8% (v/v) formaldehyde solution (37 - 40% stock solution, Minema®). The main purpose of this was to control virus contamination, as the diet was not prone to fungal contamination, even when inoculated with unsterilized egg sheets. Through trial and error, 8% (v/v) formaldehyde solution was found to acceptably control virus outbreaks without having any significant effect on egg hatch and was therefore used for all experimentation unless otherwise stated. Surface sterilization involved dipping the egg sheet into the formaldehyde solution for 1 second. The lids of the jars were removed in a biohazard chamber and the surface sterilized egg sheets placed directly onto the surface of the diet. The rearing jars were placed into an environmental chamber (Forma Scientific, Inc. USA) and maintained at 26.5°C and 65% RH for the duration of the larval development. Pupation occurred inside the uningested and desiccated diet (Figure 2.1).

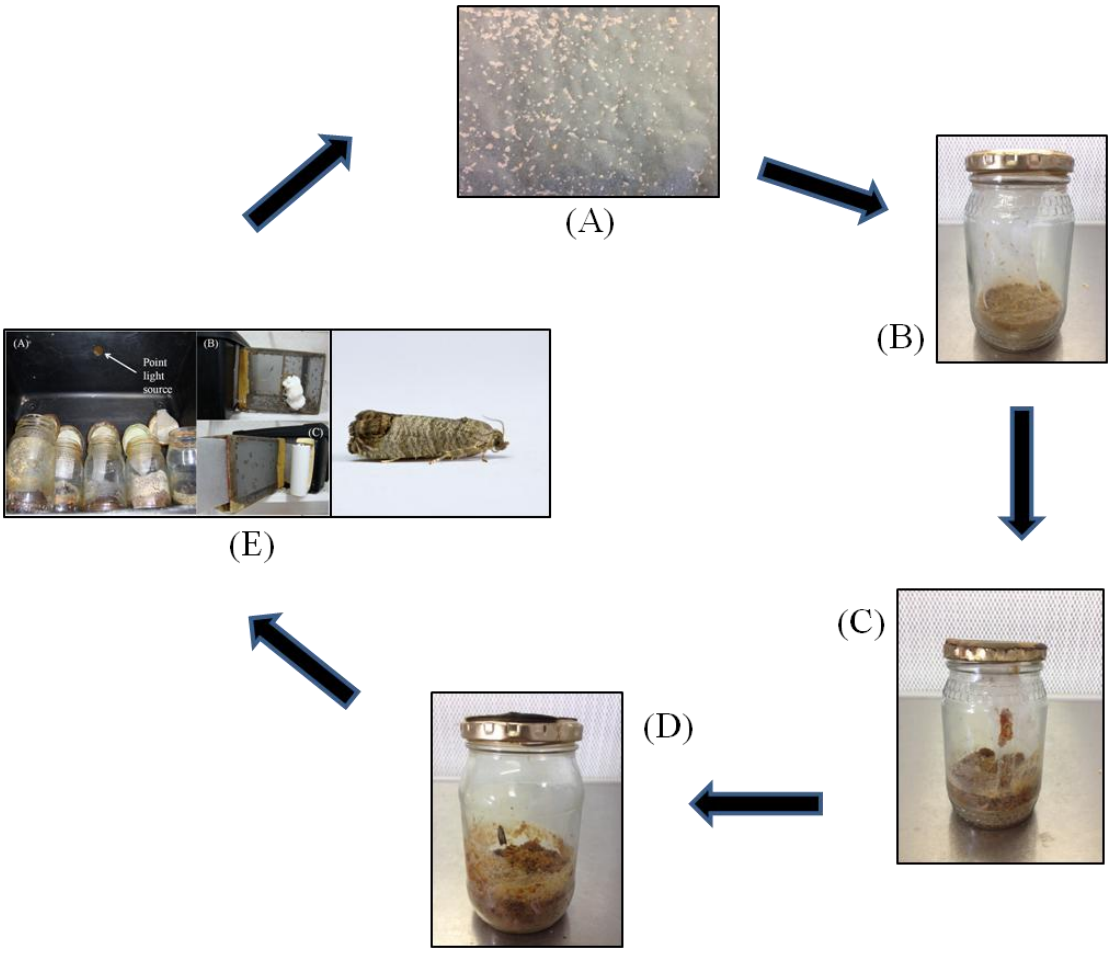


Figure 2.1 The protocol used for the establishment and maintenance of a laboratory culture from *Cydia pomonella* egg sheets. (A) Egg sheet. (B) Jars inoculated with egg sheets. (C) Larvae developing in the artificial diet. (D) First signs of eclosion (E) Eclosion box (see Fig 2.2. for larger image).

Jars were monitored daily for signs of eclosion. Once eclosion had commenced the jars were removed from the chamber and placed into an emergence box (Figure 2.2 A). On the front side of the emergence box a small hole had been cut to create a point light source which attracted emerging moths to move out of the dark chamber and congregate inside the nylon mesh emergence cage (Figure 2.2 B). The steel framework supporting the nylon mesh had a hole on one side which was plugged with wet cotton wool for moths to drink from. The cotton wool plugs were replaced daily. Wax paper was run along one side of the emergence cage and acted as an oviposition substrate (Figure 2.2 C).

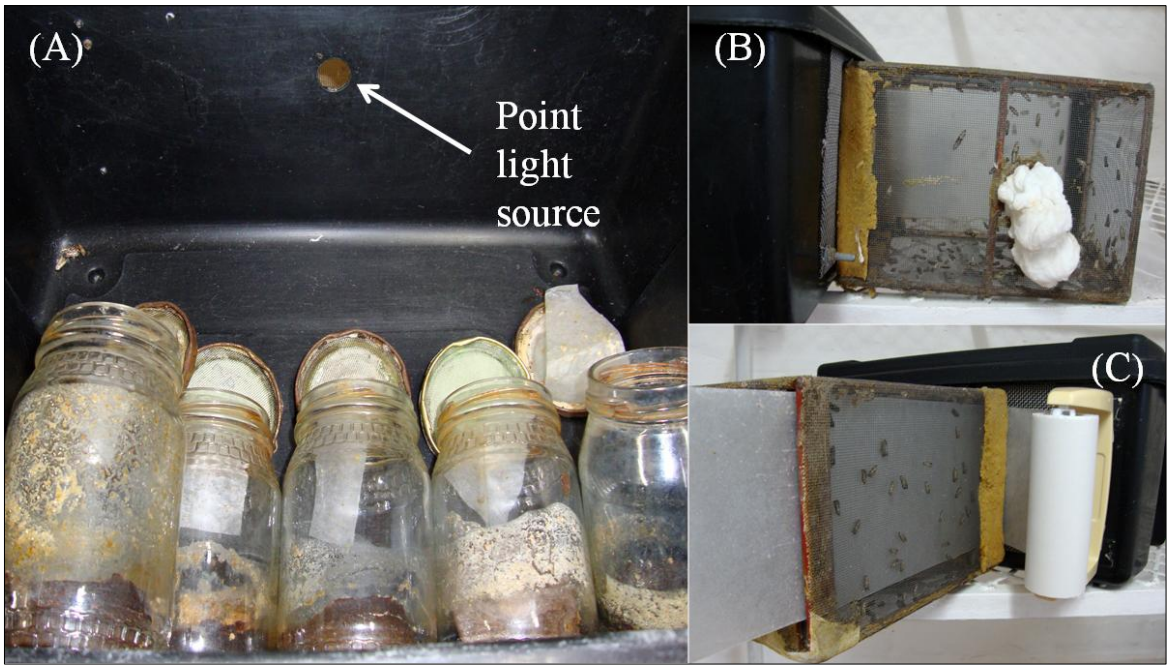


Figure 2.2 *Cydia pomonella* rearing technique. (A) Rearing jars in emergence box; (B) Nylon mesh cage; (C) Wax paper running on the inside of the emergence cage.

Moths were allowed to oviposit for 8 days after which the cage was cleaned and the next batch of jars containing pupae inserted. Egg sheets were collected every second day. The rearing process was repeated as described above for each generation. Attempts were made to establish new laboratory colonies from field collected infested fruit. Three geographically isolated orchards were chosen (Figure 2.3). An abandoned orchard near Vermaaklikheid (Table 2.2) was sampled as the orchard appeared to have been abandoned for many years and the fruit was heavily infested with *C. pomonella*. Orchards were chosen in the Langkloof, Eastern Cape Province and the Free State Province (Table 2.2) based on high pest pressure and it was also established that no previous applications of a commercial CpGV product had been applied as a control measure for *C. pomonella* in these orchards.

Table 2.2 Locations in South Africa sampled for the establishment of *Cydia pomonella* laboratory colonies.

Area	Farm	Province	Co-ordinates
Vermaaklikheid	Neglected orchard	Western Cape	34°18'19.15"S 21°01'37.51"E
Langkloof	Zondagh farms	Eastern Cape	33°44'42.91"S 23°15'24.77"E
Free State	Undisclosed location	Free State	N/A

Figure 2.3 Map of South Africa. Stars indicate areas which were sampled for CpGV.

Field collections took place in December 2011 and January 2012. Only a single collection was conducted at Vermaaklikheid in December 2011. The fruit was collected and brought back to the laboratory and dissected for the presence of larvae. All recovered larvae were placed directly onto an artificial diet plug in a glass vial as described above. The diet was prepared in 200 ml non-stick baking trays. 150 g of dry ingredients was mixed with 180 ml reverse osmosis water. The baking tray was covered with aluminium foil and cooked at 180°C for 12 minutes. Diet plugs were produced by inverting the vial and pressing it into the diet. The plugs were pushed into the vial and compressed lightly. The glass vials were placed into an incubation chamber at 26.5°C and 65% RH and subjected to a 16:8 hour day light photoperiod (D:L), then monitored daily for signs of eclosion. Once moths were visible, they were removed by placing the glass vial into a fridge at 4°C for 3 min thereby inactivating the moths and allowing one to remove them without any escaping. The moths were placed under a sieve on top of wax paper. A cotton wool plug was inserted into a tiny opening cut into the top of the mesh and wet daily to provide the moths with sufficient moisture (Figure 2.4). They were then kept in the environmental chamber and the wax paper monitored daily for the presence of eggs. The egg sheets were harvested and placed into a Petri dish and maintained in the environmental chamber until hatching. Larvae were collected using a paint brush (size 000) and placed directly onto the diet surface. Rearing jars were prepared as described above.

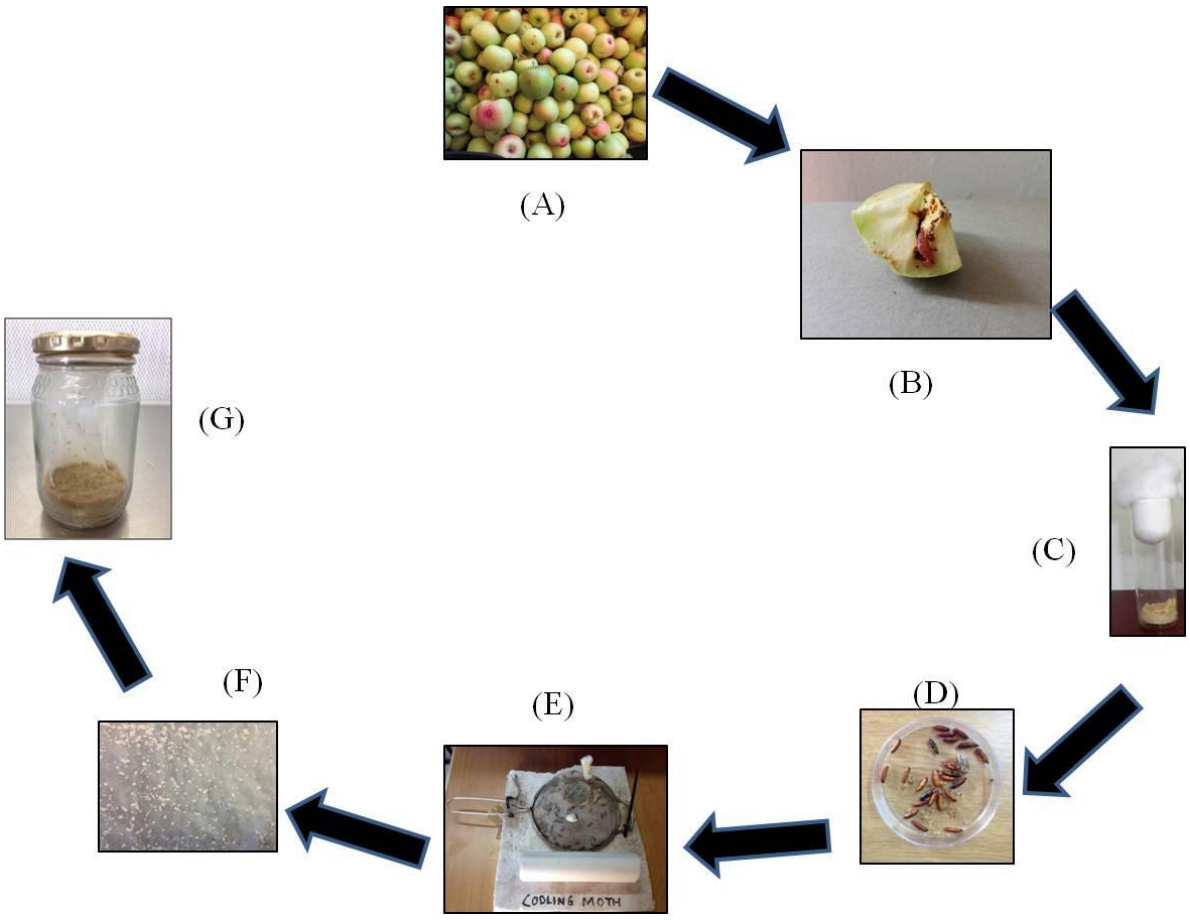


Figure 2.4 Protocol used for the attempted establishment of laboratory colonies of *Cydia pomonella* from field collected infested fruit. (A) Field collection of infested fruit. (B) Dissection of fruit. (C) Individual vials filled with artificial diet, with each holding a single larva. (D) Collection of pupae. (E) Adult moths placed under sieve. (F) Eggs sheets. (G) Neonates in diet.

2.2.1.2 *Thaumatotibia leucotreta* rearing

The *T. leucotreta* culture was established by Sean Moore in 1996 using field collected material from Citrusdal (Western Cape Province), Zebediela (Limpopo Province) and the Eastern Cape (Opoku-Debrah *et al*., 2014). The culture has been maintained by River Bioscience Pty Ltd. for over 200 generations estimated at a rate of one generation per month. The culture is therefore considered to be a mixed culture. *Thaumatotibia leucotreta* was maintained on an artificial diet and reared using a protocol described by Moore *et al*.

(2014). 45 g of Moore's diet was measured into a 352 ml glass honey jar (Consol, South Africa) (Table 2.3). 43 ml of reverse osmosis water was dispensed onto the surface of the diet. A lid containing a breathable membrane was then tightly fastened. The jars were placed into autoclave bags and cooked 121°C for 15 min, after which the jars were left overnight to cool. This also allowed for the excess moisture within the jars to evaporate leaving no water droplets on the sides of the jars.

Table 2.3 Dietary ingredients used per rearing 352 ml jar in the preparation of *Thaumatotibia leucotreta* diet.

Ingredient	Weight or volume per jar
Sifted maize meal	38.17g
Wheat germ	3.82g
Brewers yeast	1.91g
Full cream milk powder	0.29g
Methyl paraben	0.70g
Sorbic acid	0.12g
Reverse osmosis or distilled water	43ml

Thaumatotibia leucotreta egg sheets were cut up into squares containing approximately 400 eggs in a biohazard chamber and surface sterilized in 25% (v/v) formaldehyde (37-40% stock solution, Minema®) for 1 s, after which they were placed directly onto the surface of the diet. The jars were then moved into an environmental chamber and maintained at 26.5°C and 65% RH for the duration of the larval development. A photoperiod of 16:8 hour L:D was set. Approximately 10 days post inoculation, the jars were removed from the environmental chamber and placed under a laminar flow bench where the lids were removed and a sterile cotton wool stopper inserted. The jars were then returned to the environmental chamber for a further 12 days to allow the late instar larvae to move out of the diet and pupate in the cotton wool (Figure 2.5D). After 22 days the cotton wool stoppers, containing the pupae, were removed from the jars and placed into a wooden emergence box (Figure 2.5E). At the front end of the emergence box, a small door containing an exit hole was closed. This created a point light source which attracted

emerging moths to move out of the dark chamber and congregate and mate inside the stainless steel mesh emergence cage. The stainless steel mesh had a hole punctured on one side which was plugged with wet cotton wool for moths to drink from. The moths were fed with water daily. Wax paper was run along one side of the emergence cage and acted as an oviposition substrate. The mated female moths oviposited on the wax paper sheet for up to 6 days after which the cage was cleaned and the next batch of pupae inserted. Egg sheets were collected daily and inoculated onto Moore's diet for the subsequent rearing of the next generation.

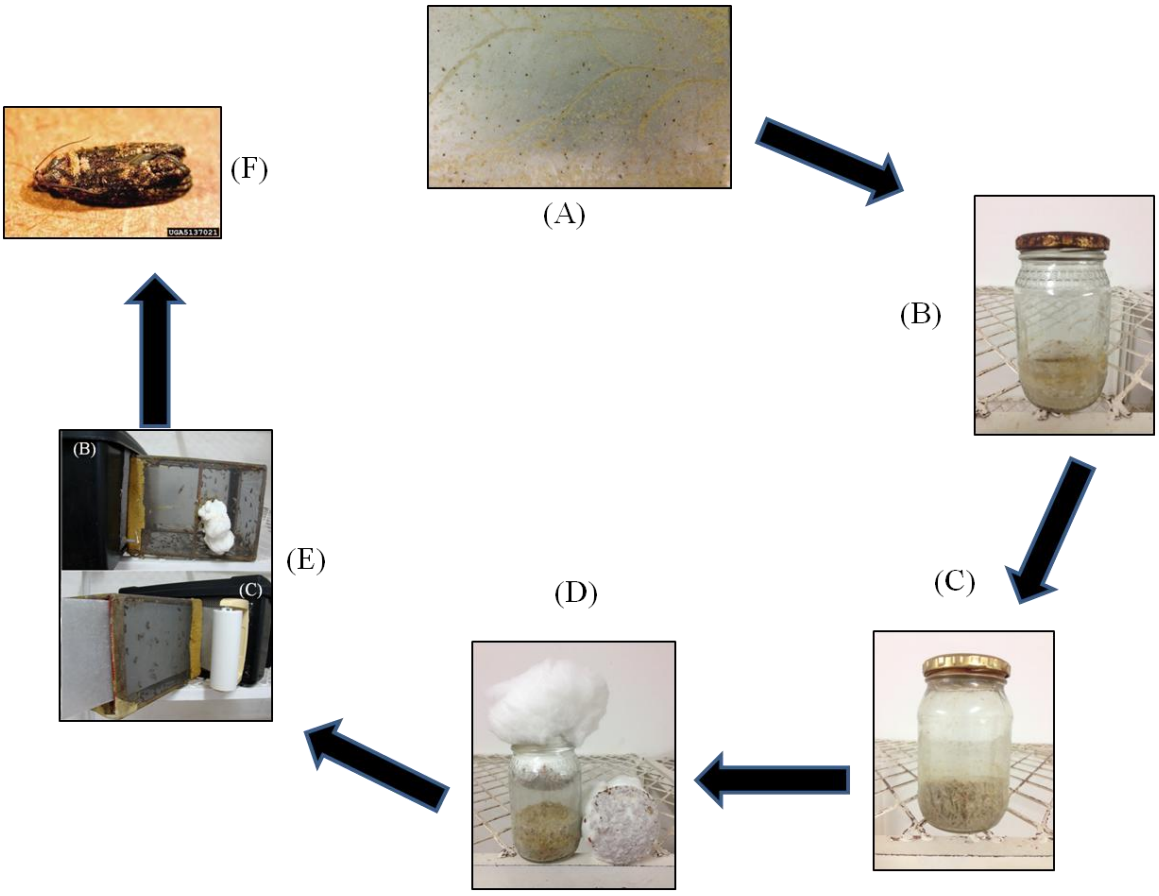


Figure 2.5 The protocol used for the establishment and maintenance of a laboratory culture of *Thaumatotibia leucotreta*. (A) Egg sheet. (B) Jars inoculated with sterilized egg sheets. (C) Larvae developing within the artificial diet. (D) Cotton wool plug containing pupae. (E) Eclosion box. (F) Adult *T. leucotreta*.

2.2.1.3 Temperature and humidity

Temperature and humidity were controlled using an environmental chamber. The environmental chamber was supplied with distilled water for increasing the humidity above ambient when required. Rearing conditions were set at 26.5°C ± 0.5 and 65% RH respectively for the duration of the host biology studies, except where otherwise stated. A 16:8 hour L:D photoperiod was set for the egg hatch and larval development. Temperature and humidity readings were periodically recorded using an OPT 506H data logger (Optirion Coprporation, USA) and the data visualized using the OptiView interface programme (ver. 1.30) to ensure the temperature and humidity was within the set parameters.

2.2.1.4 Quality control

Strict hygiene control practices were adopted for all rearing techniques to ensure that insects were reared in a disease free environment. All insectary cages, including the environmental chamber, were sterilized monthly using a 5% v/v bleach solution (14% sodium hypochlorite, Protea Chemicals, South Africa). Once moths from a particular batch had finished ovipositing, the moth emergence boxes were washed with detergent and disinfected with bleach. The cotton wool stoppers used by pupating insects were also either discarded or recycled by removing the empty pupal cases and autoclaving at 121°C for 15 minutes. All other materials used in the rearing process were either sterilized by autoclaving or bleaching. Blue Death (Perimethrin 3 g/kg Carbaryl 15 g/kg, Tiger Consumer Brands Ltd, South Africa) was used to prevent ants from damaging the colonies by feeding on larvae, pupae or moths. Moths that had escaped from the emergence boxes were killed using an electronic inset killer (Ultrablitz, South Africa). The floor of the insectary was mopped daily using PinSAN® (3 - 6% alkyl dimethyl ammonium chloride, Dynachem, RSA) antiseptic liquid diluted with water (5% v/v).

2.2.2 Host Biology

2.2.2.1 Fecundity and time to oviposition

Eighty male, and female pupae from both the *C. pomonella* and *T. leucotreta* cultures were collected and kept separately at 26°C ± 0.5 until sufficient moths had eclosed. Male and female moths of each species were collected and introduced into a ventilated plastic container and left for 8 hours to allow for coupling. Coupled moths were selected and placed onto wax paper discs and covered with a 9 cm diameter stainless steel sieve (Figure 2.6). The moths were given water twice daily by soaking the cotton wool plug in ddH_2O. Oviposition was recorded every 8 hours by removing and replacing the wax paper discs. The number of eggs oviposited was recorded by viewing the egg sheets under an Olympus dissecting microscope (Olympus SZ61) at 2.5 x magnification. Recording of oviposition continued until the death of the female, after which the mesh sieve was inspected for the presence of eggs. If any were found, they were counted and added to the final count. Three coupled moths were selected from each species and the experiment was repeated nine times. A two sample Student's *t*-test, assuming equal variances, was used to compare oviposition between *C. pomonella* and *T. leucotreta*.

Figure 2.6 Paired moths contained under a 9 cm diameter stainless steel sieve.

2.2.2.2 Percentage hatch

Jars (*C. pomonella*) or cotton wool stoppers (*T. leucotreta*) containing pupae were placed into a separate eclosion chambers at 26.5 ± 0.5°C. The eclosion chambers were monitored daily until sufficient moths had eclosed and egg production was at a maximum. A fresh sheet of wax paper was placed in the eclosion box and the moths allowed to oviposit for 8 hours. Both *C. pomonella* and *T. leucotreta* egg sheets were cut up into squares containing approximately 30 to 50 eggs per sheet. Egg sheets were surface sterilized by dipping them into a formaldehyde solution, 25% (v/v) for *T. leucotreta* and 8% (v/v) for *C. pomonella* (37% stock solution, Minema®) for 1 s. An 8% solution was used for *C. pomonella* as the diet was not prone to fungal contamination and dilution was sufficient to control viral contamination without having a detrimental effect of egg hatch. Smaller sheet sizes were chosen for increased accuracy of the counts and only egg sheets with evenly distributed oviposition were selected. Three egg sheets were used per repetition and the experiment was repeated twelve times. Each egg sheet was counted using a dissecting microscope and placed into a sterile Petri dish. The egg sheets were kept in the environmental chamber at 26.5°C and 65 ± 5.5% RH for 120 hours and subjected to a 16:8 hour L:D photoperiod. Egg sheets were then removed and examined under the dissecting microscope. The egg was considered to have hatched if an exit hole was visible and/or the chorion was transparent. Eggs containing visible live larvae were recorded as unhatched. Percentage hatch was compared between *C. pomonella* and *T. leucotreta* using a non-parametric Mann-Whitney-U-test (StatSoft, 2013).

2.2.2.3 Egg developmental times

Egg sheets were collected and prepared as in section 2.2.2.2, however they were not subjected to any form of surface sterilization. Egg sheets were placed into Petri dishes and maintained in an environmental chamber at 25°C and 65% RH with a photoperiod if 16:8 hour L:D. Egg hatch was recorded every 8 hours by examining the egg sheets under the dissecting microscope and counting all hatched eggs. Hatched eggs were counted using the same criterion established in section 2.2.2.2. To avoid counting the same egg twice each

egg was marked with a super fine tip marker once counted. The experiment was terminated after 6 days. Each experiment consisted of 21 egg sheets and was repeated 3 times. Mean time to hatch was compared between *C. pomonella* and *T. leucotreta* using a Student's *t*-test.

2.2.2.4 Larval developmental times

Moths were allowed to oviposit for 8 hours before removing the egg sheets. Egg sheets were placed in a glass honey jar and incubated at 26°C in an environmental chamber and monitored for egg hatch. Only larvae hatching within the first 4 hours were used. Rearing jars were prepared as in section 2.2.1.1 (*C. pomonella*) and section 2.2.1.2 (*T. leucotreta*). Twenty five larvae were transferred onto the surface of the diet using a 000 paint brush. Ninety jars for both *C. pomonella* and *T. leucotreta* were inoculated. The jars were maintained in an environmental chamber at 26°C and 65% RH for the duration of the experiment and subjected to a 16:8 hour L:D photoperiod. Every 8 hours a jar was removed, and the diet carefully broken apart to determine larval survival and larval developmental stage. Dyar's rule states that there is a robust relationship between larval instar and head capsule width when the measurements are plotted on a logarithmic scale (Dyar, 1890). This rule was used to determine the larval stage in both *C. pomonella* and *T. leucotreta*. Larval head capsule widths were measured using a dissecting microscope. Head capsule ranges for each instar have been described by Blomefield (2003) and Blomefield & Giliomee (2009) for *C. pomonella* and Daiber (1979) for *T. leucotreta*. These data were used to determine larvae instar at each sample. The number of recovered larvae was also recorded. The experiment was conducted twice. The mean time taken to reach each instar was compared between *C. pomonella* and *T. leucotreta* using a Student's *t*-test in Statistica v12 (StatSoft, 2013).

2.2.2.5 Efficiency of rearing

Moths were allowed to oviposit for 8 hours before removing the egg sheets. Only egg sheets with evenly distributed oviposition were used in the experiment. Egg sheets were

cut according to the number of eggs required and counted under a dissecting microscope. 150, 300, 450, 600 and 750 eggs were placed into each jar respectively. The jars were maintained at 26°C and 65% RH, with a 16:8 hour L:D photoperiod, for 10 days before assessing larval survival. This was considered sufficient time for the larvae to have developed to a size that would be easily recoverable within the diet. Twelve jars were inoculated per replicate and 3 replicates were conducted. Differences in the number of larvae recovered when different numbers of eggs were placed onto the surface of the diet were compared using a General Linear Model (GLM) and a post-hoc Tukey HSD test in Statistica V12.

2.2.3 Economics of producing CpGV in *Cydia pomonella* compared with *Thaumatotibia leucotreta*

An economic comparison was conducted comparing the rearing costs of *C. pomonella* and *T. leucotreta*. The data presented were calculated on figures obtained in September 2014 and took into account dietary costs and larval yield per gram of diet. The use of labour and the cost thereof was also taken into consideration. All prices quoted are in ZAR (South Africa Rands) for the year 2014 and include 14% value added tax (VAT) (USD 1 = ZAR 11.18).

2.3 RESULTS

2.3.1 Establishment and maintenance of *Cydia pomonella* and *Thaumatotibia leucotreta* populations

The *C. pomonella* culture was successfully established on a modified diet as described in 2.2.1.1 from egg sheets supplied by ENTOMON technologies. In 2003, the culture was established from *C. pomonella* larvae collected from various regions in the Western Cape, South Africa, but wild moths were introduced into the culture for genetic diversity (Stenekamp, pers. comm.). The culture was considered to be of heterogeneous origin or a mixed culture.

A closed rearing system was preferred to that of an open tray rearing system to significantly lower the risk of contamination. All rearing was conducted in glass honey jars which were sealed with a lid containing a breathable membrane. This PFTE membrane allowed for sufficient airflow and served as an impermeable membrane to fungal spores. This was tested by pouring nutrient agar into the jar and sealing with the lid containing the breathable membrane. Jars were placed at various positions throughout the insectary and left for a period of 2 weeks. No sign of fungal growth was observed on the surface of the nutrient agar and in most cases it completely desiccated if left for long periods. The open tray rearing method is used by a number of commercial companies. ENTOMON and the Okanagan-Kootenay sterile insect release programme in Osoyoos (British Columbia, Canada) have successfully reared *C. pomonella* on a commercial scale using this technique. However impeccable sanitation and a sterile air environment is an essential part to this rearing process, as any airborne contaminant can result is the loss of rearing trays. For the purpose of this study a rearing system of this magnitude was not possible and therefore the closed rearing system was preferred so as to reduce the risk of contamination.

The *T. leucotreta* culture was successfully established for the trial purposes using egg sheets obtained from River Bioscience Pty Ltd. Various quality control data had been established by River Bioscience as was made available for use as a reference in this study. The data were used to compare the fitness of the individuals reared under the laboratory conditions to that of the larvae produced under commercial rearing conditions. Table 2.4 shows the quality control data generated by the River Bioscience production facility for 2013.

Table 2.4 Biological data from 2013 for the rearing of *Thaumatotibia leucotreta* under commercial rearing conditions at River Bioscience Pty Ltd (Addo, South Africa). Larvae reared at 27ºC ± 2; 35% ± 20 RH. Biological data was recorded from a single production batch per week.

Measurable	Mean	SE
Average number of eggs inoculated onto the diet	888.48	14.25
Egg hatch (%) (surface sterilized)	90.80	1.36
Number of larvae per jar at fifth instar	221.39	7.31
Larval weight (fifth instar) (g)	0.038	0.01
Weight of male pupae (g)	0.022	0.00
Weight of female pupae (g)	0.037	0.01
Pupal sex ratio (male : female)	1:1.05	0.05
% Flight[1]	90.0	1.18
Moth weight – fresh (g)	0.021	0.01
Fecundity (eggs/female)	368.15	15.72

NB: Data generated from 2013 quality control data

[1] flight tests were conducted by placing 30 pupae into a tightly sealed 60l black container with a point light source in the lid. After 8 day number of moths remaining in the box was determined and the percentage flight determined.

Throughout the study the laboratory reared culture, used for all trial work, showed no significant variations from the quality control data presented in Table 2.1. In many instances the data generated during the study showed improvements in a number of the measurable outcomes.

2.3.2 Attempted establishment of laboratory colonies from field collected *Cydia pomonella*

Infested fruit were collected on a number of occasions during the study period from the areas described in Table 2.1 (section 2.2.1.1). Infested fruit was collected and dissected in the laboratory. Trees were also banded with convoluted cardboard strips which were placed

around the tree trunk approximately 30 cm from the ground. The cardboard strips left in the orchard for three weeks after which they were collected and inspected in the laboratory for the presence of pupating larvae. During the initial collection of larvae in these areas, some virus infected larvae were identified by chance. It was considered important to bulk up these viruses in the same genetic population from which the viruses were originally isolated, in order to preserve the genetic integrity of the isolate and avoid possible contamination by expression of any covert virus that may be present in the established laboratory colonies. Attempts to replicate these viruses in the established laboratory culture could have triggered a covert virus resulting in a mixed virus suspension and loss of the genetic integrity of the isolate, assuming the laboratory culture had a genetically different isolate of the CpGV isolate.

Field collections took place in December 2011 and January 2012 at the Free State collection site. Infested apples were taken back to the laboratory and dissected. A total of 89 larvae of various instars were recovered in 2011 and 97 in 2012 (Table 2.5).

Table 2.5 *Cydia pomonella* larval instars identified from field collected infested apples from the Free State province, South Africa.

	Larval instars					
Date	**1**	**2**	**3**	**4**	**5**	**Total**
December 2011	31	17	1	21	19	89
January 2012	7	8	29	25	28	97

However, this method proved unsuccessful as the larvae could not be enticed to feed upon the diet regardless of the instar recovered. A number of the fifth instar larvae pupated without feeding. No viable oviposition was recorded, in either year, from eclosed moths. The failure of the larvae to feed and complete their life cycle may be attributed to a sudden change in environment and or diet. This change from the insect's natural surroundings to an artificial diet possibly acted as a stress trigger. It has been noted that the initial difficulties with insects adapting to the diet may result in larval mortality (Sishuba, 2003; Opoku-Debrah, 2008). In order to increase the percentage of larval survival from a field

collected sample, it was suggested that high numbers of matured larvae and pupae, which are in an advanced physiological state and less susceptible to stress, be sought during field collections (Stenekamp, pers. comm).

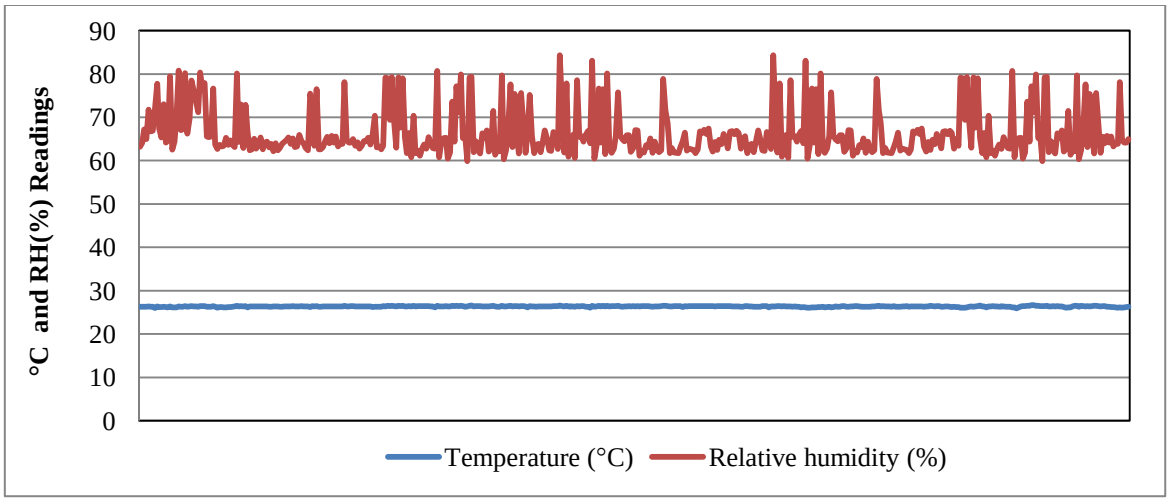
In December 2013 a third collection was conducted at the Free State collection site. The larvae were allowed to complete their life cycle within the apples. Tightly rolled convoluted cardboard strips were placed on and within the infested apples. The cardboard acted as a pupation site for the exiting larvae. Trees were also banded with convoluted cardboard (Figure 2.7). Fifty four fifth instar larvae and 31 pupae were collected from the Free State collection site. No further collections were attempted in the Langkloof and Vermaaklikheid as the preliminary genetic studies of the CpGV isolates indicated them to be identical to the strain of CpGV identified in the established laboratory culture (see Chapter 3). The pupae were allowed to eclose. Eggs sheets were harvested for a period of 5 days after which no oviposition took place. A total of 49 rearing jars were inoculated using the identical rearing protocol to that used for the established laboratory culture. However, only 11 larvae survived to reach the fifth instar stage with 7 successfully eclosing. No coupling or viable oviposition was recorded.

A final collection took place in March 2014 at the Free State collection site. The same methodology was followed as described above. From this collection 73 fifth instar larvae and 17 pupae were collected. Successful oviposition resulted in the inoculation of 62 individual rearing vials.

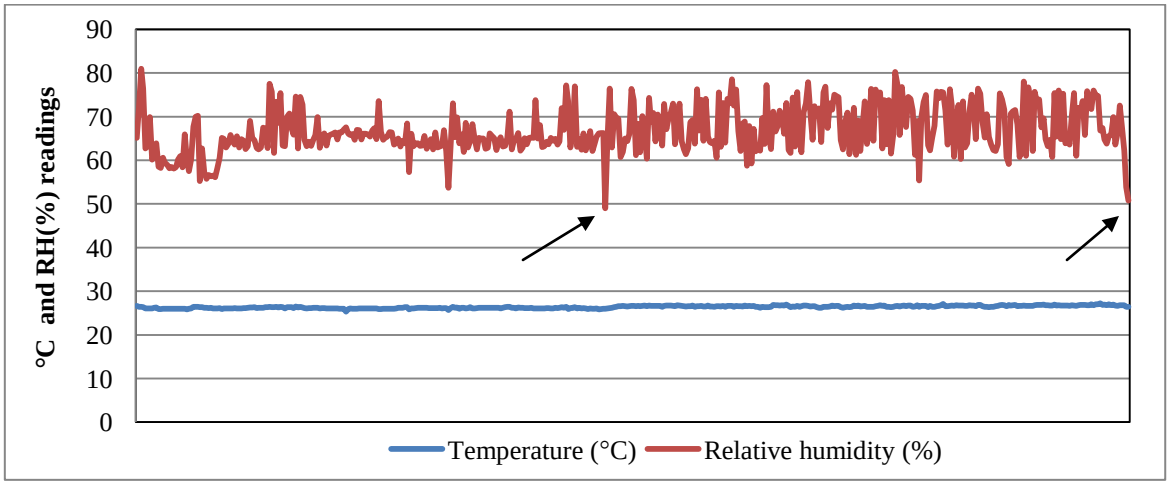
Figure 2.7 Revised collection method used in attempting to establish a laboratory culture from field collected *Cydia pomonella* larvae and pupae. (A) Tightly rolled convoluted cardboard on the surface of an apple sample. (B) Trees wrapped with a convoluted cardboard strip.

2.3.3 Temperature and humidity

The mean temperature and RH readings from the environmental chamber were 26.35 ± 0.11°C and 66.40 ± 5.48% respectively in May and June 2012. In 2013, the environmental chamber was again monitored for a period of 3 months beginning in May 2013. The mean temperature and RH readings were 26.41 ± 0.22°C and 66.72 ± 5.26 % respectively. The temperature, on both occasions, was found to be constant with little fluctuations. Elevated RH readings were recorded on a number of accasions. This was due to the environmental chamber not having the capacity to dehumidify if the environmental RH rose above the set point of the unit (Figure 2.8 A).



(A)



(B)

Figure 2.8 Temperature and humidity data. (A) Temperature and humidity readings of the environmental chamber from March 2012 to May 2013. (B) Temperature and humidity readings of the environmental chamber from May 2013 to July 2013. Arrows indicate a significant drop in the RH as a result of inadequate water supply.

The environmental chamber was continually monitored for accuracy throughout the study period. No significant fluctuations were noted. On a few occasions the unit ran out of distilled water, which the environmental chamber used to increase humidity above ambient. This resulted in a reduction of the RH but had no effect on temperature (Figure 2.8 B). In these instances the problem was quickly rectified and therefore should not have had any influence on the life studies.

2.3.4 Fecundity and time to oviposition

The average number of eggs per female recorded for *C. pomonella* was 225.33 ± 13.62. The highest and lowest numbers of eggs per female were 397 and 97, respectively. The average was significantly higher than reported by Carter (1984) and Bloemfield & Gliomee (2012). Bloemfield & Gliomee (2012) reported 137.2 and 159.3 eggs per female for spring and summer moths respectively. Cater (1984) reported oviposition ranging from 10 to over 100 eggs per female. Figures for fecundity of *C. pomonella*, sited in the literature, for both laboratory reared and feral female moths are highly variable (Geier, 1963; Ferro *et al*., 1975; Howell, 1991). The mean oviposition of *C. pomonella* females was significantly less than what was recorded for *T. leucotreta* (t_{52} = 11.63, P < 0.0001). A mean of 500.56 ± 19.34 eggs was oviposited with the highest recorded value being 745 eggs per individual *T. leucotreta* female.

Oviposition begun 8 hours after coupled pairs had separated with the highest number of eggs oviposited after 32 hours for both species. This coincided with the period from 15h00 to 23h00. Both pairs of moths were subjected to a 16:8 hour L:D cycle. Figure 2.9 shows that the majority of the oviposition occurred during the dark phase of the cycle with progressively fewer eggs oviposited as the moths aged. The *C. pomonella* and *T. leucotreta* oviposition trends were similar with the exception that *C. pomonella* produced significantly fewer eggs during the study period.

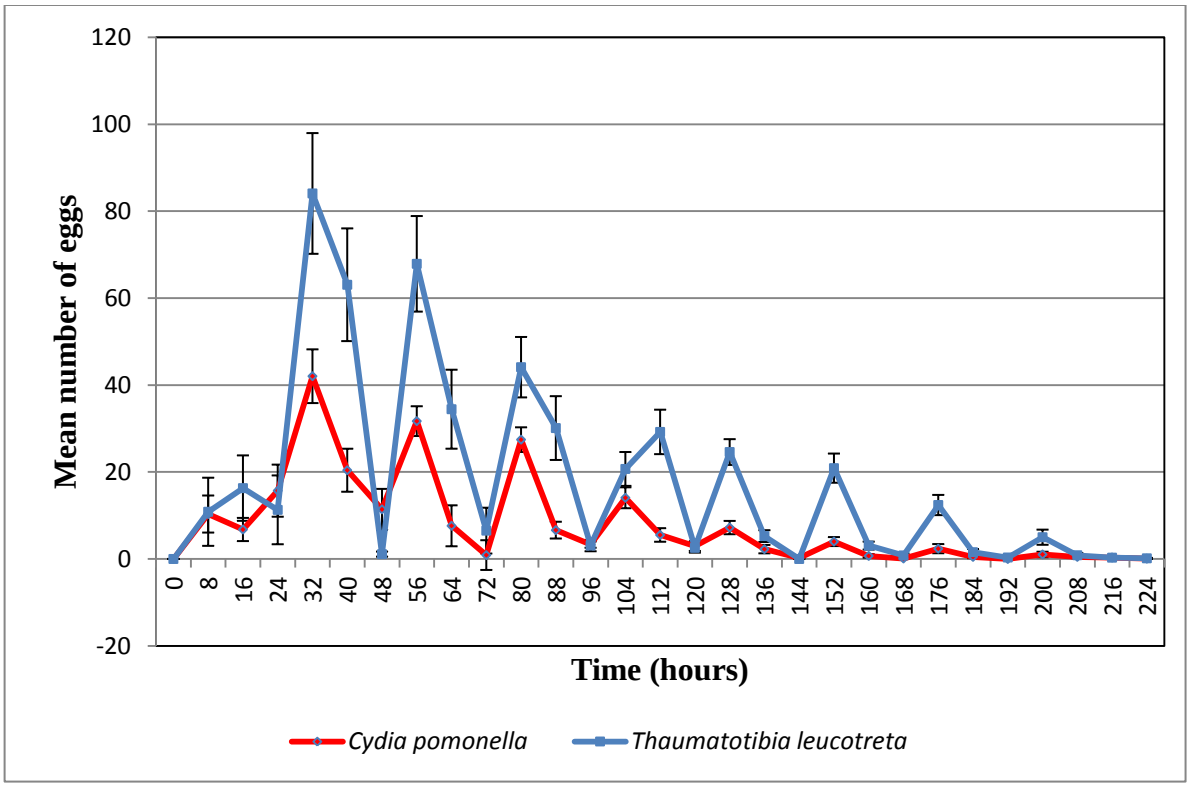


Figure 2.9 Mean number of eggs oviposited at eight hour intervals for both *Cydia pomonella* and *Thaumatotibia leucotreta*.

2.3.5 Percentage hatch

A total of 5189 *C. pomonella* eggs were counted of which 4297 successfully hatched during the allowed time frame resulting in an 83.73% ± 0.62 hatch. For *T. leucotreta*, 5206 eggs were counted with 4779 hatching resulting in a 91.8% ± 0.44 hatch. A significantly higher number of *T. leucotreta* eggs hatched then *C. pomonella* eggs ($U = 1683$, $p < 0.0001$).

2.3.6 Egg developmental times

The first substantial hatch in *T. leucotreta* was recorded 80 hours post oviposition whereas this was only evident in *C. pomonella* at 96 hours. 88.6% of the *T. leucotreta* larvae hatched within a 24 hour period between 72 to 96 hours post oviposition (Figure 2.10) 82.4% of the *C. pomonella* larvae hatched over a 32 hour period between 88 and 120 hours

post oviposition. The total cumulative percentage hatch for *C. pomonella* and *T. leucotreta* was 85.4 and 90.3% respectively. The mean time to hatch was 100.7 ± 2.5 hours for *C. pomonella*. This was significantly slower than *T. leucotreta* which was recorded at 84.4 ± 0.02 hours ($t_4 = 19.83$; $P < 0.0001$).

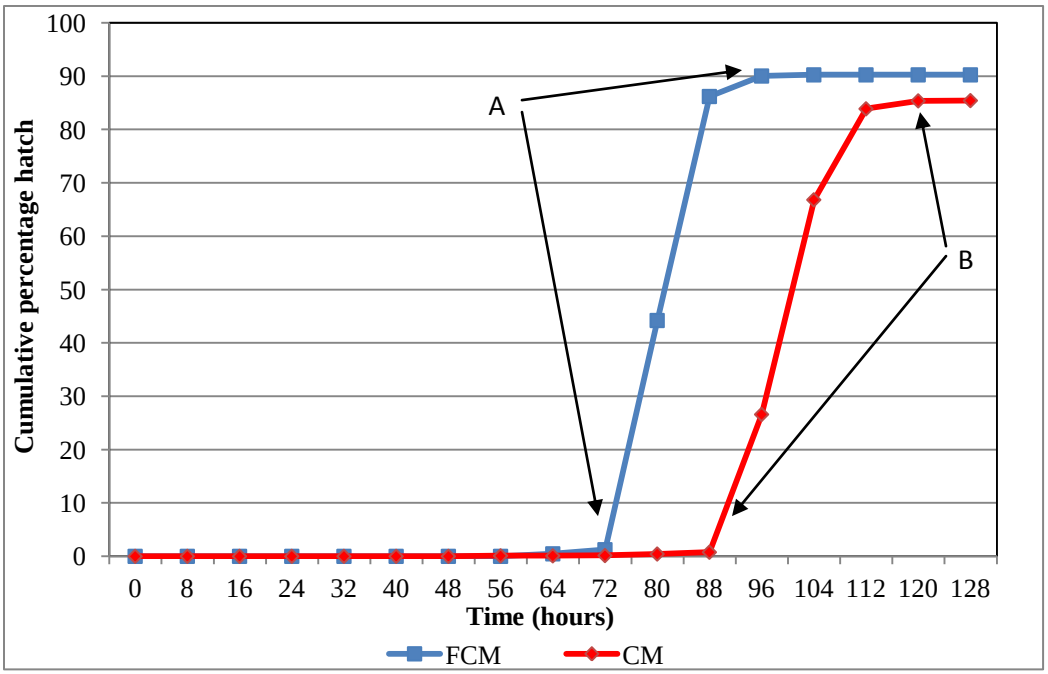


Figure 2.10 Time from oviposition to hatch for *Cydia pomonella* and *Thaumatotibia leucotreta*. A: arrows indicate the hatch period of *T. leucotreta*; B: arrows indicate the hatch period of *C. pomonella*.

At 96 hours post oviposition, 90.1% of the *T. leucotreta* eggs had hatched. The egg developmental time between *T. leucotreta* and *C. pomonella* differed by 24 hours with 85.4% of the *C. pomonella* eggs hatching after 120 hours.

2.3.7 Larval development times

The developmental times of each instar were recorded and compared with the results indicating a significant difference between *C. pomonella* and *T. leucotreta* development ($P < 0.0001$) (Table 2.6).

Table 2.6 Developmental times for first to fifth instar larvae of *Cydia pomonella* and *Thaumatotibia leucotreta*.

	Time (hours)[1]			
	Cydia pomonella	***Thaumatotibia leucotreta***		
Instar	**Mean ± (SE)**	**Mean ± (SE)**	**t**	**P**
1	-	-	-	-
2	118.39 ± 2.37	61.63 ± 1.00	25.20	< 0.0001
3	192.08 ± 2.64	104.13 ± 1.16	30.54	< 0.0001
4	263.69 ± 3.58	157.99 ± 2.23	25.04	< 0.0001
5	415.23 ± 3.33	237.52 ± 1.95	46.08	< 0.0001

[1] Larval growth was assessed every 8 hours.

Under the established rearing conditions it took 263.69 ± 62.89 and 415.23 ± 89.56 hours for the *C. pomonella* larvae to reach the fourth and fifth instar stage respectively. The mean larval mortality which was recorded at 264 and 424 hours post inoculation was 42% ± 6 and 48% ± 4 respectively. The developmental time for *T. leucotreta* was significantly less, with larval development reaching the fourth and fifth instar life stage after 157.99 ± 39.02 and 237.52 ± 47.85 hours, respectively. The mean larval mortality recorded at 160 and 240 hours post inoculation was 8% ± 0 and 24% ± 12, lower than that of *C. pomonella*.

Both fourth and fifth instar stages were considered as potential life stages for further investigation into virus production. There is a 77.01 hour (3.2 day) difference between developmental time at fourth instar and 151.51 hour (6.3 day) difference at fifth instar for the two species. In both instances *C. pomonella* developed at a significantly slower rate than *T. leucotreta*.

2.3.8 Efficiency of rearing

The mean number of *C. pomonella* larvae produced from 450 eggs was 72.22 ± 2.80. Increasing the number of eggs did not result in a substantial increase in larval numbers. Reducing the number of eggs did however result in a substantial decrease in the number of

larvae recovered. The percentage survival was very low in all treatments with an average of 16.05% surviving in the 450 egg treatment (Table 2.7).

Table 2.7 Number of *Cydia pomonella* and *Thaumatotibia leucotreta* larvae reared per jar from different numbers of egg inoculation and percentage survival.

	C. pomonella			***T. leucotreta***		
Eggs inoculated	**Mean[1]**	**SE**	**% survival**	**Mean[1]**	**SE**	**% survival**
150	21.47 d	1.88	14.31	94.00 b	3.38	62.67
300	45.50 e	2.95	15.17	193.50 f	6.03	64.50
450	72.22 a	2.80	16.05	231.75 c	6.36	51.50
600	81.39 ab	3.22	13.56	235.67 c	8.42	39.28
750	85.61 ab	3.32	11.41	236.14 c	5.94	31.49

[1] Values followed by different letters indicate significant difference. $F_{(4,350)} = 28.52$; $P < 0.001$, Tukey HSD test.

A significantly higher number of *T. leucotreta* than *C. pomonella* larvae survived in the 450 egg treatment. An average of 231.75 ± 6.36 *T. leucotreta* larvae was produced from the 450 egg treatment with no significant increase in larval numbers noted with increased number of eggs inoculated. Unlike *C. pomonella,* an increase in the percentage survival was noted with a reduction in the numbers of eggs inoculated. However in the case of commercial production, increased larval production is preferable. For both species the inoculation of 450 eggs per jar proved to be the best compromise between eggs placed and larvae reared. Therefore these figures were used as the standard for further trial work and for economic comparisons. It was noted that a reduction in the percentage survival occurred as the number of eggs inoculated increased. This was possibly due to larval competition for dietary resources in order to complete its life cycle.

2.3.9 Economics of producing CpGV in *Cydia pomonella* compared with *Thaumatotibia leucotreta*

There are a number of factors that need to be taken into consideration when comparing the rearing costs of insect populations. The main factors contributing to the cost of production would include the cost of the dietary ingredients, the staff compliment required, and infrastructural requirements as well as a sanitation component. The cost to produce a single jar of *C. pomonella* diet was ZAR 0.48. This cost was less for *T. leucotreta*, which was calculated at ZAR 0.29 (Table 2.8).

Table 2.8 Cost of dietary ingredients for *Cydia pomonella* and *Thaumatotibia leucotreta* diets based on pricing obtained in May 2014.

	Thaumatotibia leucotreta			***Cydia pomonella***		
Ingredients	**Cost/kg**	**Weight per jar (g)**	**Cost per jar[2]**	**Cost/kg**	**Weight per jar (g)**	**Cost per jar[2]**
Maize	R 3.65	38.17	R 0.139			
Whole wheat flour				R 8.00	28.41	R 0.227
Wheat germ	R 6.77	3.82	R 0.026	R 6.77	7.15	R 0.048
Brewers yeast	R 13.68	1.91	R 0.026	R 13.68	7.61	R 0.104
Methyl Paraben	R 96.05	0.70	R 0.067	R 96.05	0.36	R 0.035
Sorbic acid	R 91.50	0.12	R 0.011			
Ascorbic Acid				R 54.00	1.0035	R 0.054
Benzoic Acid				R 23.10	0.4635	R 0.011
Full cream milk powder	R 77.15	0.29	R 0.022			
Reverse osmosis water[1]	R -	43	R 0.000	R -	45	R 0.000
Totals		88g	R 0.29		90g	R 0.48

[1] The cost of producing a litre of reverse osmosis water was considered to be negligible and therefore not taken into account [2] All prices listed include 14% VAT

When each rearing jar was inoculated with 450 eggs the average larval production per jar was 72.20 and 231.75 for *C. pomonella* and *T. leucotreta* respectively (Section 2.3.8). Therefore the cost to produce 1000 larvae was calculated at R6.64 for *C. pomonella* as opposed to R1.26 for *T. leucotreta*. The dietary component of the total cost comparison is 60.4% more expensive for *C. pomonella* than *T. leucotreta*.

Consideration needs to be given not only to the dietary cost component but also the labour requirement in producing the 1000 larvae. For every 4.31 jars produced, 1000 *T. leucotreta* larvae are reared. In order to produce the equivalent number of *C. pomonella* 13.85 jars would be required. This would result in a threefold increase in both the labour requirement as well as the associated sanitation component. Although an infrastructural requirement is a once off capital expenditure, it should also be considered, as the rearing of *C. pomonella* in

the proposed manner would require 3.21 times more rearing space in an insectary. For commercial rearing purposes an open tray rearing method for *C. pomonella* is preferred.

2.4 DISCUSSION

Laboratory colonies of both species were established from eggs supplied by commercial entities and were successfully maintained using the described rearing protocols for a period of 3 years. Both the colonies remained disease free for the duration of the study and provided a good source of healthy material for further trial work.

The primary objective of this study was to compare the rearing potential of *T. leucotreta* and *C. pomonella*. This involved generating data for fecundity, egg developmental times, larval developmental times and the dietary cost of producing the larvae. This study aimed to establish whether there would be any possible benefits to producing CpGV in *T. leucotreta* as opposed to the homologous host, *C. pomonella*, taking into consideration that a natural relationship exists between CpGV and *C. pomonella*. Therefore production of the virus in its natural host should be the most economically viable means of CpGV production unless *T. leucotreta* can be shown to be significantly easier and more efficient to produce virus in.

The following rearing characteristics have been noted as desirable traits for production of a virus in a potential insect host. Female moths need to be highly fecund, the larvae should efficiently utilize the dietary medium and show tolerance to crowding with low mortality throughout the larval instars; the host should have a short life cycle and a late instar stage which is susceptible to virus infection and results in good virus production. The results of this study showed *T. leucotreta* oviposited 45.1% more eggs than *C. pomonella*, with no significant difference noted in the time from moth coupling to the onset of oviposition. The average oviposition for *T. leucotreta* was similar to that reported by Reiser *et al*. (1993), but was found to be significantly higher when compared to reports by Moore (2002) and Opoku-Debra *et al*. (2014), in which similar studies on the identical *T. leucotreta* culture were conducted. This discrepancy may be attributed to the environmental conditions under

which the both Moore's and Opoku-Debrah's *et al*.'s studies were conducted. Not only did *T. leucotreta* oviposit more eggs in an equal time period but the egg incubation period was 24 hours shorter in *T. leucotreta* than that which was observed in *C. pomonella*.

Fourth and fifth instar larval stages were considered to be important life stages for virus production. Significant larval development time differences were observed between *C. pomonella* and *T. leucotreta*. *Cydia pomonella* took 3.2 and 6.3 days longer than *T. leucotreta* to reach the fourth and fifth instar, respectively. Taking into consideration that *C. pomonella* eggs took 24 hours longer to hatch meant that the difference in the developmental time was extended to 4.2 and 7.3 days respectively. Therefore, *T. leucotreta* developed 57.1% faster from neonate to fourth instar stages and 59.9% faster to fifth instar when compared to *C. pomonella*. The significance of this observation is an increase in the number of generations produced in a calendar year resulting in an increase in the potential virus production per unit time assuming equal viral yields could be achieved from virus production. The effective utilization of a dietary medium is an important economic consideration. The commercial production facility at ENTOMON, South Africa, produces 0.4 to 0.5 larvae per gram of diet, i.e. 2 to 2.5 g of diet required per larva (Stenekamp, pers. comm.). Hansen & Andersen (2006) reported *C. pomonella* production to approach maximum efficiency at a density of one larva per 4.8 ml of diet. However, further studies conducted post 2002 showed it was possible to reduce the volume to 4.5 ml diet per larva. In this study 0.8 *C. pomonella* larvae were produced per gram of diet. Although higher than the 0.5 achieved by ENTOMON, it was still substantially less than what was achieved for *T. leucotreta*. The study showed it was possible to product 2.6 *T. leucotreta* larvae from a single gram of diet, 3.25 times more larvae per gram of diet compared to *C. pomonella*. The literature indicates that yields of *C. pomonella* are highly variable, ranging from 0.17 to 0.44 pupae/kg achieved by OKSIR (Bloem *et al*., 2007). Singh (1984) suggested that artificial diets should produce an average yield of 75% adults from the initial viable eggs inoculated. It has also been noted that a good objective would be to produce 200 adults per kg or litre of diet; however the OKSIR rearing facility in Canada has been able to produce more than 400 individuals per litre of diet (Dyck, 2010).

It is important not only to consider the number of larvae produced per unit of diet but also to take into consideration the cost of such a production. For a commercial venture to be successful, the rearing process needs not only to be efficient in larval production, but cost effective. Various authors have published costs for commercial diet production, ranging from USD 0.93 to as much as USD 2.94 per 1000 larvae (Dyck, 2010). Bloem *et al.* (1997) reported that the Canadian SIR facility was producing 1200 moths at a cost of 3.00 Canadian dollars per rearing tray. With the above mentioned rearing techniques the cost to produce 1000 *C. pomonella* larvae was calculated to be R6.64 (USD 0.64), and the cost to produce 1000 *T. leucotreta* larvae was ZAR1.26 (USD 0.12). These figures are significantly less than was found in the literature and considering the relatively low cost of labour in South Africa, production of a CpGV virus could be a lucrative prospect for a commercial company.

Considering the results obtained in this study, *T. leucotreta* should be considered as an alternate production host because it has a higher yield of eggs and larvae during the rearing process. Therefore, more larvae may be produced per unit diet at a lower cost and the requirement for production space is less. This results in significantly more larvae available for production of CpGV. The host has a shorter development time which increases the amount of virus that may be produced in a calendar year. The cost of production is significantly less per larvae, compared to *C. pomonella*, making it and economically viable option if an effective and reliable virus production technique can be developed for the production of CpGV in *T. leucotreta*.

2.5 CONCLUSION

Thaumatibia leucotreta can be produced quicker, with greater efficiency and at a reduced cost compare to *C. pomonella*. Therefore the results of this study confirm the *T. leucotreta* to be an excellent candidate for further investigation into its possible use as a heterologous host for the production of CpGV on a commercial scale.

Further consideration into the CpGV production potential in *T. leucotreta* should be investigated. Virus production potential needs to be established in both fourth and fifth instar *T. leucotreta* larvae and compared to production in *C. pomonella*. Reiser *et al*. (1993) has shown that production of CpGV in *T. leucotreta* is possible. Dose and time response data needs to be estimated to test the mass production potential for commercial use of this possible production technique. It is then imperative that stringent quality control parameters need to be established to mitigate the possible risks associated with the production of a virus in a heterologous host.

3

GENETIC CHARACTERISATION AND DETERMINATION OF BIOLOGICAL ACTIVITY OF *CYDIA POMONELLA* GRANULOVIRUS ISOLATES

3.1 INTRODUCTION

In Chapter 2 it was established that *T. leucotreta* was significantly easier and more cost effective to rear than *C. pomonella* and therefore a promising alternate host for *in vivo* CpGV production. For a commercial virus product to succeed in the market place it is pertinent to identify an isolate that has the best possible characteristics for mass production as well as field performance. Therefore, it is important not only to recover novel isolates from field collected larvae but also to characterise them genetically and biologically. Isolates of the same virus can differ genotypically between geographically distinct host populations with important implications for resistance management (Opoku-Debrah *et al*., 2013). Genetic variation among baculovirus isolates may result in differences in virulence to a given host (Shapiro, 1995; Moscardi, 1999). A number of field populations of *C. pomonella* have been reported to have developed resistance to the Mexican isolate of *Cydia pomonella* guanulovirus (CpGV-M) (Fritsch *et al*., 2005; Eberle & Jehle, 2006; Sauphanor *et al*., 2006; Fritsch *et al*., 2007) and laboratory bioassays have revealed that resistance can be managed by challenging resistant insects with different virus isolates of the same species (Eberle *et al*., 2008; Berling *et al*., 2009).

Several molecular techniques can be used to establish the identity of baculovirus species as well as differentiate them genotypically. The most commonly used method is restriction endonuclease (REN) analysis of total genomic DNA. This method, also referred to as restriction fragment length polymorphism (RFLP), is based on the fact that restriction enzymes (REs) recognise a specific target sequence within the double stranded DNA (dsDNA) and cleaving it resulting in enzyme specific DNA profiles of discrete fragments with defined length which are analysed by agarose gel electrophoresis. Variation in DNA profiles can be used to establish genetic differences between virus isolates (Ahern, 1991). Examples of REs used to generate DNA profiles for baculovirus isolates include *Bam*H1, *Eco*R1, *Hin*dIII, *Kpn*1, *Pst*1, *Sac*1, *Xba*1 and *Xho*1. This method has been successfully used by various authors to indicate genetic differences between and within several baculovirus species (e.g. Jehle *et al*., 2006; Miele *et al*., 2011; Opoku-Debrah *et al*., 2013).

Alternatively, specific genes of interest can be selected for PCR amplification and sequencing using gene-specific oligonucleotides in order to characterise baculovirus isolates. The sequences are assembled and aligned in order to detect single nucleotide polymorphisms (SNPs), which can then be analysied to establish whether they result in changes to the amino acid sequence of the protein. Several baculovirus genes have been targeted for sequencing including *granulin*, *egt*, *lef*-5, *lef*-8 and *lef*-9 (Kuzio *et al*., 1999; Luque *et al*., 2001; Jehle *et al*., 2006; Rohrmann, 2013). Complete genome sequencing can also be used to compare virus isolates. Several NPV genomes have been fully sequenced, some of which include *Autographa californica* (AcMNPV) (Ayres *et al*., 1994), *Orygyia pesudotsugata* (OpMNPV) (Ahrens *et al*., 1997), *Bombyx mori* NPV (BmNPV) (Gomi *et al*., 1999), *Lymantria dispar* (LdNPV) (Kuzio *et al*., 1999), *Spodoptera exigua* (SeNPV) (Ijkel *et al*., 1999) and *Helicoverpa armigera* (HaNPV) (Chen *et al*., 2001). Examples of GVs whose genomes have been fully sequenced are *Xestia c-nigrum* GV (XcGV) (Hayakawa *et al*., 1999), *Plutella xylostella* GV (PlxyGV) (Hashimoto *et al*., 2000) and *Cydia pomonella* GV (CpGV) (Luque *et al*., 2001).

Until recently, almost all commercial formulations of CpGV made use of the original Mexican isolate, discovered near Valle de Allende in Mexico (Tanada, 1964). Further

studies identified isolates from England (CpGV-E) and Russia (CpGV-R) (Crook *et al*., 1985; Vincent *et al*., 2007; Zingg & Kessler, 2008). Recently several strains of CpGV have been isolated worldwide and compared genetically to the Mexican, English and Russian isolates (Crook *et al*., 1985; Eberle *et al*., 2009). Isolates from Georgia and Iran have been characterised by RFLP, with partial genome sequencing revealing heterogeneity in their genetic content (Rezapanah *et al*., 2008). After conducting an extensive literature search, there is no evidence of a South African CpGV isolate that has been identified and characterised genetically. Basdew (2005) described the isolation of a South African isolate based on biological studies, although no genetic comparisons were conducted to confirm these results. Therefore, it is important to identify and characterise novel isolates and then to compare biological activity with that of the Mexican strain of CpGV in order to ensure the isolate has the required characteristics for further development as a biological control agent.

The first objective of this study was to identify novel CpGV isolates through bio-prospecting in both established orchards in various regions of South Africa and from a laboratory reared culture of *C. pomonella*. Orchards were selected based on their geographic isolation as well as previous *C. pomonella* control strategies. Orchards spray records were checked to ensure no trial or commercial applications of CpGV products had been applied previously.

The second objective was is to identify and genetically characterise novel isolates of CpGV. It was also important to establish the genetic identity of each different isolate and compare it to available commercial CpGV products, namely Madex® and Carpovirusine™. For the purpose of this study REN analysis of the genomic DNA, and PCR amplification and sequencing of selected viral genomes were performed.

The third objective of this study was to evaluate the biological activity of any genetically novel isolates identified from the genetic characterisation. To justify further investigation of an isolate, it must be established whether the virus possesses any potential as a biological control agent. This can be established not only by gauging the potential of the

virus in isolation, but by comparing dose-response relationships with those of viruses considered to effectively control their hosts. It is also important that a norm for virulence be established for use as a quality control standard for the testing of all further batches of virus.

3.2 MATERIALS AND METHODS

3.2.1 Virus collection

Three areas were identified for sampling. All three areas had no previous record of any commercial applications of a CpGV virus product. The areas were selected on the basis of their geographic isolation from other orchards or productive farmlands. Table 3.1 lists the area sampled for the presence of CpGV in the host population.

Table 3.1 Area's sampled for the presence of CpGV in the *Cydia pomonella* population.

Area	Site	Province	Co-ordinates
Western Cape	ENTOMON (lab culture)	Western Cape	33°56'36.58"S; 18°52'04.62"E
Vermaaklikheid	Neglected orchard	Western Cape	34°18'19.15"S; 21°01'37.51"E
Langkloof	Zondagh farms	Eastern Cape	33°44'42.91"S; 23°15'24.77"E
Langkloof	Zondagh farms	Eastern Cape	33°44'42.91"S; 23°15'24.77"E
Free State	n/a	Free State	Undisclosed

Infested fruit were collected and brought back to the laboratory for inspection. The fruit were carefully inspected for the presence of *C. pomonella* instars and potential infected larvae. All recovered healthy larvae were placed onto artificial diet and reared at 26°C and 60% RH. The rearing vials were prepared as in Chapter 2, section 2.2.1.1. The vials were inspected daily for signs or symptoms of viral infection. All larvae showing signs of viral infection were collected. On occasion, infected larvae were discovered in fruit (Figure 3.1). Attempts were made to establish viable laboratory culture from the Langkloof and Free State populations with limited success. A laboratory culture of *C. pomonella* was

established from egg sheets obtained from Mathew Addison of Hortgro Science (the research arm of Hortgro) (Chapter 2).

Figure 3.1 CpGV infected *Cydia pomonella* larva discovered inside an infested apple.

3.2.2 Symptomatology

Larvae reared on artificial diet were periodically inspected visually and without disturbance, for any symptoms regarded as being characteristic of baculoviral infections. These included colour changes (particularly a milky appearance), flaccidity and behavioral changes such as premature exiting of the diet or hanging from substrates. Larvae showing such symptoms were collected individually and stored at -16ºC.

3.2.3 Preliminary identification by light microscopy

Insects showing symptoms of a baculovirus infection were smeared onto microscope slides. Both gut and internal organs were included in the smear. Each smear was spread thinly across the microscope slide before air drying. Buffalo Black solution was heated to 40ºC in a staining rack on a hotplate. The slide was immersed in the Buffalo Black solution for five minutes, after which it was washed under running tap water for 10 seconds. The slide was left to air dry before being examined through a light microscope at 1000X magnification under oil immersion.

3.2.4 Crude extraction of virus from infected larvae for TEM observation

Crude extractions were performed according to the method described by Hunter-Fujita (1998) and successfully used by Moore (2002). Larvae showing signs of viral infection were macerated using a micro pestle in 1 ml of 0.1% sodium dodecyl sulphate (SDS), in a 2 ml microcentrifuge tube. The tubes were centrifuged at 1400 rpm for 30 seconds. The supernatant was removed and pipetted into a JA-20 tube (Beckman Coulter). The pellet was re-suspended in 500 µl of 0.1% SDS and vortexed for 30 seconds. It was then centrifuged for 30 seconds at 1400 rpm (Beckman Coulter, JA-20). The supernatant was removed and transferred to the JA-20 centrifuge tube. This step was repeated four times. The final pellet was discarded. The JA-20 centrifuge tube containing the supernatant was then filled to the brim with 0.1% SDS and centrifuged at 13 000 rpm for 30 min at 4°C. The supernatant was removed and the pellet re-suspended in 100 µl of sterile distilled water.

3.2.5 Transmission electron microscopy (TEM)

Purified OBs were examined using the technique described by Horne & Rochetti (1974). A carbon grid was securely fastened between the points of fine forceps. A drop of purified OBs was placed directly onto the carbon grid for 1 min, ensuring the coated side was uppermost. The excess OBs were removed by gently touching the edge of the grid with

filter paper (Whatman; Lasec, South Africa). The grid was allowed to air dry after which a drop of 5% uranyl acetate was placed onto the carbon grid and left for 1 min. The excess uranyl acetate was removed from the grid using filter paper. The grid was allowed to air-dry overnight. The following day the grid was examined using a JEOL JEM 1210 transmission electron microscope operated at 100 kV. Magnification ranged from 10 000 X to 120 000 X and particle sizes were measured using the TEM computational software (ANALYSIS).

3.2.6 Purification of occlusion bodies using a glycerol gradient

Cadavers (approximately 3 g) were homogenised in 4 ml 0.1% sodium dodecyl sulphate (SDS), using a mortar and pestle. Once homogenised, the volume of homogenate was made up to 20 ml and filtered through a double layer of muslin cloth and collected in a 50 ml glass beaker. The homogenate was divided equally between two JA-20 centrifuge tubes. Each tube was filled to the brim with 0.1% SDS. Both tubes were spun for 30 min at 10000 rpm and 10°C. The pellets obtained were resuspended in 10 ml 0.1% SDS. If required the tubes were vortexed to aid the re-suspension of the pellet. After the pellet was fully resuspended the tubes were once again filled to the brim with 0.1% SDS and spun for a further 30 min at 10000 rpm. The pellets obtained were resuspended in 6 ml ddH_2O. Two continuous 30 to 80% (v/v) glycerol gradients were prepared in 38 ml SW-28 rotor tubes. The virus was carefully loaded onto the gradient using an auto pipette and spun for 15 min at 15000 rpm and 4°C. Each virus band was recovered, by pipetting, into a JA-20 tube. Tubes were filled to the top with sterile distilled water and centrifuged for 14 min at 10000 rpm. The supernatant was discarded and the pellets re-suspended in sterile distilled water. This spin was repeated twice to remove any traces of glycerol. Finally, each pellet was re-suspended in 1 ml sterile distilled water. 1000 µl aliquots were stored at -16°C.

3.2.7 Genomic DNA analysis

In order to characterise the five CpGV isolates, purified OBs of each isolate were subjected to a DNA purification protocol in order to obtain pure genomic DNA for REN analysis and

PCR amplification of specific viral genes. The PCR was used to amplify two genes of interest for sequencing, namely the *granulin* and *egt* genes. The protocols for the DNA extraction, REN analysis and PCR are described below. The two commercial isolates, Madex® and Carpovirusine™ (CpGV) were also subjected to the same analysis and served as reference isolates.

A version of the CTAB DNA extraction protocol established by Opoku-Debrah (2011) was used in this study. This protocol is similar to that described by Aspinall *et al.* (2002) and Goble *et al*. (2011). Initially, 200 µl of purified OBs was pipetted into sterile 1.5 µl microcentrifuge tubes to which 90 µl of Na_2CO_3 (1 M) was added. If the suspension did not clarify, more Na_2C0_3 was added. The microcentrifuge tubes were incubated in a water bath for 30 min at 37ºC. After incubation, the suspension was neutralized with 120 µl of Tris-HCL (1 M, pH 6.8). Thereafter, 90 µl of SDS (10 % w/v) and 50 µl of Proteinase K (25mg/ml) were added. The microcentrifuge tubes were further incubated for 30 min at 37°C. Afterwards, 10 µl of RNaseA (10mg/ml) was added to the contents of the tube and incubated for a further 30 min at 37°C. The tubes were removed and centrifuged at 14000 rpm for 3 min in a tabletop laboratory centrifuge (BIO-RAD, model 16K). The supernatant was carefully transferred into a new tube and the pellets, if any, were discarded. 400 µl of pre-warmed (to 70°C) CTAB buffer was added to the supernatant and incubated in a heating block set at 70°C for 1 hour. Every 10 min, the tubes were gently inverted several times to allow the contents to mix. 400 µl of chloroform stored at -4°C was added. The tubes were again inverted briefly and spun for 10 min at 10000 rpm. Thereafter, the upper aqueous phase was transferred into a new tube and 400 µl of cold isopropanol (stored at -25 $^{\circ}$C) was added. The tubes were incubated overnight, at -25°C. The following day the tubes were spun at 14000 rpm for 20 min. The resulting pellet was re-suspended in 1 ml of 70% cold ethanol. Thereafter, the tubes were again spun at 14000 rpm for 5 min. The ethanol was gently poured off without discarding the DNA pellet in the process and the pellet retained. The tubes were incubated in a heating block (set at 50°C) until all remaining traces of ethanol had evaporated. The DNA pellet was re-suspended overnight at -4°C in 10mM Tris-HCL (pH 8.0) buffer. DNA was stored for a few days at -4°C or for

longer periods at -25°C (or in a freezer). The resulting DNA could then be used for PCR and REN analysis.

3.2.8 Determination of DNA concentration

The DNA concentration was measured using a Nano Drop™ 2000 spectrophotometer version 1.4.2 (Thermo Scientific). Approximately, 1 µl of the genomic DNA was placed onto the optical measurement surface of the microspectrophotometer and the DNA concentration was determined.

3.2.9 Restriction enzyme digestion and agarose gel electrophoresis

Restriction enzyme digestions were conducted with the five CpGV isolates. Approximately 20 µl of DNA were digested using 3 µl of 1X RE buffer and 3 µl (30 U) each of the following REs: *Bam*H1, *Eco*R1, *Hin*dIII, *Pst*1, *Xba*1 and *Xho*1. Four µl of sterile ddH_20 was added to make up a final volume of 30 µl. The contents of the reaction were mixed in a microcentrifuge tube and spun briefly at 5000 rpm for 3 seconds. The tubes were incubated at 37°C for 4 hours for digestion of DNA.

The resulting digests were viewed using an agarose gel electrophoresis technique. DNA samples were loaded on 0.6% ethidium bromide (Ethbr) stained agarose gels in Tris-acetate (TAE) buffer, which were run at 30 V for 16 hours (see Appendix 2 for reagent preparations). Two DNA markers GeneRuler High Range and 1 Kb DNA marker were loaded onto the gels and served as a reference for measuring the molecular weights (Mwts) of the DNA fragments (Figure 3.2). The Ethbr stained gels were viewed using a UV trans-illuminator with an in-built camera (Uvitec®) and the gel images captured using UVIprochemi (version 12.4 for windows) software.

The Mexican strain (CpGV-M) was selected as a reference strain against which all isolates of CpGV were compared. The size of the DNA fragments were estimated using the scanning and imaging analysis software UVIband version 11.9 (UVItec 2004). For each of the REs mentioned above, *in silico* profiles of the Mexican strain of CpGV were obtained

using A-plasmid Editor Version 2.046 (Davis, 2013) and the fragments sizes were compared with one another.

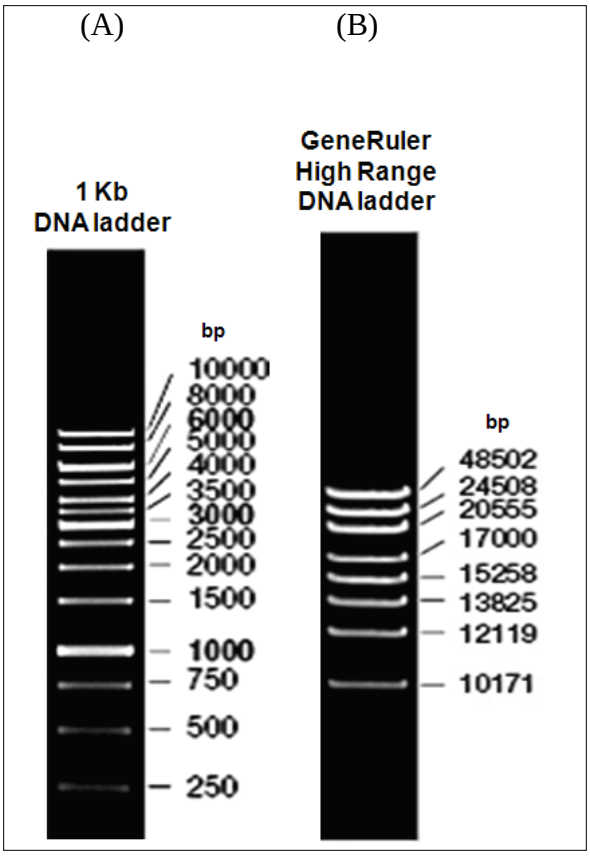


Figure 3.2 DNA fragments from the DNA markers. (A) 1 Kb DNA marker (250 bp to 10000 bp) and (B) GeneRuler High Range marker (10171 bp to 48502 bp), with their corresponding Mwts as indicated by the manufacturer, Fermentas® (Source: www.fermentas.com).

3.2.10 Amplification of the CpGV *granulin* and *egt* gene sequences using the polymerase chain reaction

The *granulin* and *egt* genes were amplified from genomic DNA of the six CpGV isolates using PCR. Oligonucleotide primers used for amplification are shown the Table 3.2 The primers were designed using the complete genome of CpGV-M (U53466.2) (Luque *et al*.,

2001). The PCR reaction was conducted according to the manufacturer's protocol by using the appropriate buffers and enzymes (Table 3.3) and a MJ Mini Personal Thermal Cycler (BIO-RAD®) with cycling parameters indicated in Table 3.4. Amplified products were analysed on 0.6% Ethbr stained gels run at 30 V for 3 hours in 10 cm gel rigs. A 1 Kb DNA marker or the DNA marker II was run along the edge of the gels in order to estimate the size of the amplified PCR products. The gels were visualised under a UV trans-illuminator. The DNA marker was also used as a guide to ensure that the PCR products obtained were indeed amplified fragments of the *granulin* and *egt* genes of CpGV. The PCR products obtained from the reaction were sent to Inqaba Biotechnical Industries (Pty) Ltd (Pretoria, South Africa) for sequencing, using the oligonucleotide primers indicated in Table 3.2.

Table 3.2 Primer sequence for the *granulin* and *egt* genes of CpGV.

Primer Name	**Sequence (5' to 3')**	**Binding site on the genome**	**Location on the genome**	**Amplicon size (nt)**
Granulin forward	ATGGGATATAACAAATCTTTG	1 - 21	1 - 747	747
Granulin reverse	TTAATAGGCTGGACCGGTG	728 - 747		
Egt forward	TCATTTACTCCAATATTTATTGC	120853 -120875	120853-122307	1455
Egt reverse	ATGGGACGATACACTCCAAATG	122284 -122307		

Table 3.3 Reagents used for PCR amplification of the *granulin* and *egt* genes of the CpGV isolates.

***Granulin* gene**	***Egt* gene**
36 µl of sterile distilled water (ddH_20)	36 µl of sterile distilled water (ddH_20)
5 µl of Taq buffer (with $MgCl_2$)	5 µl of Taq buffer (in $MgCl_2$)
2 µl of *Granulin* forward primer (10µM stock)	2 µl of *Egt* forward primer (10µM stock)
2 µl of *Granulin* reverse primer (10µM stock)	2 µl of *Egt* reverse primer (10µM stock)
2 µl of dNTPs (10mM stock)	2 µl of dNTPs (10mM stock)
2.5 µl of template (DNA)	2.5 µl of template (DNA)
0.5 µl of Taq polymerase	0.5 µl of Taq polymerase
50 µl total reaction	50 µl total reaction

Table 3.4 Amplification temperature of PCR reactants in PCR machine

		Temperature °C [1]	**Duration**	**Cycle**
Stage 1	Step 1	95°C	1:30 min	1 x
Stage 2	Step 1	95°C	0:30 min	30 x
	Step 2	50°C	0:40 min	
	Step 3	72°C	0:45 min	
Stage 3	Step 1	72°C	5 min	1 x

[1] Denaturing (95°C): Annealing (45°C): Extension or elongation (72°C)

3.2.11 Sequence alignment of *granulin* and *egt* gene sequences of CpGV isolates

Two overlapping sequence products were obtained for the *granulin* and *egt* genes, one in the forward direction and one reverse. The chromatographs were viewed using Finch TV (Version 1.4.0). Forward and reserve sequences were assembled and aligned using Molecular Evolutionary Genetics Analysis (MEGA) version 6.06. Sequences obtained were subjected to nucleotide BLAST on the National Centre for Biotechnology Information Database (NCBI) to detect single nucleotide polymorphisms (SNPs). The

nucleotide sequences of the five CpGV isolates were then translated using MEGA and subjected to protein BLAST to detect any amino acid changes in the sequence.

3.2.12 Dose-response bioassay with *Cydia pomonella* neonate larvae

Surface dose bioassays were only conducted with isolates that were shown to be genetically novel (distinct from CpGV-M) and with CpGV-M (Mexican strain purified from Madex®). The assays were conducted in 25 cell bioassay trays (Sterilin Ltd, United Kingdon) (Figure 3.3).

Figure 3.3 25-cell bioassay trays with artificial *Cydia pomonella* diet poured into each well and inoculated with 50 µl of virus suspension.

Diet was prepared by adding 6.5 g of agar to 600 ml of distilled water. The water and agar was heated to boiling point and then allowed to cool to 65°C before adding 140 g of dry ingredients. The diet was prepared in its standard proportions (Chapter 2, section 2.2.1.2) and autoclaved for 15 min at 121°C before adding ascorbic acid, methyl paraben and benzoic acid as anti-microbial agents. Each cell was filled with a layer of 6 to 8 mm of diet with agar and allowed to cool. Seven-fold dilutions of purified CpGV-5 and CpGV-M in sterile distilled water and a sterile distilled water control were used (Figure 3.4). OBs

were purified as described in section 3.2.6. Fifty larvae were treated per dose (two 25 cell bioassay trays per dose) and assays were replicated three times. A volume of 50 µl of each virus dilution and of the control was pipetted onto the centre of the diet surface in each cell using an autopipette. The fluid was spread evenly over the diet surface by tilting and rotating the tray slowly. Inoculated bioassay trays were left within the laminar flow cabinet for ± 30 min, until the surface of the diet was dry. One neonate larva was placed into each cell using a 000 point brush. All larvae were from the same batch of eggs, having all hatched on the morning that the assay was initiated. Trays were sealed by placing multiple layers of sterilised paper towel and the lid firmly pressed in place. Thick elastic bands were used to keep the lid firmly in place. The trays were marked and kept in an environmental chamber at 27ºC and 50% RH.

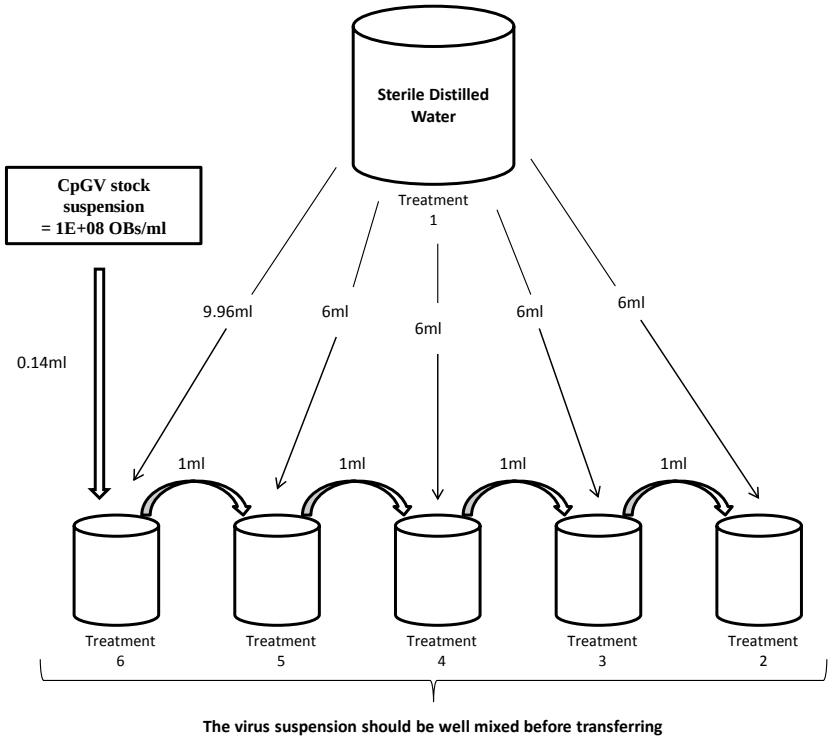


Figure 3.4 Seven-fold dilution series of CpGV for surface treatment dosage mortality bioassays with neonate *Cydia pomonella* larvae. Each treatment was thoroughly mixed by pipetting the virus suspension prior to the transferral of the 2 ml aliquot to the next lower dose. A new sterilised 1 ml pipette tip was used for each dosage.

The dosages used in the five CpGV treatments were determined from the literature (Eberle *et al*., 2008; Zichoúa *et al*., 2011). A seven fold dilution series was used with the highest concentration being 1.4 x 10^5 OBs/ml.

After eight days, the trays were opened and inspected. Larvae were recorded as alive or dead. The dose-response curve was calculated using PROBAN (Van Ark, 1995), a computer package for calculating probit analysis (Finney, 1971). PROBAN took into consideration the mortality of the control insects, and corrected the mortality of treated larvae according to Abbott's formula (Abbott, 1925). From this, LC_{50} and LC_{90} (concentration required to kill 50 and 90% of larvae in a sample respectively) were calculated for each assay. Overall values were obtained by calculating the means from the three assays.

3.3 RESULTS

3.3.1 Virus collection

Five CpGV isolates were identified from around South Africa, two from separate orchards in the Eastern Cape, two from the Western Cape and one from the Free State (Table 3.5). CpGV-1 was isolated from infected larvae from a laboratory culture established and maintained by ENTOMON technologies. All of the field collected isolates were identified from infected larvae found within infested fruit or collected during the rearing process. The four field collected isolates were identified from isolated farms and orchard blocks which, according to spray records, had never received any application of a CpGV based product (Table 3.5).

Table 3.5 Origins of the five CpGV isolates identified for testing.

Isolate	Area	Farm	Province	Co-ordinates
CpGV-1	Stellenbosch	ENTOMON (lab culture)	Western Cape	n/a
CpGV-2	Vermaaklikheid	Neglected orchard	Western Cape	34°18'19.15"S 21°01'37.51"E
CpGV-3	Langkloof	Zondagh farms	Eastern Cape	33°44'42.91"S 23°15'24.77"E
CpGV-4	Langkloof	Zondagh farms	Eastern Cape	33°44'42.91"S 23°15'24.77"E
CpGV-5	Free State	Free State	Free State	n/a

3.3.2 Symptomatology

Initial symptoms of infection were observed in larvae as a black speckling on the body surface (Figure 3.5A). Thereafter, larvae would become sluggish and usually take on a milky appearance. Some larvae appeared to swell as the infection progressed. Infected larvae usually did not lose their natural colour which was dark pink to purple in mature larvae, and white in the young instars (Figure 3.5 B). Infected larvae did not show the typical behavioural characteristic of moving out onto the surface of the diet or hanging/inverting themselves from the lid or sides of the rearing jar by their prolegs when infected (Tanada & Kaya, 1993). Rather, CpGV-infected *C. pomonella* larvae tended to remain within the diet, making them difficult to identify and remove. As the infection progressed and death occurred, the larvae lost their natural colouration and turned dark grey or brown (Figure 3.5 C).

Infected larvae which were not collected shortly after they deceased were difficult to recover from the diet. The integument became extremely weak and tended to rupture when attempts were made to collect or harvest the infected larvae (Figure 3.5 C).

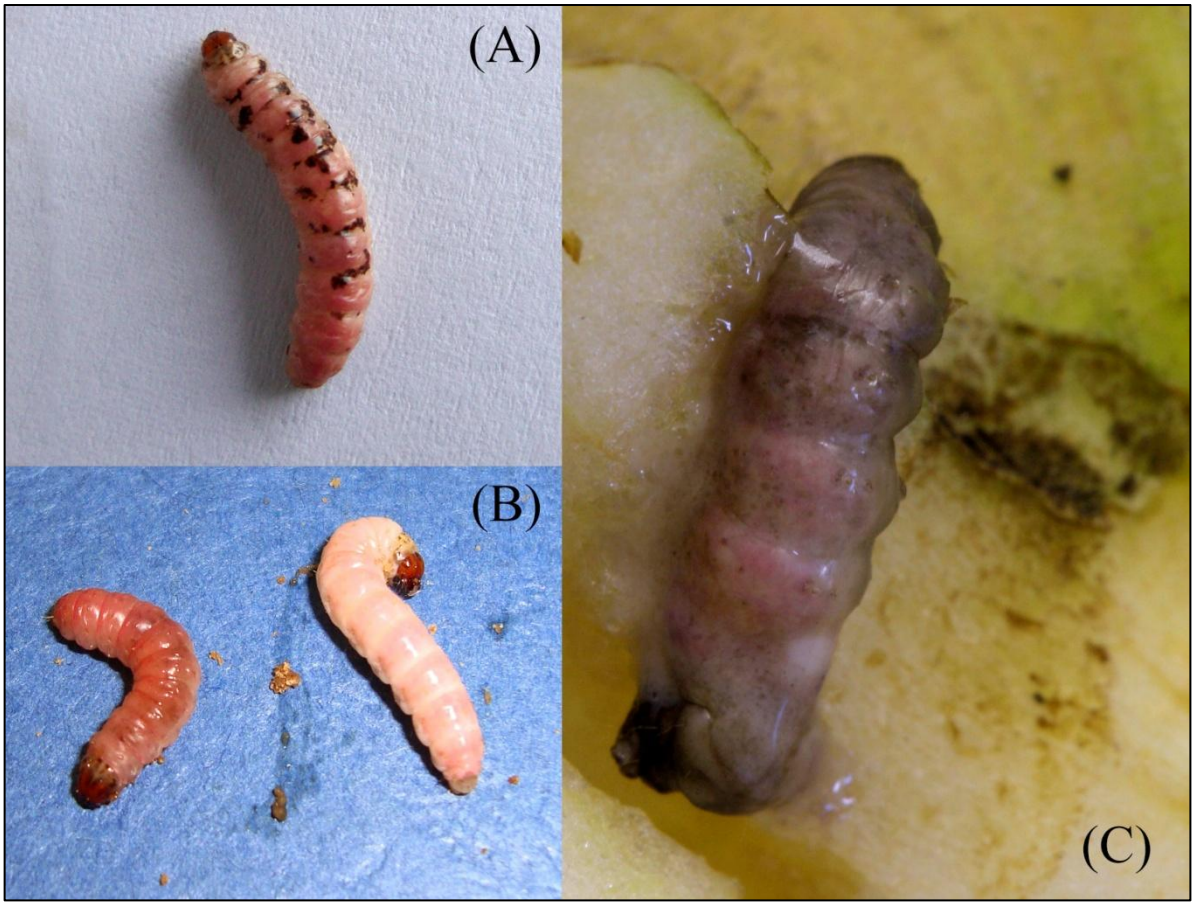


Figure 3.5 Symptomatically CpGV infected *Cydia pomonella* larvae: (A) with black speckling, (B) creamy appearance (C) and grey to brown colouration.

3.3.3 Preliminary identification of virus using light microscopy

Larval smears, stained with Buffalo Black solution, appeared infected with a possible baculovirus. This was evident by numerous microscopic virus particles which were stained grey in colour (Figure 3.6).

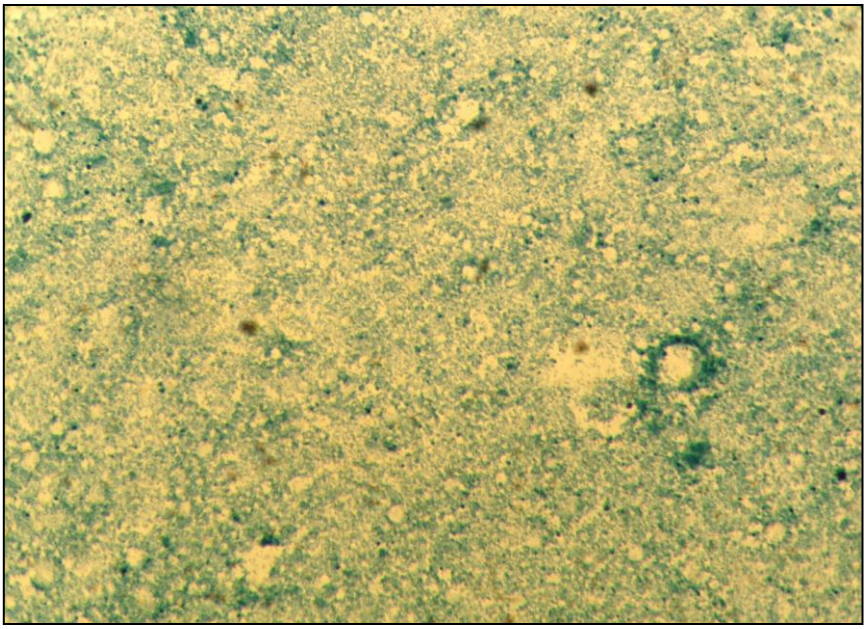


Figure 3.6 Buffalo Black stained smear of virus OBs from an infected *Cydia pomonella* larva, (1000X magnification) viewed with a light microscope.

3.3.4 Identification using transmission electron microscopy

To confirm the presence of a granulovirus, purified OBs were prepared for transmission electron microscopy using uranyl acetate staining. The TEM images confirmed the typical shape and morphology of a GV (Figure 3.8A). In a number of the OBs, a single nucleocapsid was visible (Figure 3.8B). The mean diameter and length of the OBs (n = 12) observed was 378.33 ± 6.23 nm in length and 199.94 ± 5.23 nm in width. Tanada (1964) calculated the average size of the virus capsules at 393.9 ± 4.29 nm in length and 207.7 ± 9.76 nm in width, slightly larger than what was observed in this study.

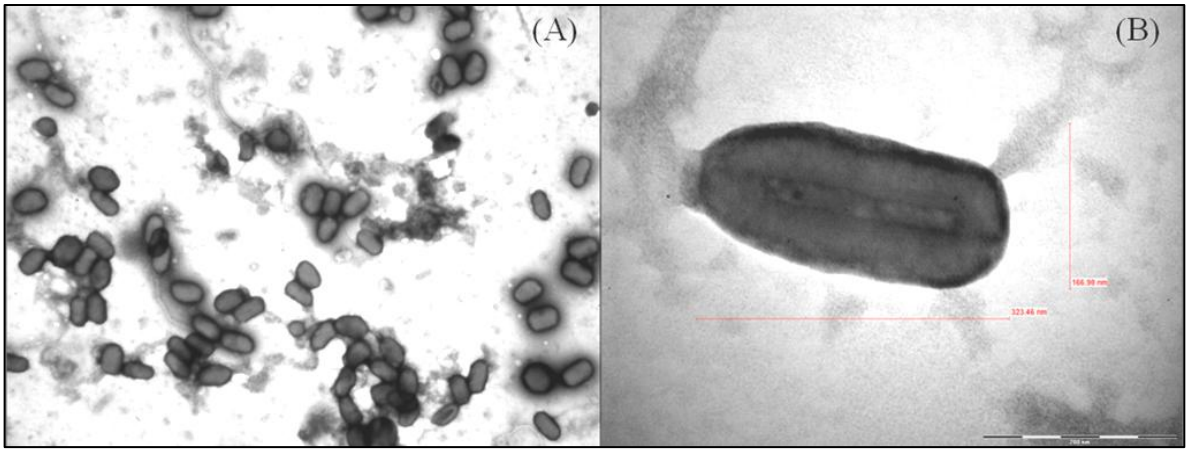


Figure 3.7 Transmission electron micrographs of CpGV: (A) numerous OBs, (B) single OB showing internal nucleocapsid.

3.3.5 Genomic DNA extraction and DNA concentration

Genomic DNA was successfully extracted from CpGV purified OBs and analysed on a 0.7% agarose gel (Figure 3.9). The DNA was observed to be of high molecular weight (Figure 3.9, lane 3). The concentration of DNA was determined according to the method described in section 3.2.8. The concentration of the three independent samples was 77.0, 71.1and 59.8 ng/ml.

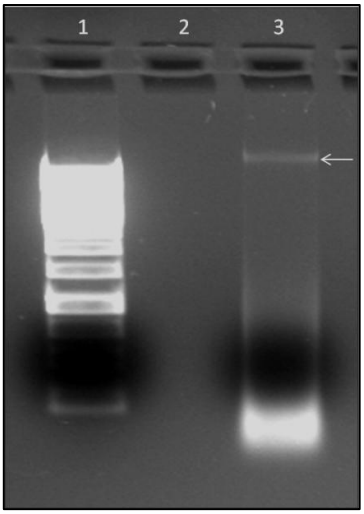


Figure 3.8 1% Agarose gel electrophoresis of CpGV genomic DNA. Lane 1 - High range DNA marker, lane 3 - CpGV genomic DNA (indicated by arrow). Electrophoresis was carried out at 85 V for 40 min.

3.3.6 Restriction endonuclease (REN) analysis of genomic DNA

REN analysis was conducted in order to compare the genomic DNA profiles of the five CpGV isolates with the genomic DNA profiles of two commercially available CpGV products, namely Madex® and Carpovirusine™. Both the commercial products available in South Africa are formulated with the Mexican strain of CpGV whose genome has been fully sequenced (U53466.2, Luque *et al*., 2001). This isolate was therefore used as a reference point for genotypic differences in virus strains.

Single REN digestion of the CpGV genomic DNA with *Bam*H1, *Pst*1, *Eco*R1, *Xba*1 and *Xho*1 produced several prominent bands and a few fainter bands. It was noted that digestion of DNA with *Hin*dIII resulted in a single band rather than a profile (data not shown). This observation provided a means for determining CpGV presence and quality control for production purposes (see Chapters 4 and 5). DNA markers were run alongside the restriction digest in order to estimate the size of the DNA fragments. Figure 3.9 shows the DNA profiles of the five CpGV isolates (Table 3.5) using *Bam*H1. The restriction digest produced 10 prominent DNA fragments. The profiles of all the isolates were identical with the exception of CpGV-5 which had only 9 prominent DNA fragments.

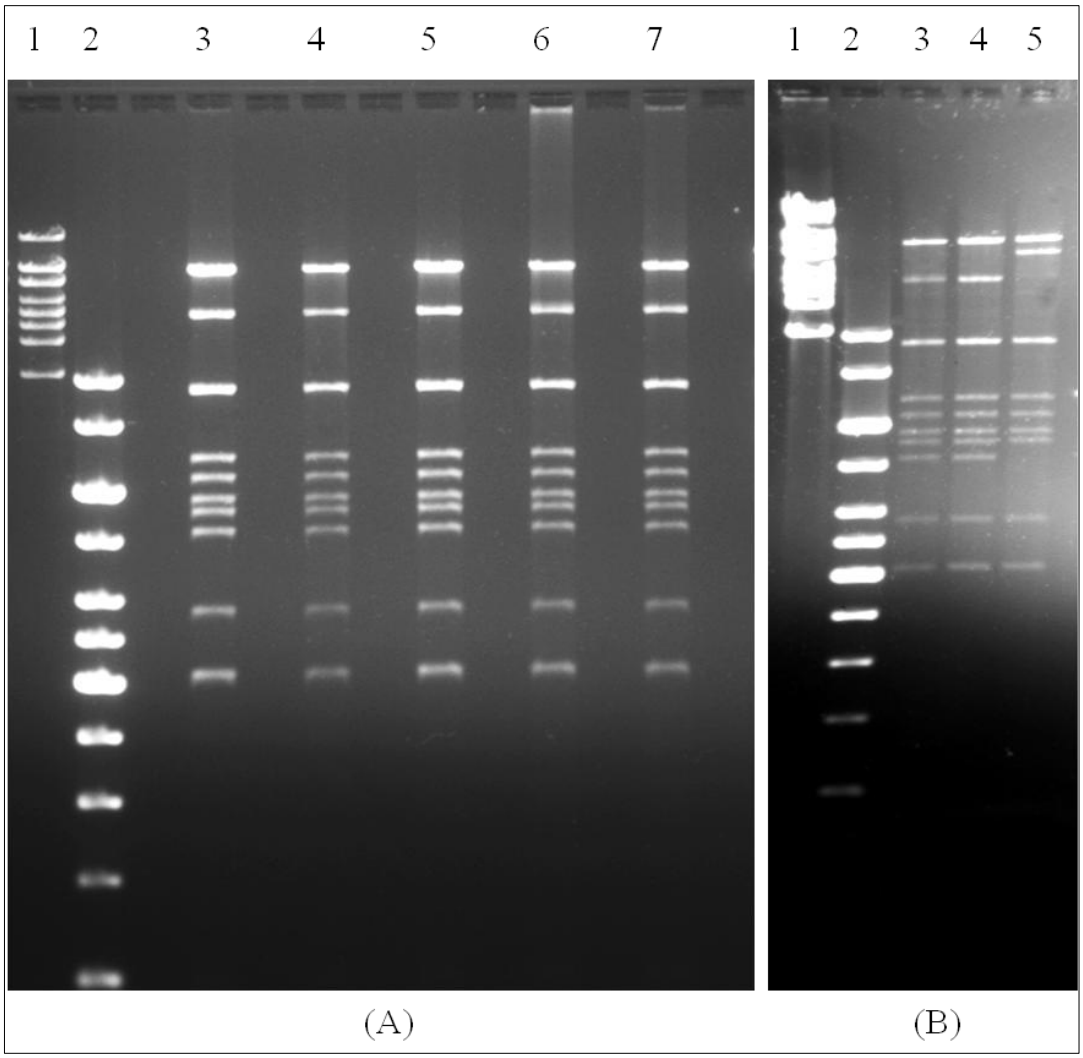


Figure 3.9 Restriction endonuclease profiles of seven isolates of CpGV digested with *Bam*HI. Electrophoresis was conducted on 0.6 % agarose gels for 16 h at 30 V. (A) Lane 1 – High range DNA marker, lane 2 – 1 Kb DNA marker, lane 3 – CpGV-1, lane 4 – CpGV-2, lane 5 – CpGV-3, lane 6 – Carpovirusine™, lane 7 – Madex®. (B) Lane 1 – DNA marker, lane 2 – 1 Kb DNA marker, lane 3 – Madex®, lane 4 – CpGV-4, lane 5 – CpGV-5.

The restriction digest with *Pst*I produced 8 prominent DNA fragments and 4 faint fragments with the exception of isolates CpGV-3, Madex® and Carpovirusine™ which produced additional faint fragments of high molecular weight (Figure 3.7). These bands are likely a result of incomplete digestion of the DNA (indicated by arrows in Figure 3.10). The restriction pattern of CpGV-5 differed from the remaining isolates. The REN profiles

of CpGV-5 produced 12 prominent DNA fragments some of which were the same size as those in the other profiles, and additional bands that were unique to this isolate.

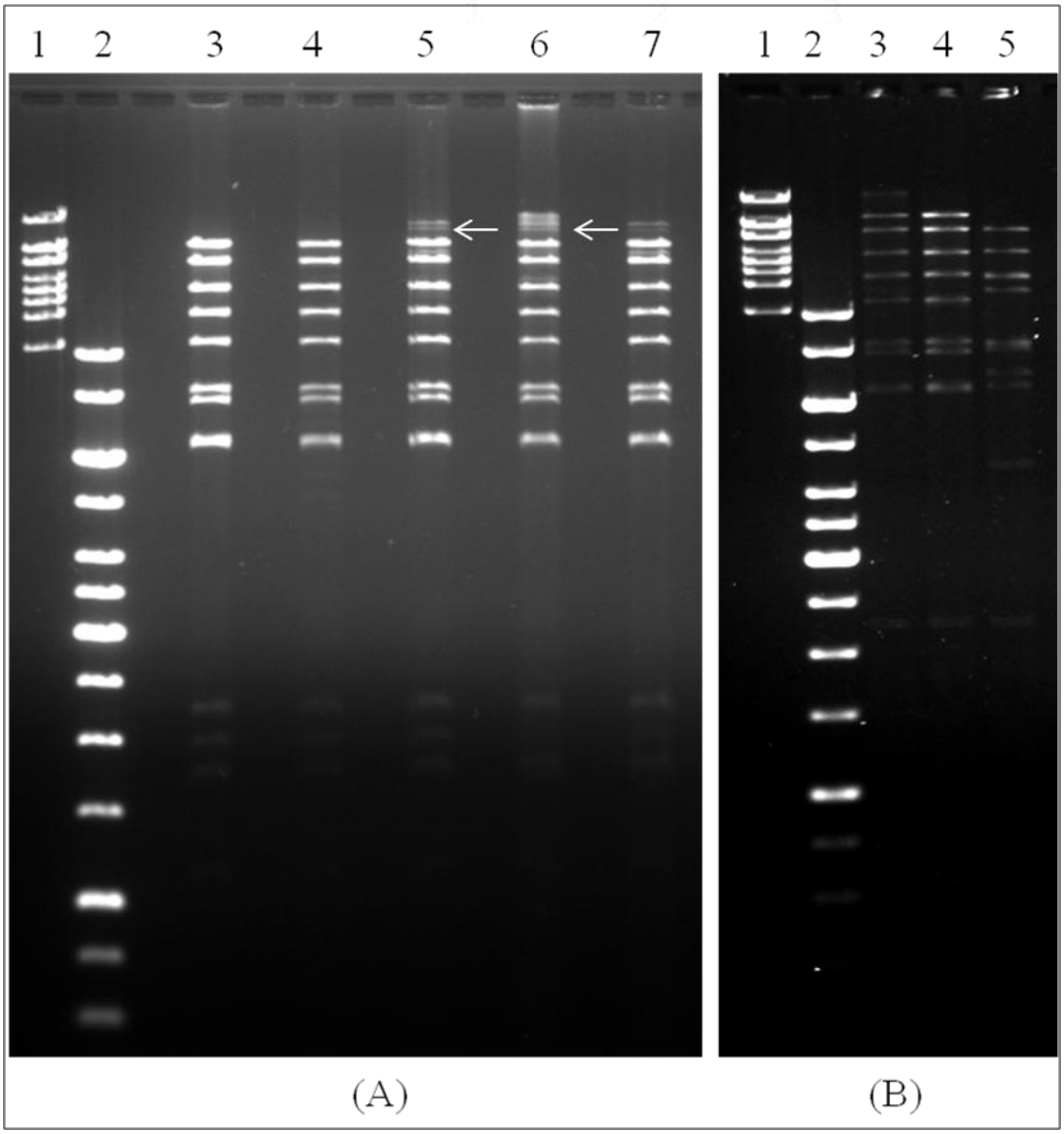


Figure 3.10 Restriction endonuclease profiles of seven isolates of CpGV digested with *Pst*I. Electrophoresis was conducted on 0.6 % agarose gels for 16 h at 30 V. (A) Lane 1 – High range DNA marker, lane 2 – 1 Kb DNA marker, lane 3 – CpGV-1, lane 4 – CpGV-2, lane 5 – CpGV-3, lane 6 – Carpovirusine™, lane 7 – Madex®. (B) Lane 1 – DNA marker, lane 2 – 1 Kb DNA marker, lane 3 – Madex®, lane 4 – CpGV-4, lane 5 – CpGV-5.

The restriction digest with *Eco*R1 produced 10 prominent DNA fragments and 1 faint fragment in all isolates (Figure 3.11). Additional high molecular weight bands were observed in the profiles for CpGV-2 and Madex®. Once again, these are likely due to incomplete digestion of the DNA (indicated by arrows in Figure 3.11).

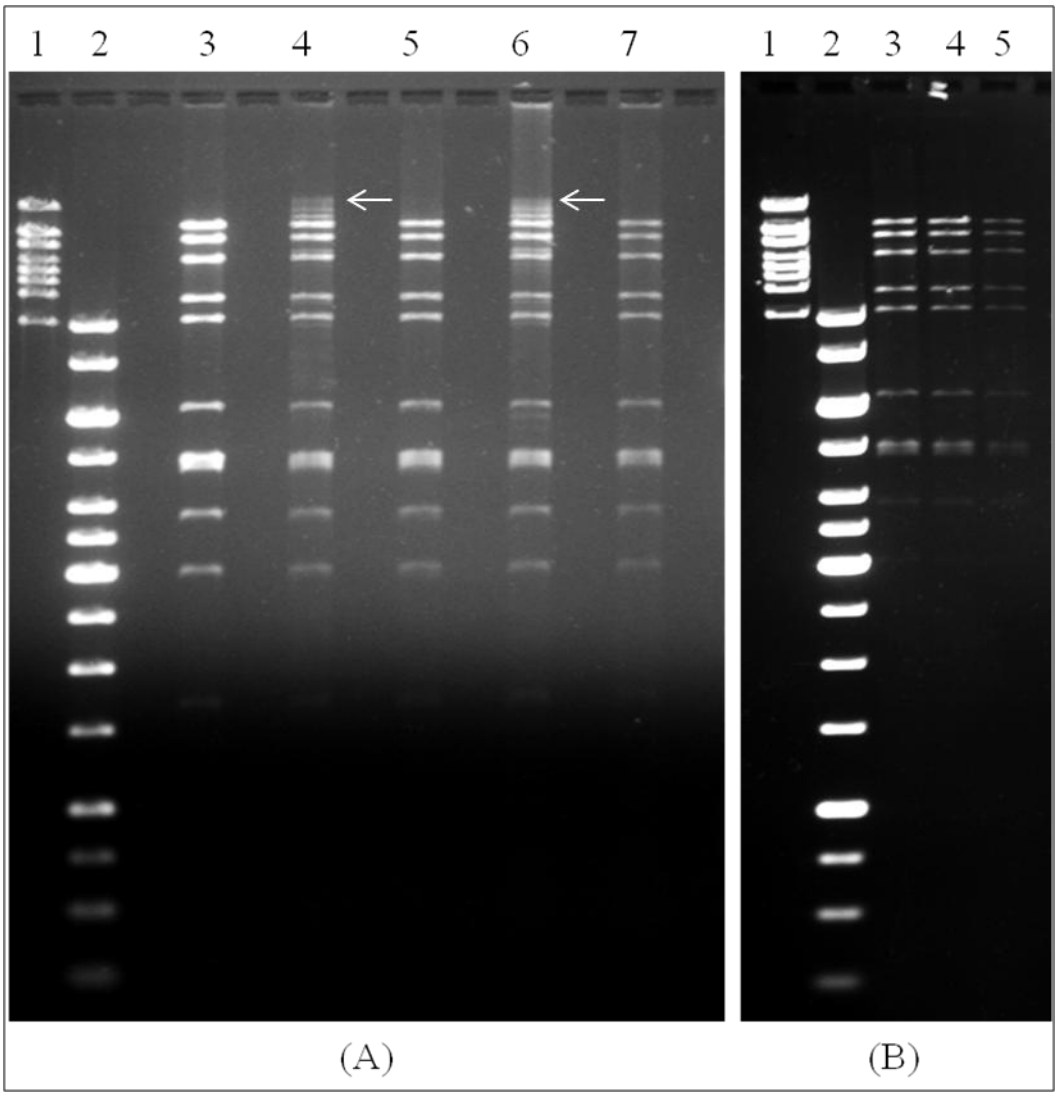


Figure 3.11 Restriction endonuclease profiles of seven isolates of CpGV digested with *Eco*RI. Electrophoresis was conducted on 0.6 % agarose gels for 16 h at 30 V. (A) Lane 1 - High range DNA marker, lane 2 – 1 Kb DNA marker, lane 3 – CpGV-1, lane 4 – CpGV-2, lane 5 – CpGV-3, lane 6 – Carpovirusine™, lane 7 – Madex®. (B) Lane 1 – DNA marker, lane 2 – 1 Kb DNA marker, lane 3 – Madex®, lane 4 – CpGV-4, lane 5 – CpGV-5.

Restriction profiles obtained by digestion with *Xba*I and *Xho*I produced identical profiles for all CpGV isolates (Figure 3.12). Due to insufficient virus stock for OB purification and DNA extraction both CpGV-4 and CpGV-5 were not subjected to REN analysis with *Xba*I and *Xho*I.

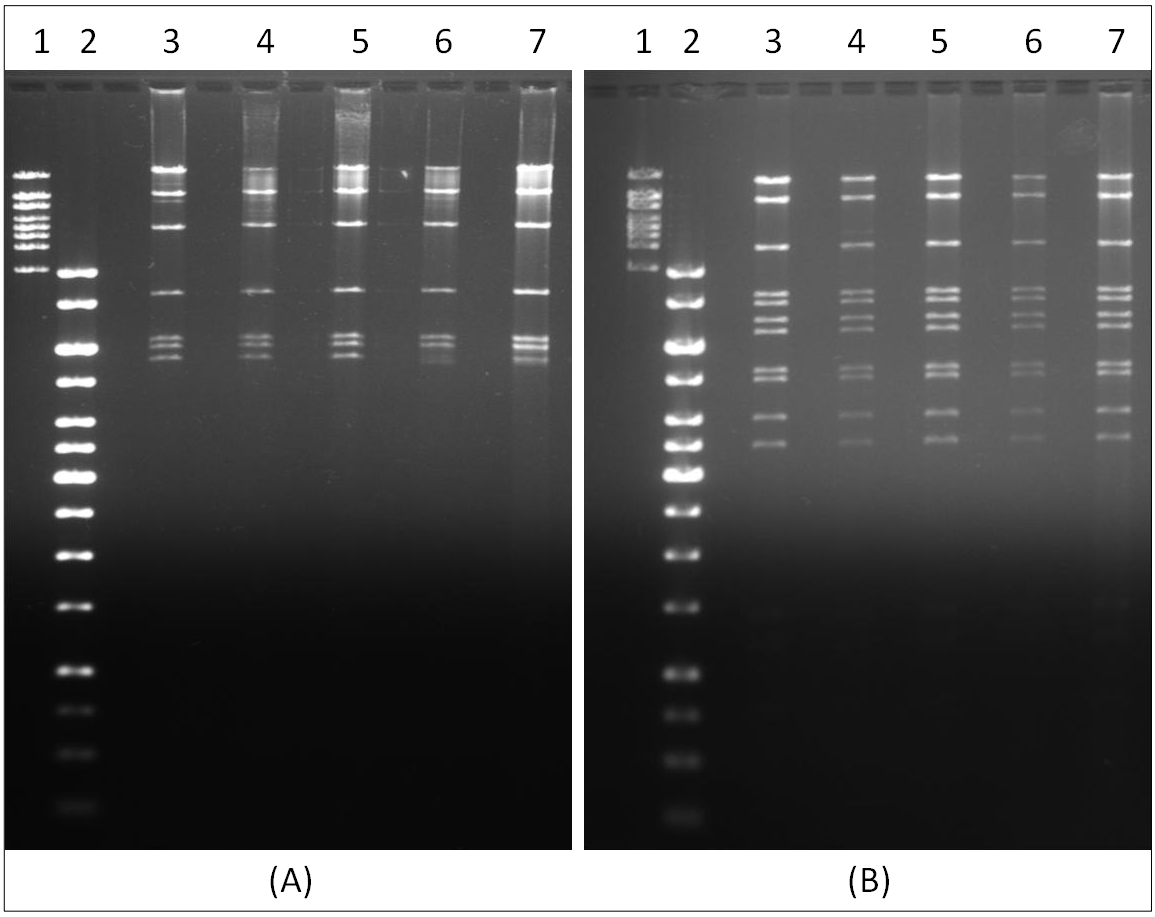


Figure 3.12 Restriction endonuclease profiles of five isolates of CpGV digested with (A) *Xba*I and (B) *Xho*I. Electrophoresis was conducted on 0.6 % agarose gels for 16 h at 30 V. (A) Lane 1 – High range DNA marker, lane 2 – 1 Kb DNA marker, lane 3 – CpGV-1, lane 4 – CpGV-2, lane 5 – CpGV-3, lane 6 – Carpovirusine™, lane 7 – Madex®.

3.3.7 Comparison of CpGV-M, CpGV-1 and CpGV-5 restriction profiles

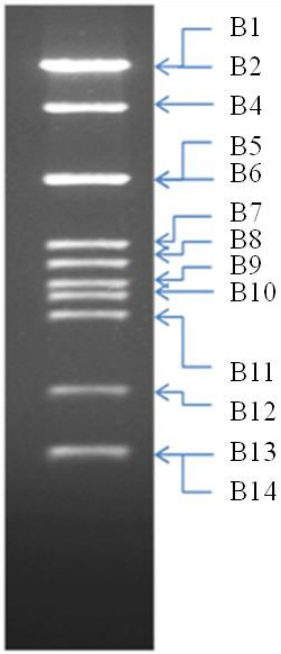
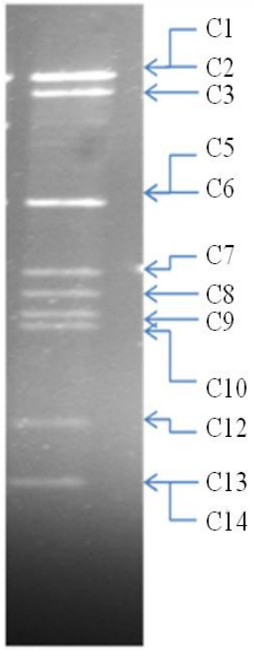
From the RE analysis it is evident that CpGV-1, CpGV-2, CpGV-3 and CpGV-4 DNA profiles are similar if not identical to that of the Mexican strain of CpGV found in both

commercial CpGV products distributed in South Africa. However, CpGV-5 showed unique *Bam*H1and *Pst*1 profiles. Because the profiles for CpGV-1 to 4 were identical to each other and to CpGV-M for each enzyme, only the profiles for CpGV-1 and CpGV-5 were selected for further comparison with those of CpGV-M generated *in silico* as described in the next section.

3.3.7.1 Comparison of CpGV-1 and CpGV-5 *Bam*HI profiles

In silico digestion of CpGV-M genomic DNA with *Bam*H1 produced 15 fragments ranging from 24501 to 989 nt and a total genomic size of 123500 nt (Table 3.6) Ten DNA fragments were observed in CpGV-1 which were estimated using a scanning and imaging analysis software UVIband version 11.9 (UVItec 2004). These ranged from approximately 23660 nt to 3077 nt with a total genome size of 121322 nt. Nine DNA fragments were observed in CpGV-5. The fragment sizes were estimated using scanning and imaging analysis software UVIband version 11.9. These ranged from approximately 23417 nt to 3122 nt with a total genome size of 121461 nt. A comparison of the profiles showed that all fragments present in CpGV-M were also present in CpGV-1 and CpGV-5 with the exception of fragment A10 which was absent in CpGV-5. There was also an additional fragment (C3) observed in CpGV-5 was estimated to be 20332 nt and not present in CpGV-1 or CpGV-M. Fragment A15 shown in the *in silico* analysis was not present in either isolate possibly due to its low molecular weight.

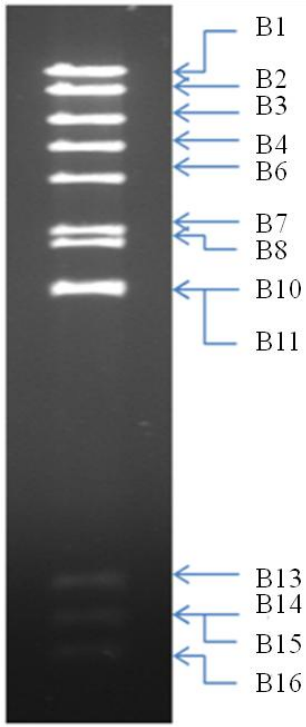
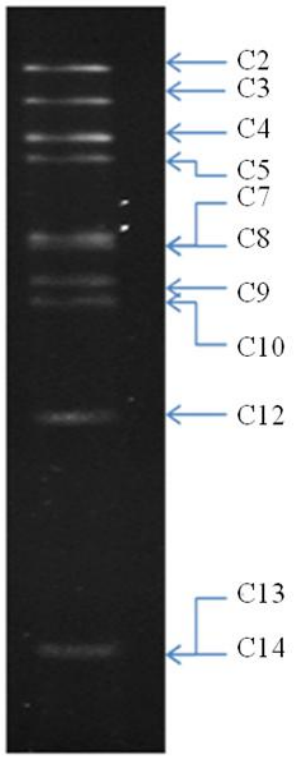
Table 3.6 *Bam*H1 DNA restriction profiles of CpGV-M, CpGV-1 and CpGV-5.

CpGV-M		CpGV-1			CpGV-5		
Fragment	**Size (nt)**	**Fragment**	**Size (nt)**	**Gel**	**Fragment**	**Size (nt)**	**Gel**
A1	24501	B1	± 23660[1]		C1	± 23417[1]	
A2	24162	B2	± 23660[2]		C2	± 23417[2]	
A3	-	-	-		C3	± 20332	
A4	15261	B4	± 14943		C4	-	
A5	9425	B5	± 9589[1]		C5	± 9755[1]	
A6	9374	B6	± 9589[2]		C6	± 9755[2]	
A7	6847	B7	± 6939		C7	± 6960	
A8	6344	B8	± 6369		C8	± 6288	
A9	5808	B9	± 5818		C9	± 5819	
A10	5559	B10	± 5577		C10	± 5587	
A11	5143	B11	± 5168		C11	-	
A12	3866	B12	± 3856		C12	± 3887	
A13	3121	B13	± 3077		C13	± 3122[1]	
A14	3100	B14	± 3077		C14	± 3122[2]	
A15	989	B15	-		C15	-	
Number of fragments	14		13			12	
Total	123500		121322			121461	

3.3.7.2 Comparison of CpGV-1 and CpGV-5 *Pst*I profiles

In silico digestion of CpGV-M genome DNA with *Pst*1 produced 13 fragments ranging from 27231 to 1160 nt and a total genomic size of 123500 nt (Table 3.7). Eleven DNA fragments were observed in CpGV-1 and nine in CpGV-5. Fragment sizes of CpGV-1 ranged from ± 25096 nt to 1002 nt with a total genome size of ± 120353 nt. Fragment sizes for CpGV-5 ranged from ± 22279 to ± 2291 with a total genome size of ± 120531 nt. A comparison of the profiles showed that all fragments present in CpGV-M were also present in CpGV-1. Fragments A1, A6 and A11 did not appear in CpGV-5 (Table 3.7). There were also three additional fragments observed in the CpGV-5 profile (C5, C9 and C12) of estimates sizes 11581, 7021 and 4506 nt respectively. It was suspected that fragments C15 and C16 were present but due to their low molecular weight were not visible on the gel.

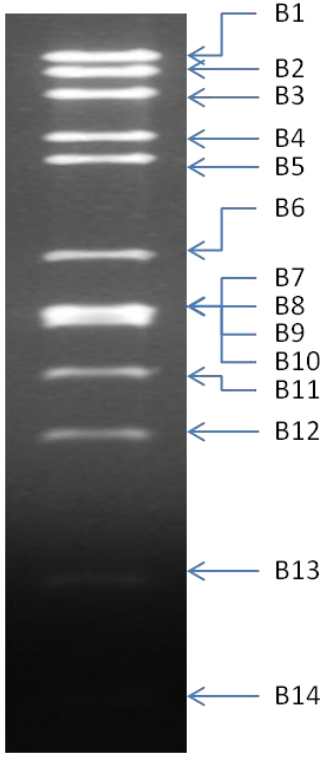
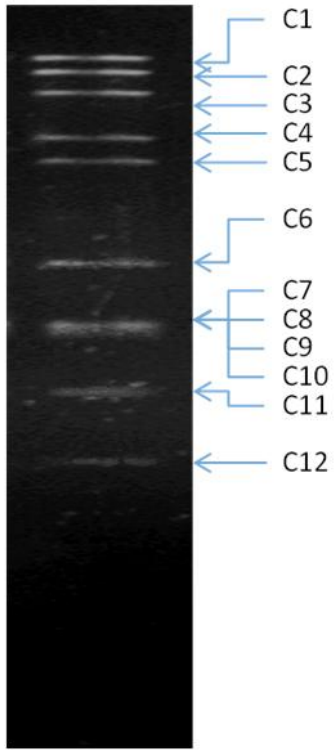
Table 3.7 *Pst*1 DNA restriction profiles of CpGV-M, CpGV-1 and CpGV-5.

CpGV-M		CpGV-1			CpGV-5		
Fragment	**Size (nt)**	**Fragment**	**Size (nt)**	**Gel**	**Fragment**	**Size (nt)**	**Gel**
A1	27231	B1	± 25096		C1	-	
A2	21205	B2	± 20286		C2	± 22279	
A3	15841	B3	± 15429		C3	± 16537	
A4	12741	B4	± 12749		C4	± 13173	
A5	-	B5	-		C5	± 11581	
A6	10518	B6	± 10352		C6	-	
A7	8118	B7	± 8118		C7	± 8314	
A8	7662	B8	± 7912		C8	± 8000	
A9	-	B9	-		C9	± 7021	
A10	6423	B10	± 6513[1]		C10	± 6565	
A11	6333	B11	± 6513[2]		C11	-	
A12	-	B12	-		C12	± 4506	
A13	2264	B13	± 2381[1]		C13	± 2291[1]	
A14	2222	B14	± 2381[2]		C14	± 2291[2]	
A15	1782	B15	± 1621		C15	-	
A16	1160	B16	± 1002		C16	-	
Number of fragments	13		13			12	
Total	123500		± 120353			± 120531	

3.3.7.3 Comparison of CpGV-1 and CpGV-5 *Eco*RI profiles

In silico digestion of CpGV-M genome DNA with *Eco*R1 produced 15 fragments ranging from 27551 to 177 nt and a total genomic size of 123500 nt (Table 3.8). Ten DNA fragments were observed in CpGV-1. These ranged from ± 26223 nt to ± 839 nt with a total genome size of ± 122992 nt. Nine DNA fragments were observed in CpGV-5. These ranged from ± 27327 nt to ± 3106 nt with a total genome size of ± 120531 nt. A comparison of the two profiles showed that all fragments present in CpGV-M were also present in CpGV-1 and CpGV-5. Fragments B15 in CpGV-1 and fragments C13, C14 and C15 in CpGV-5 were not visible on the gel possibly due to their low molecular weights but are suspected to be present.

Table 3.8 *Eco*R1 DNA restriction profiles of CpGV-M, CpGV-1 and CpGV-5.

CpGV-M		CpGV-1			CpGV-5		
Fragment	**Size (nt)**	**Fragment**	**Size (nt)**	**Gel**	**Fragment**	**Size (nt)**	**Gel**
A1	27551	B1	± 26223		C1	± 27327	
A2	21821	B2	± 21931		C2	± 22553	
A3	16830	B3	± 16790		C3	± 12730	
A4	11748	B4	± 11687		C4	± 12011	
A5	10124	B5	± 10230		C5	± 10319	
A6	6264	B6	± 6475		C6	± 6319	
A7	4973	B7	± 5039[1]		C7	± 5000[1]	
A8	4913	B8	± 5039[2]		C8	± 5000[2]	
A9	4817	B9	± 5039[3]		C9	± 5000[3]	
A10	4711	B10	± 5039[4]		C10	± 5000[4]	
A11	3843	B11	± 3924		C11	± 3913	
A12	3061	B12	± 3086		C12	± 3106	
A13	1698	B13	± 1651		C13	-	
A14	969	B14	± 839		C14	-	
A15	177	B15	-		C15	-	
Number of fragments	15		14			12	
Total	123500		± 122992			± 120531	

3.3.8 Amplification of the CpGV-5 *granulin* and *egt* gene sequences

The *granulin* and *egt* genes were successfully amplified by PCR (Figure 3.13). The amplified products were analysed by 0.6% agarose gel electrophoresis. The *granulin* gene amplicon produced a bright band resolving at a size of approximately 750 nt (Figure 3.13 A) and the amplified product of the *egt* gene formed a thick bright band of approximately ± 1500 nt (Figure 3.13 B). The sizes of the *granulin* and *egt* genes are 747 and 1455 nt respectively (Luque *et al*., 2001).

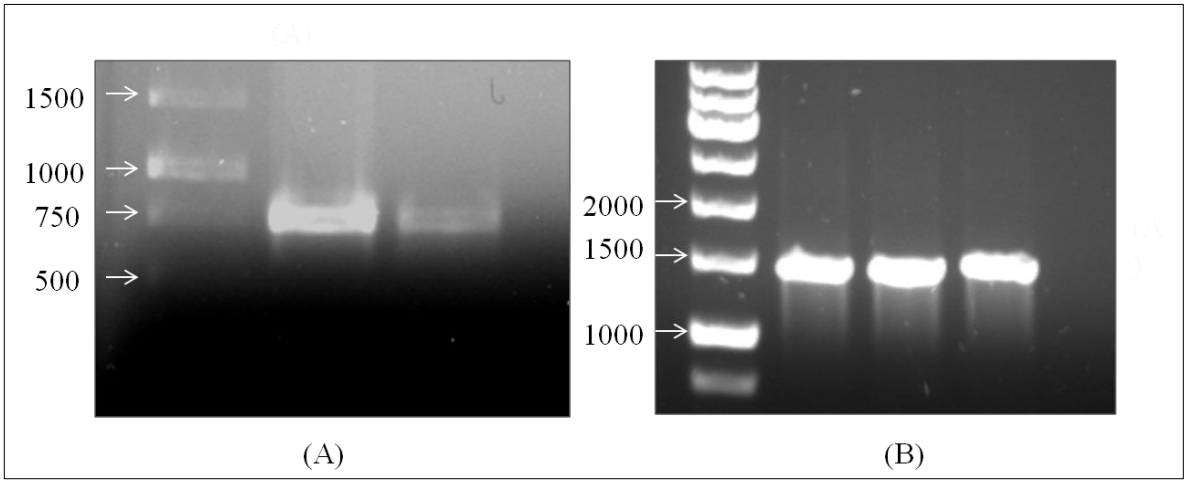


Figure 3.13 Agarose gel electrophoresis of the amplified products of *granulin* and *egt*, genes from CpGV-1 (A) *granulin* gene amplicon, (B) *egt* gene amplicon.

3.3.9 Analysis of nucleotide and amino acid sequences of the CpGV isolates' *granulin* genes

The forward and reverse sequences of each gene were assembled and edited using MEGA 6.06 analysis software. Because the oligonucleotides were designed within the gene sequence, only partial *granulin* sequences were obtained. The following partial sequences were subjected to BLAST in order to determine the closest match sequence: CpGV-1, 37 – 738, CpGV-2, 34 – 737, CpGV-3, 31 – 737, Madex®, 31 – 717 and Carpovirusine™, 25 – 717. BLAST analysis of the *granulin* gene sequences of each isolate revealed a 100%

nucleotide identity with CpGV-M. Alignment of the *granulin* gene sequence with CpGV-M showed no nucleotide substitutions within this gene for all of the isolates tested.

3.3.10 Analysis of nucleotide and amino acid sequences of the CpGV isolates' *egt* gene

The forward and reverse sequences of each gene were assembled and edited using MEGA 6.06 analysis software. Because the oligonucleotides used for amplification were designed within the gene only partial sequences were obtained. The following partial sequences were subjected to BLAST in order to determine the closest match sequence: CpGV-1, 120894 – 122256, CpGV-2, 120911 – 122255, CpGV-3, 120899 – 122266, CpGV-4, 120899 – 122269, CpGV-5, 120897 – 122269, Madex®, 120895 – 122275 and Carpovirusine™, 120895 – 122292. BLAST analysis of the *egt* gene sequences of each CpGV isolate revealed a 100% nucleotide identity to CpGV-M for CpGV-1 to CpGV-4. CpGV-5 showed a 99% nucleotide identity to CpGV-M. Only a single SNP was observed in the CpGV-5 sequence (Table 3.9). This single SNP did not result in an amino acid change in the protein.

Table 3.9 Single nucleotide polymorphisms (SNPs) found in the CpGV-5 *egt* gene after alignment with CpGV-M.

Nucleotide position/codon	Isolate		Amino acid
	CpGV-M	CpGV-5	
196	TTC	**G**TC	Valine

Letters in red bold colour indicate single nucleotide polymorphisms.

3.3.10 Surface-dose bioassays with *Cydia pomonella* neonate larvae

Due to the results obtained in the genetic analysis CpGV-5 will be referred to as CpGV-SA for the remainder of this thesis. Surface dose bioassays were conducted against *C.*

pomonella neonate larvae in 25 cell bioassay trays. The LC values of the Mexican strain of CpGV were compared to the South African strain, CpGV-SA, isolated from the Free State area in South Africa.

The regression lines fitted to the data in Table 3.10 for the three replicates with the CpGV-M isolate had the equations y = 4.3309 + 0.4773 x (SE of slope = 0.0813), y = 2.6301 + 0.7321 x (SE of slope = 0.0924) and y = 3.2980 + 0.5963 x (SE of slope = 0.0764). Deviations for the lines for all three replicates were estimated to be homogenous.

The three replicates of the CpGV-SA isolate (Table 3.10) had the equations y = 4.2944 + 0.4380 x (SE of slope = 0.0809), y = 2.8684 + 0.6755 x (SE of slope = 0.0810) and y = 4.0120 = + 0.4730 x (SE of slope = 0.0655) (Table 4.2). Deviations for the lines for all three replicates were estimated to be homogenous. The slopes of the lines in all neonate bioassays were below 1. Jones (2000) reported that slopes of between 1 and 2 should be considered as the norm for virus assays.

Table 3.10 Mortality of *Cydia pomonella* neonate larvae in dose-response bioassays with six concentration of CpGV-M.

Treatment (CpGV in OBs/mm²)	Replicate 1			Replicate 2			Replicate 3 [1]		
	Larval mortality (%)	Mortality corrected for control mortality (%)	Empirical probit	Larval mortality (%)	Mortality corrected for control mortality (%)	Empirical probit	Larval mortality (%)	Mortality corrected for control mortality (%)	Empirical probit
Distilled water control	10.00			12.00			2.00		
8.33×10^0	18.00	8.89	3.65	24.00	13.64	3.90	43.75	42.60	4.813
5.83×10^1	24.00	15.56	3.99	38.00	29.55	4.46	56.25	55.36	5.135
4.08×10^2	34.00	26.67	4.38	54.00	47.73	4.94	72.92	72.36	5.594
2.86×10^3	54.00	48.89	4.97	56.00	50.00	5.00	81.25	80.87	5.873
2.0×10^4	70.00	66.67	5.43	84.00	81.82	5.91	93.75	93.62	6.524
1.4×10^5	98.00	97.78	7.01	96.00	95.45	6.69	*	*	*

[1] 100% mortality was achieved in the highest concentration and therefore it was omitted from the analysis

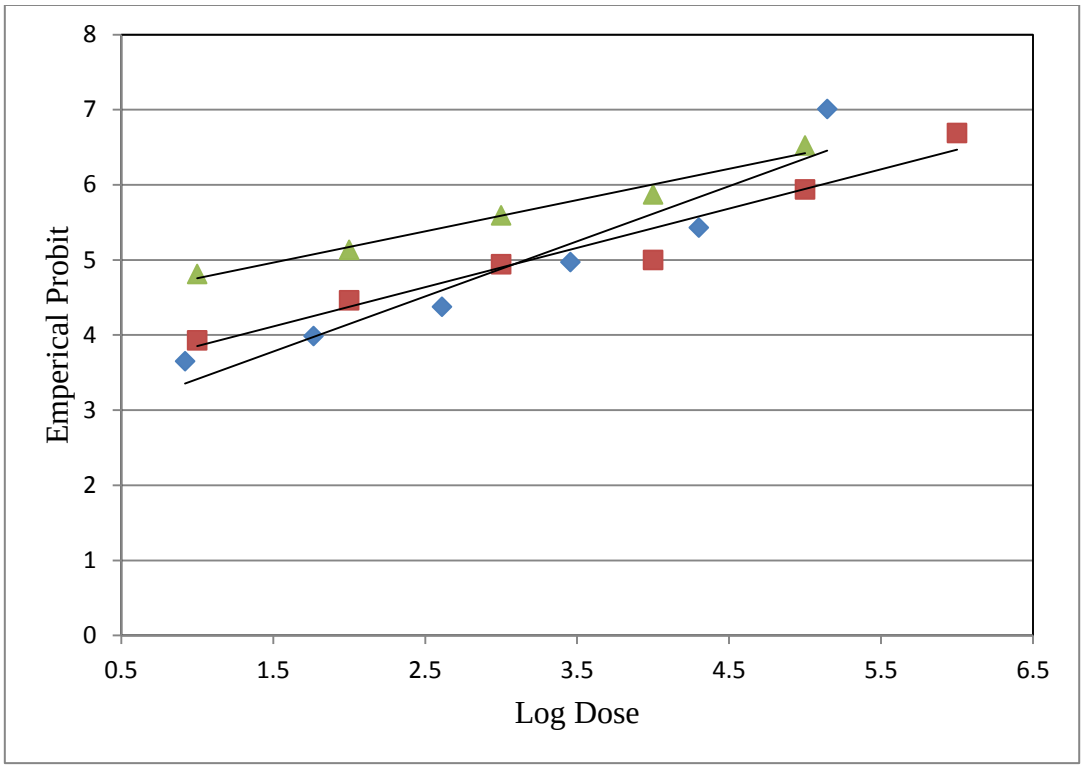


Figure 3.14 Dose-mortality probit lines for CpGV-M with *Cydia pomonella* neonate larvae.

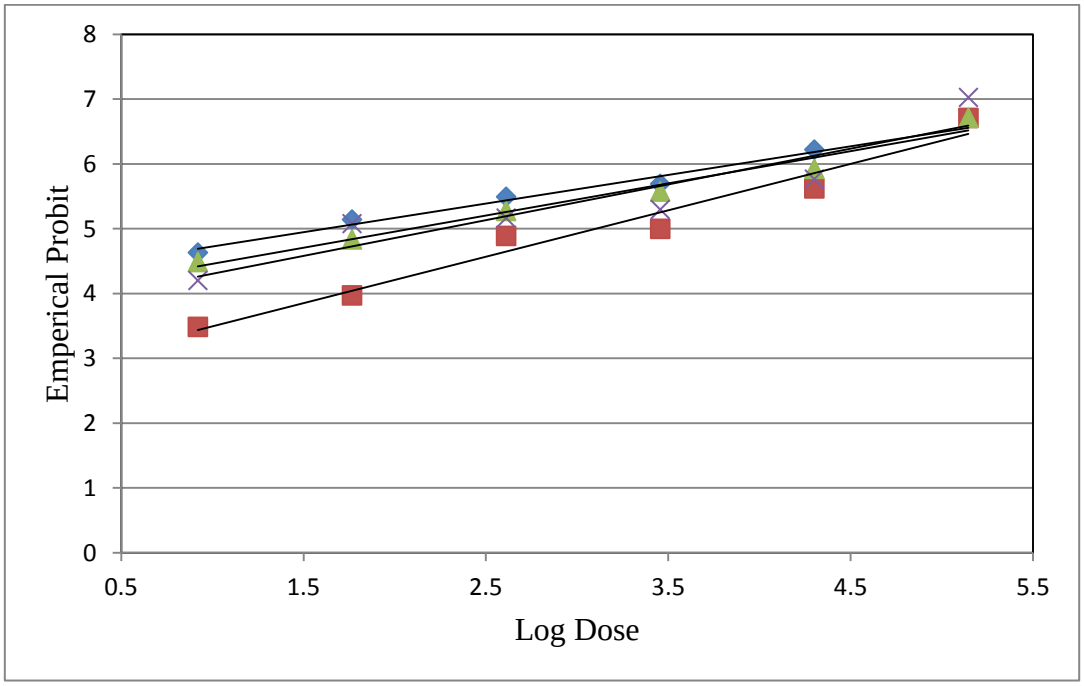


Figure 3.15 Dose-mortality probit lines for CpGV-SA with *Cydia pomonella* neonate larvae.

Table 3.11 Mortality of neonate *Cydia pomonella* larvae in dose-response bioassays with six concentration of CpGV-SA in series dilution.

Treatment (CpGV in OBs/mm²)	**Replicate 1**			**Replicate 2**			**Replicate 3**		
	Larval mortality (%)	**Mortality corrected for control mortality (%)**	**Empirical probit**	**Larval mortality (%)**	**Mortality corrected for control mortality (%)**	**Empirical probit**	**Larval mortality (%)**	**Mortality corrected for control mortality (%)**	**Empirical probit**
Distilled water control	8.00			8.00			6.00		
8.33×10^0	14.00	6.52	3.49	36.00	33.43	4.49	26.00	21.28	4.20
5.83×10^1	22.00	15.22	3.97	48.00	43.48	4.84	56.00	53.19	5.08
4.08×10^2	50.00	45.65	4.89	64.00	60.87	5.28	59.18	56.58	5.17
2.86×10^3	54.00	50.00	5.00	74.00	71.74	5.58	64.00	61.70	5.30
2.0×10^4	75.51	73.38	5.62	83.67	82.25	5.93	79.17	77.84	5.77
1.4×10^5	96.00	95.65	6.71	96.00	95.65	6.71	98.00	97.87	7.03

G for fiducial limits was calculated to be 0.1114, 0.0611 and 0.0631 for the three bioassay replicates with the Mexican isolate of CpGV. The G for fiducial limits value for the three replicate of the CpGV-SA was calculated to be 0.0553, 0.0737 and 0.0703. Figures of greater than 0.025 indicate a large variation in mortality between treatments; however experimental procedures or the value of the probit line should only be questioned if the G value exceeds 0.25 (Van Ark, 1995). From the above data, the calculated LC_{50} and LC_{90} doses are presented in Table 3.12.

Table 3.12 Comparison of the LC_{50} and LC_{90} values for both CpGV-M and CpGV-SA with fiducial limits.

		Mean Dose (OBs/ml)	**SE**	**Fiducial limits**	
				Upper ± SE	**Lower ± SE**
CpGV-M	LC_{50}	7.21×10^2	3.54×10^2	1.99×10^3 (1.29×10^3)	5.11×10^2 (3.61×10^2)
	LC_{90}	7.33×10^4	1.77×10^4	3.97×10^5 (1.40×10^5)	3.01×10^4 (1.43×10^4)
CpGV-SA	LC_{50}	3.18×10^3	2.10×10^2	8.87×10^3 (6.14×10^2)	2.09×10^2 (1.71×10^2)
	LC_{90}	8.21×10^4	3.62×10^4	5.84×10^5 (7.30×10^4)	2.42×10^4 (7.52×10^4)

The slope of the regression lines was compared using PROBAN to test for significant differences in the LC concentrations of each isolate. PROBAN found the residual variances of the two lines (CpGV-M and CpGV) to be homogenous as per the F test for homogeneity between two samples ($F_{4,4} = 23.16$; $P = 0.638$). The slopes of the two lines were thus comparable. Using the chi-squared test, the lines were found to be parallel so the elevations could be compared ($\chi^2 = 3.841$, df = 1; $P = 0.481$). Their elevations did not differ significantly from one another ($F_{1,9} = 5.12$; $P = 0.289$).

3.4 DISCUSSION

The aim of this chapter was first to bioprospect for novel South African isolates of CpGV in the field. Three deciduous fruit growing areas in South Africa where no previous record of a commercial application of CpGV was evident were sampled. Initial investigations

involved observing larvae for signs and symptoms of virus infection. In this study, indication of an infection was observed as a black speckling on the body surface after which the larvae became sluggish, swelled and usually took on a milky appearance. The observations of viral infection in this study support the presence of virus and are similar to the observations of CpGV infection reported by Lacey & Thomson (2004). Transmission electron microscopy analysis of purified OBs confirmed the presence of a GV similar in size to that reported in previous studies (Tanada, 1964; Lacey *et al*., 2008a)

Following morphological characterisation, it was necessary to determine the genetic identity of the virus. Genomic DNA was successfully extracted for downstream applications using the CTAB method. A total of 25 µl of DNA at a concentration of 485 µg/ml was obtained from 200 µl of purified OBs. DNA extraction was repeated at least three times for each isolate in order to provide enough genomic DNA for restriction endonuclease analysis and PCR amplification of gene sequences. Similar concentrations of DNA were obtained for each batch of DNA extracted, showing that the CTAB method of DNA extraction used in this study was reproducible and reliable. This result correlated with reports from Abdulkadir *et al*. (2013), Opoku-Debrah *et al*. (2013) and Jukes *et al*. (2013) in which the CTAB method was used for the extraction of genomic DNA from baculoviruses.

Single restriction endonuclease analysis of genomic DNA with a variety of enzymes was used to compare the genetic identities of five field collected CpGV isolates. Commonly used enzymes were chosen so that profiles could be compared against published data if novel isolates were obtained. The DNA profiles obtained by agarose gel electrophoresis were also compared with those found within two commercially available CpGV products formulated with the Mexican strain of CpGV. Reports of the comparative analysis of the *Bam*H1, *Pst*1, *Eco*R1 and *Xho*1 fragment profiles of CpGV-M with profiles obtained for various CpGV isolates using agarose gel electrophoresis has revealed differences in fragment patterns between isolates (Harvey & Volkman, 1983; Crook *et al*., 1997; Eberle *et al*., 2008; Rezapanah *et al*., 2008; Berling *et al*., 2009).

An accurate and reliable method for comparison of DNA profiles of novel isolates is complete genome sequencing. However this methodology is both time consuming and expensive and was considered to be beyond the scope of this study. Only a single complete genome sequence of CpGV is available to date and was described by Luque *et al*. (2001). This complete sequence was used to generate *in silico* profiles for comparison with those of the five CpGV isolates in this study. *In silico* digestion of the CpGV-M sequence produces exact band sizes, whereas the measurement of band sizes for CpGV-1 and CpGV-SA is an estimation. The latter profiles were generated by single restriction enzyme digestion of genomic DNA followed by AGE, and the band size estimates using computer based software. Comparison of the *Bam*H1, *Pst*1, *Eco*R1, *Xba*1 and *Xho*1 fragments sizes of the CpGV isolates with the *in silico* profile of CpGV-M revealed that only CpGV-SA showed variation in banding patterns between isolates.

Restriction analysis with *Hin*dIII followed by AGE produced no visible fragment patterns for any of the isolates tested and the DNA was observed as a single, high molecular weight fragment. *In silico* digestion of the CpGV-M genome with *Hin*dIII produced two DNA fragments of 83474 and 40055 in size. Restriction analysis of CrleGV with *Hin*dIII produces multiple DNA fragments of varying molecular weights (Opoku-Debrah, 2008; Opoku-Debrah *et al*., 2013). Therefore this technique was considered as an initial diagnostic step in confirming the presence of CpGV, CrleGV or a mixture of the two in a virus sample. A total genome size of 123500 nt was obtained for CpGV-M following *in silico* digestion with *Bam*H1, *Pst*1 and *Eco*R1. The total genome size estimated for CpGV-SA varied from 120631 to 121461 with the range of REs used. This discrepancy in the genome size is expected as the measurement of band size by REN analysis is an estimation and therefore the calculated genome size can be less than or more than the actual genome size.

It was important to gain more information regarding the genetic identity of the CpGV isolates. This was attempted by PCR amplification and sequencing of the *granulin* and *egt* genes for each of the isolates. The *granulin* gene of CpGV is located in open reading frame (ORF) 1 of the CpGV genome and encodes for 249 amino acids (Luque *et al*., 2001). The

granulin gene is highly conserved and was used in this study to identify CpGV in diseased insects. Sequence alignment of the *granulin* gene of CpGV-1, CpGV-2, CpGV-3, Madex® and Carpovirusine™ isolates with that of CpGV-M revealed no SNPs. This result is not unexpected as the *granulin* gene is reported to be highly conserved (Federici, 1997) and any change in the amino acid sequence of the protein could result in increased susceptibility of the virus OBs to adverse environmental conditions. Opoku-Debrah *et al.* (2013) showed several SNPs in the *granulin* gene of different isolates of CrleGV in South Africa. However, none of these SNPs resulted in amino acid changes within the protein. Similar observations were observed in studies on PhopGV and PlxyGV (Abdulkadir *et al*., 2013; Jukes *et al*., 2013). The *egt* gene is an auxiliary gene that encodes an enzyme which mediates the inactivation of the moulting hormone in insects and is not considered to be an essential gene for the virus (O'Reilly *et al*., 1992). In CpGV, the gene is encoded in ORF 141 and encodes for 483 amino acids (Luque *et al*., 2001). The expression of the *egt* gene has some significant advantages as it is thought to allow the virus to delay the development of the larva by preventing moulting or pupation. This potentially increases the feeding time of the larva which is beneficial to the virus as it enables the virus to replicate for a longer period, thus producing more virus (Tumilasci *et al*., 2003; Ferrelli *et al.*, 2012). Deletion of the *egt* gene may result in an improved speed of kill, as the host dies much quicker (O'Reilly *et al*., 1992). This has been the focus of studies on genetically engineered baculoviruses (Black *et al*., 1997; Hilton *et al*., 2008). Only a single SNP in isolate CpGV-SA was observed after comparison between CpGV-M, which did not result in an amino acid change in the protein. This result was surprising as the *egt* gene nucleotide sequence has been found to vary between virus isolates in other baculoviruses. For example, Eberle *et al*. (2008; 2009) identified a number of SNPs in highly conserved genes when comparing various isolates of CpGV to the Mexican strain, CpGV-M. Opoku-Debrah *et al*. (2013) reported several amino acid substitutions in the *egt* sequence between geographically different CrleGV isolates. Similar results have been shown for the *egt* gene sequence for PlxyGV (Abdulkadir *et al*., 2013) and PhopGV (Espinel-Correal *et al*., 2010; Jukes *et al*., 2013) when compared between isolates. It is acknowledged that both genes chosen for amplification in this study are highly conserved genes and that further work in

identifying genetic differences between CpGV-SA to other strains of CpGV should make consider the use of less conserved genes.

All commercial preparations of CpGV registered in South Africa have been formulated with the Mexican strain of CpGV. The commercial products, Madex® and Carpovirusine™, have been distributed widely around the South African deciduous fruit growing regions and applied by many growers as part of an IPM based control programme. Therefore, the fact that only a single isolate (CpGV-SA) was identified to be genetically different from CpGV-M was not surprising. The orchard from which the CpGV-SA was isolated is geographically isolated with the nearest possible codling moth population approximately 16 km away. The restriction profiles generated with *Bam*HI and *Pst*I indicated that CpGV-SA differs genetically form the Mexican strain of the virus. It must be noted that subsequent to this section of the study being completed, further work has been conducted by Rhodes University on the genetic novelty of CpGV-SA. A complete genome analysis has been completed and the results of this study confirm that isolate CpGV-SA is indeed genetically different from the Mexican strain of CpGV. This would be a first report of a South African isolate of CpGV that is genetically different from CpGV-M.

Comparative surface dose bioassays were conducted for both the CpGV-SA and CpGV-M against *C. pomonella* neonate larvae. The results obtained in this study indicate CpGV-SA to be highly pathogenic to *C. pomonella* larvae producing a LC_{50} value of 3.18×10^3 OBs/ml and an LC_{90} value of 7.33×10^4 OBs/ml. In comparison, the CpGV-M isolate produced an LC_{50} value of 7.21×10^2 OBs/ml and an LC_{90} value of 8.21×10^4 OBs/ml. The Mexican strain of the virus is seen as the benchmark for comparisons as it is the isolate most commonly used in all commercial CpGV based products. The LC values determined in this study compare favourably to those found in the literature. Small variations in the calculation of the LC values can be attributed to experimental design. Moreover, different populations of *C. pomonella* could possibly vary in their susceptibility to a CpGV isolate. Several studies have conducted bioassays of CpGV preparations against *C. pomonella* neonate larvae (Keller, 1973; Sheppard & Stairs, 1977; Laing & Jaques, 1980; Huber,

1981; Glen & Payne, 1984; Lacey *et al*., 2002; Lacey *et al*., 2005a). Laing & Jaques (1980) developed a bioassay technique in which the larva was placed onto a small, restricted area of diet which was surface-treated with the virus inoculum, and the LC_{50} were estimated to be 17 OBs per neonate larva per 28 mm². Glen & Payne (1984) reported similar LC_{50} values of 2.4×10^3 OBs per ml of diet with a mean slope of the dose-mortality response of 1.38 ± 0.34. Lacey *et al*. (2005a) reported LC_{50} and LC_{95} values of 35 and 540 granules per mm^2 for neonate *C. pomonella* larvae. Resistance or partial resistance in *C. pomonella* populations to CpGV has been well documented (Jehle, 2008; Jehle *et al*., 2010; Schmitt *et al*., 2013). Therefore, it is important that novel pathogenic strains of CpGV be discovered and tested to manage resistance and provide continued control of *C. pomonella* populations. Jehle *et al*. (2010) stated that diversity in CpGV genotypes is the capital for the further development and improvement of CpGV based products. It was also recommended that applications should include not only single genotypes but rather a mix of different isolates or, alternatively, different genotypic isolates in each application. The biological activity of the CpGV-SA isolate is encouraging from a commercial perspective and its unique genetic identity potentially makes it an important tool in a CpGV resistance management programme. Interestingly, it has been shown that resistance in *C. pomonella* to CpGV is isolate specific and related to an insertion in the *pe38* gene which is not only essential for the infectivity of CpGV, but also a key factor in managing CpGV resistance (Gebhardt *et al*., 2014). Currently Rhodes University is conducting a study to genetically characterise the CpGV-SA isolate. Preliminary results have shown that the 24 nt insertion that occurs in the CpGV-M strain is absent in CpGV-SA (personal communication, Boitumelo Motsoeneng). Therefore the CpGV-SA isolate could be an important tool for use against host resistance in *C. pomonella* populations. What remains to be determined is if the pathogenicity of the isolate will be sufficient to warrant further investigation into the use of CpGV-SA development on a commercial scale.

3.5 CONCLUSION

Several CpGV isolates were recovered from field populations of *C. pomonella* and genetically characterized. Comparison of DNA profiles using a variety of REs confirmed

that only one isolate, CpGV-SA, was genetically different from the Mexican strain of the virus which used in the two commercially available products in South Africa. However, the data obtained from the sequencing of selected viral genes (*granulin* and *egt*) did not support this. Complete genome sequencing and analysis is currently underway to confirm the genetic novelty of CpGV-SA which may be an important tool in resistance management in *C. pomonella* populations. The biological activity data generated in this study indicates that isolate CpGV-SA has potential as a biological control agent. It is also important to further evaluate the biological activity of the virus isolate against the host population in terms to determine median lethal time.

The main objective of this study was to develop and test a methodology for the production of CpGV in *T. leucotreta*. Chapter 4 compares virus production in fourth and fifth instar *C. pomonella* and *T. leucotreta* larvae when inoculated with various concentrations of CpGV-M. Dose-response and time-response bioassays are conducted to establish LC_{90} and LT_{90} values for CpGV inoculation of fourth and fifth instar *T. leucotreta* larvae which can be used for the development of mass production methodologies.

4

COMPARISON OF THE VIRUS YIELDS AND PATHOGENICITY OF *CYDIA POMONELLA* GRANULOVIRUS PRODUCED IN *CYDIA POMONELLA* AND *THAUMATOTIBIA LEUCOTRETA*

4.1 INTRODUCTION

In Chapter 2, the rearing characteristics of *Cydia pomonella* and *Thaumatotibia leucotreta* were compared under laboratory conditions and it was concluded that the latter host had potential for efficient CpGV production. Subsequently, Chapter 3 described the identification of a novel isolate of CpGV identified from a field population of *C. pomonella* which was genetically different from the commercial strain, CpGV-M. Dose response bioassays determined that CpGV-SA had comparable virulence to the Mexican strain of CpGV. With mass production of CpGV being the ultimate goal of this study, virus yield studies and dose- and time-response bioassays were necessary to determine the LC (lethal concentration) and LT (lethal time) estimates for mass production of CpGV in the heterologous host, *T. leucotreta*.

Bioassays are used to determine dose-response and time-response relationships, and are an effective method of assessing the biological activity of baculoviruses (Hughes *et al*., 1986; Hughes & Shapiro, 1997; Jones, 2000; Opoku-Debrah, 2011). Dose-responses with viruses differ from those using chemical insecticides in that they take into account the fundamental notion that virus particles can act independently of one another, with a single virus particle

capable of initiating an infection that could possibly result in mortality (Ridout *et al*., 1993). Increasing the inoculum dosage results in an increased response, although this increase will be as a result of the increased probability of one or more virus particles successfully passing through the natural defences of the host and not due to an additive effect as seen in chemical assays (Ridout *et al*., 1993).

Baculoviruses isolated from genetically distinct insect populations vary genotypically and may display differences in virulence towards the host (Parnell *et al*., 2002; Eberle *et al*., 2008; Opoku-Debrah *et al*., 2013). The use of the correct bioassay technique to assess the pathogenicity of CpGV is essential in understanding the biology of the virus as well as the development of effective *in vivo* mass production systems (Evans, 1994). Various bioassay methods have been used to assess the pathogenicity of viruses against their hosts. These methods include, surface dose, diet incorporation, droplet feeding and egg dipping techniques. Diet incorporation methodology is used for internal or cryptic feeders such as fruit borers like *C. pomonella* (Fritsch *et al*., 2007). However, this method is considered impractical when comparing this technique to feeding habits of the insect in its natural environment. In the field there is little or no chance that the larva will ingest virus particles once inside the fruit. Virus ingestion is exclusive to the fruit surface and will occur as the larva tunnels to gain access into the fruit (Moore, 2002). The droplet feeding method was first developed by Hughes & Wood (1981). Recently this technique has been developed for bioassaying *T. leucotreta* neonate larvae (Pereira-da-Conceicoa *et al*., 2012). In the droplet bioassay method the virus is presented to the larvae in droplets of virus suspension containing Brilliant Blue R dye. These bioassays have a high level of accuracy due to the increased probability that the larvae ingest the virus and the easy identification of those that have consumed the virus suspension. Since the volume ingested by a larva remains virtually constant, or at least measurable, this allows an accurate estimation of the LD value from a given LC and is therefore considered appropriate for LT studies (Jones, 2000). This technique was initially indentified for use in this study. However sufficient numbers of larvae could not be enticed to drink from the droplets and a large percentage of those that did became entrapped in the droplet and drowned. The egg dip method was developed for larvae that consume the entire chorion upon hatching (Jones, 2000). This

behaviour is not exhibited by *C. pomonella* or *T. leucotreta* and therefore this method was not considered. For the purpose of this study, surface dose bioassays were preferred. This method involves applying a known concentration of purified virus to the surface of an artificial diet or leaf surface. Sufficient virus should be applied to cover the entire surface of the diet. The inoculum is then allowed to dry. Usually a single larva is placed onto the diet surface and allowed to feed until death or pupation occurs (Hughes & Shapiro, 1997; Hunter-Fujita *et al*., 1998).

The assessment of any dose-response assay will depend upon the data on which it is based. For example, any experiment in which the dose levels are either too low to show any response, or so high that they all show 100% response, will result in little or poor information on the dose-response of the target organism to the stimulus. Any study, properly designed to provide adequate information on the relationship between dose and response, will entail the consideration of many factors (Jerne & Wood, 1949; Finney, 1964). Probit analysis (Finney, 1971) is the most frequently used statistical analysis tool used to describe the dose-mortality relationship of insect controlling agents with log-dose or concentrations as the explanatory variable (Sporleder *et al*., 2005). It is applied in situations where there is a cumulative quantitative response with increasing dosage or concentration. Probit analysis transforms data from sigmoid to linear after which a regression is run on the relationship, from which the regression LC doses may be calculated. Biologically meaningful statistical parameters are the expression of LC_{50} (lethal concentration required to cause 50% mortality) or LC_{90} (lethal concentration required to cause 90% mortality) and the slope of the regression line (Sporleder *et al*., 2005).

One overall aim of this study was to determine whether an alternate host would be suitable for the production of CpGV. For this purpose, *T. leucotreta* was chosen, as it is closely related to *C. pomonella* (both tortricids) and its susceptibility to CpGV has already been demonstrated (Reiser *et al*., 1993). Furthermore, a tested and reliable protocol for the *in vivo* production of CrleGV has been established by Moore (2002), and is currently used in the production of a commercial GV for the control of *T. leucotreta* (Moore *et al*., 2014). The methodology developed by Moore (2002) is both efficient and economically viable on

a commercial scale. It is with this in mind that this study investigates the potential for *in vivo* production of CpGV in a heterologous host, *T. leucotreta*. Reiser *et al*. (1993) showed that production of CpGV in *T. leucotreta* larvae was possible. However, their study did not determine dose and time response data for production of the virus in late instar *T. leucotreta* larvae. This information will be necessary for mass production of CpGV in late instar larvae.

The objectives of this chapter were three-fold. Firstly, to determine the quantity of virus produced per larva, in both *T. leucotreta* and *C. pomonella*, when inoculated with various concentrations of CpGV. Secondly, to determine the concentration of CpGV inoculum which will be required for virus production and the incubation time required to maximise virus yields. The dose-response and time-response data generated in this section of the study will be used in mass production trials to determine if CpGV can effectively be produced in *T. leucotreta* on a large scale. The final aim was to determine if the heterologous virus, CpGV, infected and replicated effectively within *T. leucotreta* larvae. REN analysis with *Hin*dIII was used to generate profiles of the viral genomic DNA produced in fourth and fifth instar *T. leucotreta* larvae during the time-response bioassays as the first step in quality control of virus samples

4.2 MATERIALS AND METHODS

4.2.2 Propagation of virus in *Thaumatotibia leucotreta* and *Cydia pomonella* larvae

4.2.2.1 Propagation of virus in fourth and fifth instar *Thaumatotibia leucotreta* and *Cydia pomonella* larvae

Dry *T. leucotreta* diet was mixed using the same methodology as described in Chapter 2, section 2.2.1.2; 140 g of dry ingredients, in their standard proportions, was mixed with 150 ml of sterile distilled water. This made a mixture which was sufficiently moist to settle in an even layer over the bottom of the dish. The mixed diet was poured into non-stick baking trays measuring 260 x 160 x 35 mm to a depth of 6 to 8 mm. Each dish was sealed with a layer of aluminium foil and baked in an oven at 180ºC for 15 min. The cooked diet was

placed into a laminar flow cabinet to cool. Dry *C. pomonella* diet was mixed using the same methodology described in Chapter 2, section 2.2.1.1. The diet was prepared as described above.

The bioassay was conducted in 22 ml glass pill vials rather than bioassay trays (Figure 4.1). It was found to be easier to inspect for larvae showing signs or symptoms of viral infection. Using this method, it was not necessary to open and close the vials in order to inspect the larvae, therefore limiting any factors and reducing the risk of contamination. Round diet plugs were produced by inverting a sterilized vial and pressing it into the diet. Vials were washed in 0.5% sodium hypochlorite solution, rinsed and autoclaved at 121°C for 15 min. The vial was slowly rotated until the diet plug was freed from the non-stick surface. The plug was pushed down the vial until it sat flush on the bottom and was then compressed lightly to ensure the plug fitted securely against the sides and bottom on the vial. The surface area of the diet plug was 380 mm². Eight concentrations of CpGV were tested against *T. leucotreta* and *C. pomonella* larvae. The concentrations tested ranged from 10^2 to 10^9 OBs per surface area and an untreated control.

Figure 4.1 Glass vials used in propagation bioassay to determine virus yield in *Thaumatotibia leucotreta* and *Cydia pomonella* larvae.

A sample of 10 individuals was removed from a batch which appeared to be at a fourth instar stage. Larvae were placed into a freezer (-16ºC) for a few minutes until they ceased moving. Head capsule size was measured under a light microscope, using a calibrated eye-piece. If head capsule size fell within the acceptable range (0.84 to 1.19 mm) for fourth instar *T. leucotreta* larvae (Daiber, 1979), or 0.92 to 1.23 mm for *C. pomonella* (Weitzner & Whalon, 1987; Bloemfield & Giliomee, 2009) then larvae from that particular batch were used for the bioassay.

Propagation of CpGV in fifth instar *T. leucotreta* and *C. pomonella* larvae was conducted using the same protocol described above. Ten day old larvae were selected based on the results obtained from Chapter 2, section 2.3.7. Head capsule size was measured to determine if they were within the acceptable range as above, then larvae from that particular batch were used for the bioassay.

Thirty glass vials were inoculated per treatment with each treatment being repeated three times. 200 µl of virus was pipetted onto the surface of the diet. Each vial was tilted and rotated to ensure the virus suspension evenly covered the entire surface of the diet. The vials were left on a laminar flow bench until sufficiently dry (± 30 min). A single fourth instar larva was placed onto the surface of the diet. Each vial was sealed by inserting a tightly fitting cotton wool stopper. The vials were placed at 27°C, with 60% RH and 16:8 (L:D) for the duration of the trial. After 16 hours, the vials were inspected one by one for any dead larvae. Thereafter, the vials were inspected twice daily until all larvae had either died or pupated. Infected larvae were collected and placed individually in sterile 1.5 ml Eppendorf tubes. Each tube was labelled and stored in the freezer at -16°C.

4.2.2.2 Purification and enumeration

Five infected cadavers were selected from each of the eight treatments described in section 4.2.2.1 and weighed. The larvae were subjected to the same methodology described in Chapter 3, section 3.2.6 using a glycerol gradient to purify the viral OBs. Once the purified OBs had been obtained, it was then possible to enumerate the purified virus using a Helber

bacteria Thoma counting chamber (cell depth 0.02mm, Hawksley, United Kingdom) at 400 X magnification under dark field. Before this was done, the virus (in a 2 ml Eppendorf tube) was placed in an ultra-sonic water bath for 60 seconds to separate any clumped OBs (Hunter-Fujita *et al.*, 1998). A 1000 X dilution of virus was then prepared (the dilution was adjusted if required). The counting chamber and coverslip were cleaned with 70% ethanol and lens tissue. The chamber was about three quarters covered with the coverslip, when a few microlitres of the virus were pipetted into the edge of the chamber until it overflowed into the outer ring. The coverslip was slid to fully cover the chamber. Pressure was applied on either side of the coverslip until Newton's rings were visible on both sides. The slide was then left for 5 min to allow the virus to settle. OBs were counted in the four large corner squares of the chamber and one inner square selected at random, each of which comprised of 16 smaller squares (Figure 4.2). The concentration of virus (OBs per ml) was calculated by the formula in which D is the dilution of the suspension, X is the number of OBs counted, N is the number of small squares counted and V is the volume in millilitres:

$$(D \times X) \div (N \times V)$$

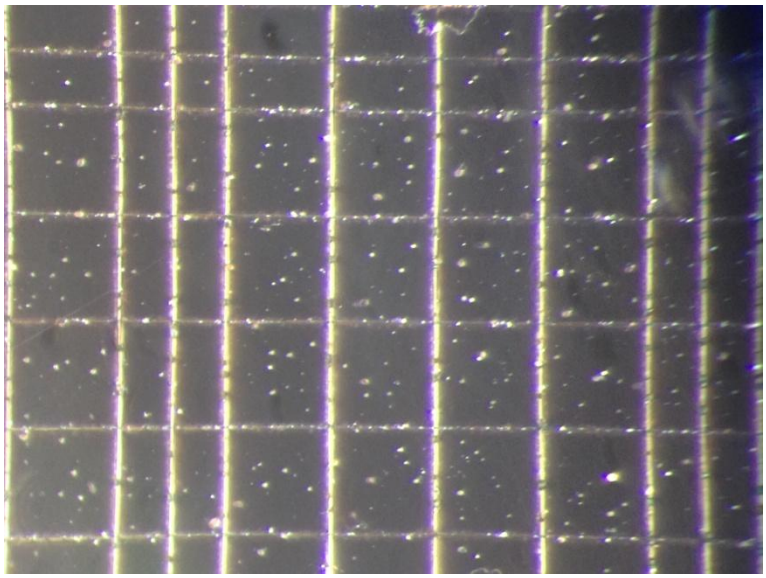


Figure 4.2 Occlusion bodies of CpGV in the small squares of a 0.02 mm depth Thoma bacterial counting chamber. 400 X under dark field light microscopy.

4.2.3 Dose-response bioassay with fourth and fifth instar *Thaumatotibia leucotreta* larvae

Fourth and fifth instar larvae were selected as possible life stages for the *in vivo* production of CpGV. It was not only important to determine the susceptibility but also to establish the potential for virus production. A highly susceptible life stage that results in a low viral yield would not be commercially beneficial. Conversely, a resistant life stage with minimal infection but high virus yield may not be suitable. Therefore establishing which life stage is not only susceptible but also results in a high viral yield would be optimal for virus production on a commercial scale. With this in mind, CpGV was tested against both fourth and fifth instar *T. leucotreta* larvae, as these are the life stages that have been used for the commercial production of CrleGV. The dosages used in the five CpGV treatments were determined by conducting several trial and error bioassays with one tray (25 cells) of larvae per treatment. The results obtained in section 4.2.2 were used as a starting reference. Treatments were prepared in either a seven or 10 fold dilution series. Small samples of larvae were selected from each treatment in order to confirm death had occurred as a result of a viral infection. This was done by macerating two to three larvae in 10 ml of distilled water. The sample was then sieved through a 100 micron nylon mesh, diluted 100 X and viewed using dark field microscopy at 400 X for the presence of viral particles.

4.2.3.1 Dose-response bioassay with fourth instar *Thaumatotibia leucotreta* larvae

Surface dose bioassays were conducted against fourth instar *T. leucotreta* larvae. Seven day old larvae were selected based on the results obtained from Chapter 2, section 2.3.7. The assays were conducted in the 25 cell bioassay trays. Six non-stick baking trays of diet were required to fill 12 bioassay trays: two bioassay trays were pressed into each dish.

Purified CpGV (see Chapter 3, section 3.2.6 for purification protocol) was diluted in sterile distilled water in a seven-fold dilution series of five treatments and a sterile distilled water control was used as a sixth treatment (Figure 4.3). Fifty larvae were treated per dose (two 25 cell bioassay trays per dose) and assays were replicated three times. Therefore, a total of

150 larvae were treated with each dose. A volume of 200 µl of each treatment was pipetted onto the diet surface in each cell (using an autopipette), ensuring that it was evenly dispersed over the diet surface. It was previously ascertained that 200 µl was sufficient to fully cover the surface of each square of diet. Inoculated bioassay trays were left in the laminar flow cabinet for ± 30 min, until the surface of the diet had adequately dried. One fifth instar larva was then placed into each cell. Trays were sealed in the manner previously described (Chapter 3, section 3.2.12), marked and kept at 27ºC and 60% RH.

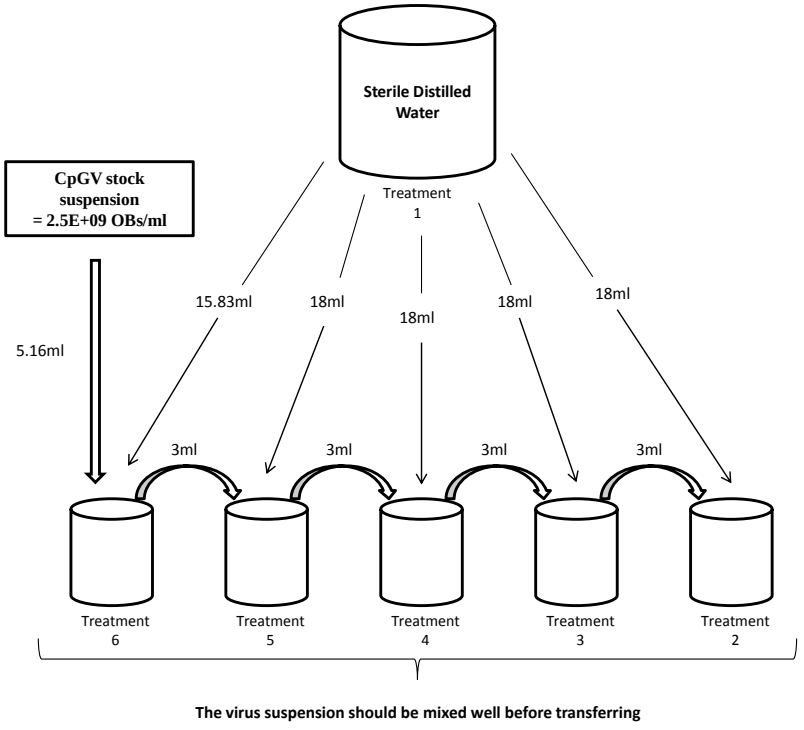


Figure 4.3 Seven-fold dilution series of CpGV for surface treatment dose-response bioassays with fourth instar *Thaumatotibia leucotreta* larvae. Each treatment was thoroughly mixed with its own sterilized stainless steel spatula prior to transferral of the 3 ml aliquot to the next lower dose. A clean pipette tip was used for each transferral.

The dosages used in the five CpGV treatments were determined by the results obtained in 4.2.2.1. After 10 days, trays were opened and inspected. Larvae were recorded as alive or dead. The dose-response curve was calculated as in Chapter 3, section 3.2.12.

4.2.3.2 Dose-response bioassay with fifth instar *Thaumatotibia leucotreta* larvae

The same methodology was used as described in section 4.2.3.1. Purified CpGV was diluted in sterile distilled water in a ten-fold series of five treatments and a sterile distilled water control was used as a sixth treatment (Figure 4.4). Ten day old larvae were selected based on the results obtained from Chapter 2, section 2.3.7. A sample of 10 individuals was removed from the batch and placed into a freezer (± -16 ºC) for a few minutes until they ceased moving. Head capsule size was measured under a light microscope, using a calibrated eye-piece. If head capsule size fell within the acceptable range, 1.25 to 1.49 mm for fifth instar T. *leucotreta* larvae, established by Daiber (1979) then larvae from that particular batch were used for the bioassay. After 10 days, trays were opened and inspected. Larvae were recorded as alive or dead.

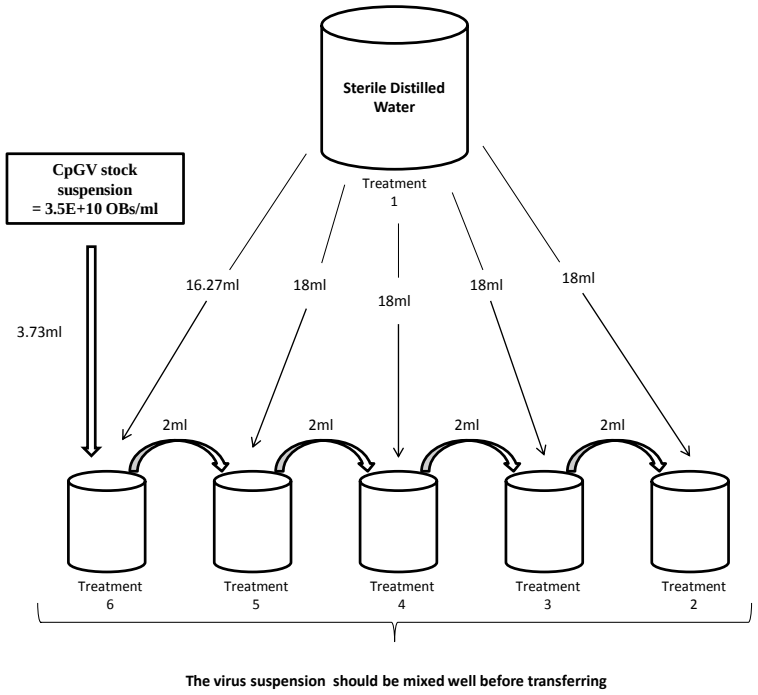


Figure 4.4 Ten-fold dilution series of CpGV for surface treatment dose-response bioassays with fifth instar *Thaumatotibia leucotreta* larvae. Each treatment was thoroughly mixed with its own sterilized stainless steel spatula prior to transferral of the 3 ml aliquot to the next lower dose. A clean pipette tip was used for each transferral.

4.2.3.3 Statistical analysis of dose-response bioassays

The dose-response curve was calculated using PROBAN (Van Ark, 1995), a computer package for calculating probit analysis (Finney, 1971). PROBAN took into consideration the mortality of the control insects, and corrected the mortality of treated larvae according to Abbott's formula (Abbott, 1925). From this, LC_{50} and LC_{90} (concentration required to kill 50 and 90% of larvae in a sample respectively) were calculated for each assay. Overall values were obtained by calculating the means from the three assays.

4.2.4 Time-response bioassays with fourth and fifth instar *Thaumatotibia leucotreta* larvae

Surface dose bioassays were conducted in 22 ml glass pill vials rather than bioassay trays. It was found to be easier to inspect for larvae showing signs or symptoms of viral infection (Figure 4.5). Using this method alleviated the necessity to open and close the vials in order to inspect the larvae therefore reducing any stress factors and therefore also limited the chances for contamination. The diet was prepared as described in section 4.2. Round diet plugs were produced by inverting the vial and pressing it into the diet. The diet plug was pushed down the vial until it sat flush on the bottom. The diet plug was then compressed lightly to ensure the plug was secure and fitted tightly.

Figure 4.5 Glass pill vials used to conduct the time-response bioassays. (A) Numerous vials, each containing a single larva, (B) Virus infected larvae on the surface of the diet.

Fifty glass vials were inoculated with 200 µl of sterile distilled water and the remaining 50 vials were inoculated with the LC_{90} (calculated from the dose-response bioassays) solution of purified CpGV. The vials were left on a laminar flow bench until sufficiently dry (± 30 min). One fourth instar larva was placed into each vial. Larval instar was determined by head capsule size as described in section 4.2.2.1. Vials were sealed with cotton wool stoppers. Bioassays were replicated three times. After 16 hours, the vials were inspected one by one for any dead larvae. Thereafter, the vials were inspected every eight hours (three times a day): at 07h00, 15h00 and 23h00, until all larvae had either died or pupated.

4.2.4.1 Statistical analysis of time-response bioassays

Time-response relationships were evaluated using a logistic version (logit) of a probit analysis (Bliss, 1935), suitable for multiple observations over time. Logit is a commonly accepted technique for analysis of time-response data, particularly when the same batch of insects is evaluated over time due to limited resources (Jones, 2000; Lacey *et al.*, 2002; Throne *et al.*, 1995). Abbott's formula was used to correct for control mortality (Abbott, 1925). The analysis was conducted using STATISTICA version V12 (StatSoft, 2013). The LT_{50} (time to kill 50% of larvae in a sample) and LT_{90} (time to kill 90 % of larvae in a sample) values were calculated from these analyses. The slope of the regression line was

compared using PROBAN to test significant difference between LC values obtained for CpGV produced in *T. leucotreta* and CpGV-M produced in *C. pomonella* CpGV-M (dose-response data obtained from Chapter 3, section 3.3.10).

4.2.5 Confirmation of virus production using REN analysis

Restriction digestion of CpGV genomic DNA with *Hin*dIII results in two DNA fragments of high molecular weight, Chapter 3, section 3.3.6. The DNA profiles of CpGV and CrleGV (numerous DNA fragment produced), generated by REN with *Hin*dIII, differ allowing this methodology to be used to determine if pure CpGV or a mixed virus suspension was produced in late instar *T. leucotreta* when inoculated with an LC_{90} concentration of CpGV. Infected larvae from both the fourth and fifth instar time-response bioassays were collected and randomly grouped in batches of ten according to instar. Three batches of ten larvae were selected from the fourth instar and two from the fifth instar bioassays and subjected to the OB purification protocol as described in Chapter 3, section 3.2.6. Genomic DNA was extracted from the purified OBs as described in Chapter 3, section 3.2.7 and restriction enzyme digestion using *Hin*dIII was conducted on the six DNA samples as described in Chapter 3, section 3.2.9. The DNA samples were loaded on a 0.6% Ethbr stained agarose gel in TAE buffer. The gels were viewed using a UV trans-illuminator with an in-built camera. The gel image was captured using UVIprochemi software.

4.2.6 Dose-response bioassay with CpGV inoculum produced in *Thaumatotibia leucotreta* against *Cydia pomonella* neonate larvae

The virus samples from section 4.2.5 which were subjected to *Hin*dIII analysis (section 4.2.5). From this analysis a sample containing pure CpGV was selected for comparison against CpGV-M. A dose-response bioassay was conducted with the CpGV produced in *T. leucotreta*. The same protocol was followed as described in Chapter 3, section 3.2.12. A seven fold dilution series was used with the highest concentration of 1.4×10^5 OBs/ml. The resulting LC data was compared to the data generated for CpGV-M in Chapter 3, section

3.3.11. Comparison of slopes of the probit regression lines for the two samples was conducted using PROBAN (Van Ark, 1935). Significant differences between slopes were established at $P < 0.01$.

4.3 RESULTS

4.3.1 Propagation of virus in *Thaumatotibia leucotreta* and *Cydia pomonella* larvae

The average yield of CpGV was examined in relation to the inoculum concentration. Both fourth and fifth instar larvae were inoculated with 8 concentrations of CpGV (Table 4.1 and Table 4.2).

The lowest inoculum dose resulting in larval mortality in fourth instar *T. leucotreta* was 1 x 10^3 OBs/380 mm^2 in which 18.3% mortality was recorded. In order to produce virus infected mortality in fifth instar larvae, a 10000 X higher concentration was required. A mortality of 17.3% was recorded at 1 x 10^7 OBs/380 mm^2. The study also concluded that *C. pomonella* neonates required approximately 10000 X lower concentration of virus inoculum resulting in a LC_{50} value of 10 to 50 OB per larvae. Virus induced mortality in *C. pomonella* was recorded in all inoculum concentration for both fourth and fifth instars. A mortality of 98.8% was recorded at 1 x 10^9 OBs/380 mm^2 in fourth, compared to 94.7% in fifth instar larvae for *C. pomonella* larvae. Reiser *et al*. (1993) reported the lowest inoculum dosage to result in larvae mortality to be 1 x 10^5 OBs per 6300 mm^2, a six times higher concentration than was required in this study. The highest yield of OBs per larva was similar for both species (7.58 x 10^{10} in *C. pomonella*, 8.78 x 10^{10} in *T. leucotreta*). The results of this study showed higher yields of virus for both CpGV produced in *C. pomonella* and *T. leucotreta* when compared to yield data reported by Reiser *et al*. (1993). Reiser *et al*. (1993) reported maximum viral yields of 1.7 x 10^{10} and 1.8 x 10^{10} OBs per larva in fifth instar *C. pomonella* and *T. leucotreta* respectively.

Table 4.1 Yield of virus and percentage mortality in relation to inoculation dosage for fourth instar *Cydia pomonella* and *Thaumatotibia leucotreta*.

Treatment (OBs/380mm²)	*Cydia pomonella*				*Thaumatotibia leucotreta*			
	Mean OBs per larva[1]	**SE**	**Mean mortality (%)**	**SE**	**Mean OBs per larva[1]**	**SE**	**Mean mortality (%)**	**SE**
Control			9.0	2.1			5.3	1.7
10^2	2.97×10^{10}	9.58×10^9	22.4	4.8	-	-	-	
10^3	5.18×10^{10}	6.22×10^9	58.3	7.5	4.09×10^{10}	2.11×10^{10}	18.3	4.4
10^4	5.81×10^{10}	1.34×10^9	60.0	2.9	6.67×10^{10}	2.11×10^{10}	18.4	8.7
10^5	4.96×10^{10}	4.61×10^9	91.7	3.3	4.76×10^{10}	8.22×10^9	21.7	3.3
10^6	6.45×10^{10}	8.48×10^9	80.0	5.8	6.51×10^{10}	1.11×10^{10}	38.3	10.9
10^7	5.01×10^{10}	5.14×10^9	88.2	3.3	5.71×10^{10}	6.12×10^9	54.0	8.3
10^8	5.16×10^{10}	2.86×10^9	93.3	1.7	5.68×10^{10}	6.13×10^9	66.3	6.7
10^9	5.87×10^{10}	6.77×10^9	98.8	1.7	5.55×10^{10}	5.69×10^9	71.7	4.4

[1] OBs were harvested from infected cadavers

Table 4.2 Yield of virus and percentage mortality in relation to inoculation dosage for fifth instar *Cydia pomonella* and *Thaumatotibia leucotreta*.

Treatment (OBs/380mm²)	***Cydia pomonella***				***Thaumatotibia leucotreta***			
	Mean OBs per larva[1]	**SE**	**Mean mortality (%)**	**SE**	**Mean OBs per larva[1]**	**SE**	**Mean mortality (%)**	**SE**
Control			6.7	1.3			8.3	3.5
10^2	4.39×10^{10}	-	18.7	3.5	-	-	-	-
10^3	4.79×10^{10}	7.20×10^9	37.3	5.8	-	-	-	-
10^4	6.27×10^{10}	1.00×10^9	29.3	1.3	-	-	-	-
10^5	5.20×10^{10}	1.64×10^9	42.7	10.4	-	-	-	-
10^6	5.78×10^{10}	1.07×10^9	73.7	9.3	4.10×10^9	4.10×10^9	2.7	2.7
10^7	5.84×10^{10}	6.44×10^9	81.3	6.1	7.64×10^{10}	6.35×10^9	17.3	7.1
10^8	4.78×10^{10}	7.01×10^9	86.7	10.1	6.43×10^{10}	4.22×10^9	44.0	8.0
10^9	7.58×10^{10}	1.85×10^9	94.7	5.8	8.21×10^{10}	5.56×10^9	48.0	4.6

[1]OBs were harvested from infected cadavers.

4.3.2 Dose-response bioassay with fourth and fifth instar *Thaumatotibia leucotreta* larvae

4.3.2.1 Dose-response bioassay with fourth instar *Thaumatotibia leucotreta* larvae

The regression lines fitted to the data (Figure 4.6) in Table 4.3 have the equations y = 1.1595 + 0.9396x (SE of slope = 0.1302), y = 2.3653 + 0.7953x (SE of slope = 0.1088) and y = 2.0492 + 0.8299x (SE of slope = 0.1075). Deviations for the lines, calculated from the data for all three replicates, were estimated to be homogenous. The slopes of the lines (0.9396, 0.7953 and 0.8299) are nearing 1. Slopes of between 1 and 2 are considered the norm for virus assays (Jones, 2000).

G for fiducial limits was calculated to be 0.0737, 0.0719 and 0.0645 for the three replicates respectively. Figures of greater than 0.025 indicate a large variation in mortality between treatments; however experimental procedures or the value of the probit line should only be questioned if the G value exceeds 0.25 (Van Ark, 1995). The mean LC_{50} and LC_{90} (for the three replicates) were 5.96 x 10^3 OB/mm² and 1.64 x 10^5 OBs/mm² respectively. The 95% fiducial limits of the LC_{50} and LC_{90} concentrations ranged from 3.03 x 10^3 to 1.06 x 10^4 and 7.67 x 10^4 to 5.25 x 10^5 OBs/mm² respectively.

Table 4.3 Mortality of fourth instar *Thaumatotibia leucotreta* larvae in dose-response bioassays with five concentration of CpGV-M.

Treatment (CpGV in OBs/ 380mm²)	Replicate 1			Replicate 2			Replicate 3		
	Larval mortality (%)	Mortality corrected for control mortality (%)	Empirical probit	Larval mortality (%)	Mortality corrected for control mortality (%)	Empirical probit	Larval mortality (%)	Mortality corrected for control mortality (%)	Empirical probit
Control	14.00			12.00			10.00		
2.55×10^2	20.00	6.98	3.52	38.00	29.55	4.46	24.00	15.56	3.99
1.79×10^3	24.00	11.63	3.81	48.98	42.02	4.80	46.00	40.00	4.75
1.25×10^4	58.00	62.79	5.33	74.00	70.45	5.54	72.00	68.89	5.49
8.75×10^4	80.00	76.74	5.73	93.88	93.04	6.48	90.00	88.89	6.22
6.13×10^5	94.00	93.02	6.48	98.00	97.73	7.00	96.00	95.56	6.70

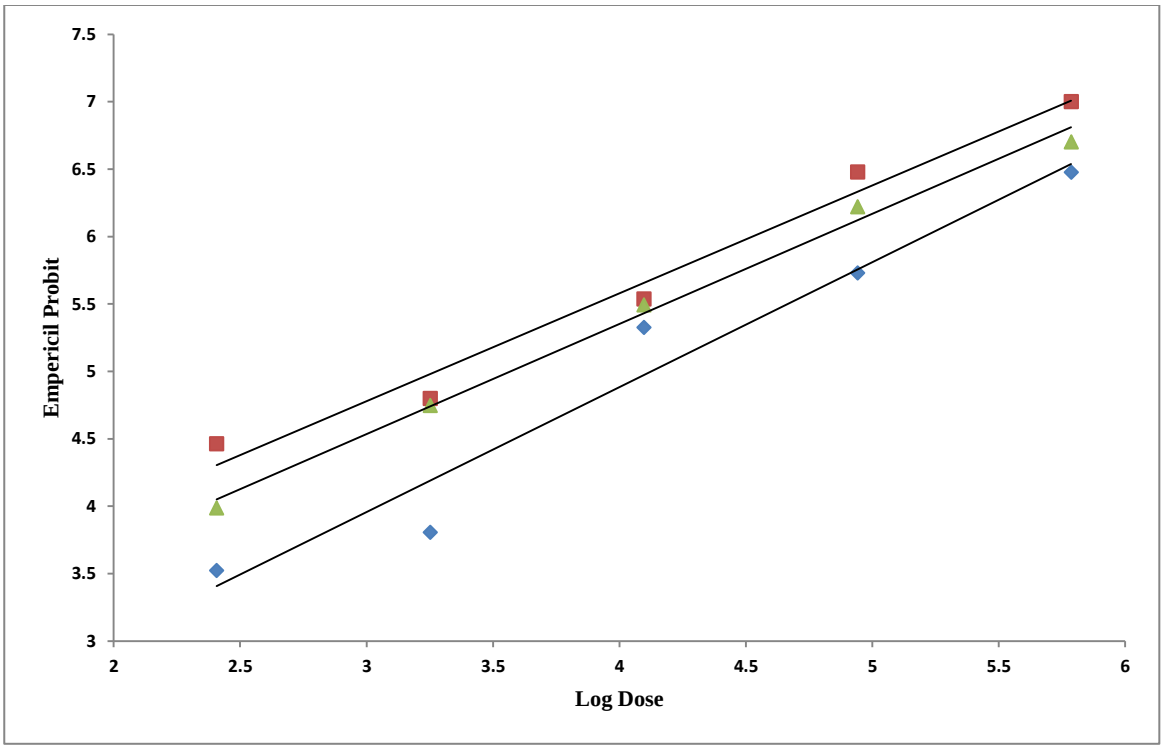


Figure 4.6 Dose-mortality probit lines for CpGV-M against fourth instar *Thaumatotibia leucotreta* larvae.

4.3.2.2 Dose-response bioassay with fifth instar *Thaumatotibia leucotreta* larvae

The regression lines fitted to the data (Figure 4.7) in Table 4.4 had the equations y = 2.7414 + 0.4952x (SE of slope = 0.0721), y = 2.4799 + 0.5404x (SE of slope = 0.0694) and y = 0.7936 + 0.8258x (SE of slope = 0.0903). Deviations for the lines, calculated from the data for all three replicates, were estimated to be homogenous. The slopes of the lines (0.4952, 0.5404 and 0.8258) were rather gradual with the exception of the third replicate in which the slope was approaching 1.

Table 4.4 Mortality of fifth instar *Thaumatotibia leucotreta* larvae in dose-response bioassays with five concentrations of CpGV-M.

Treatment (CpGV in OBs/mm²)	Replicate 1			Replicate 2			Replicate 3		
	Larval mortality (%)	Mortality corrected for control mortality (%)	Empirical probit	Larval mortality (%)	Mortality corrected for control mortality (%)	Empirical probit	Larval mortality (%)	Mortality corrected for control mortality (%)	Empirical probit
Control	6.00			0.00			2.00		
2.50×10^3	30.00	25.53	4.34	28.00	28.00	4.42	14.00	12.24	3.84
2.50×10^4	54.17	51.25	5.03	36.00	36.00	4.64	20.00	18.37	4.10
2.50×10^5	72.00	70.21	5.53	70.00	70.00	5.52	66.00	65.31	5.39
2.50×10^6	74.00	72.34	5.59	84.00	84.00	6.00	88.00	87.76	6.16
2.50×10^7	96.00	95.74	6.72	92.00	92.00	6.41	96.00	95.92	6.74

G for fiducial limits was calculated to be 0.0815, 0.0634 and 0.0460, all of which are less than 0.25. The mean LC_{50} and LC_{90} (for the three replicates) were 6.88 x 10^4 and 9.78 x 10^6 OBs/mm² respectively. The 95% fiducial limits of the LC_{50} and LC_{90} concentrations ranged from 3.31 x 10^4 to 1.34 x 10^5 and 3.20 x 10^6 to 6.12 x 10^7 OBs/mm² respectively.

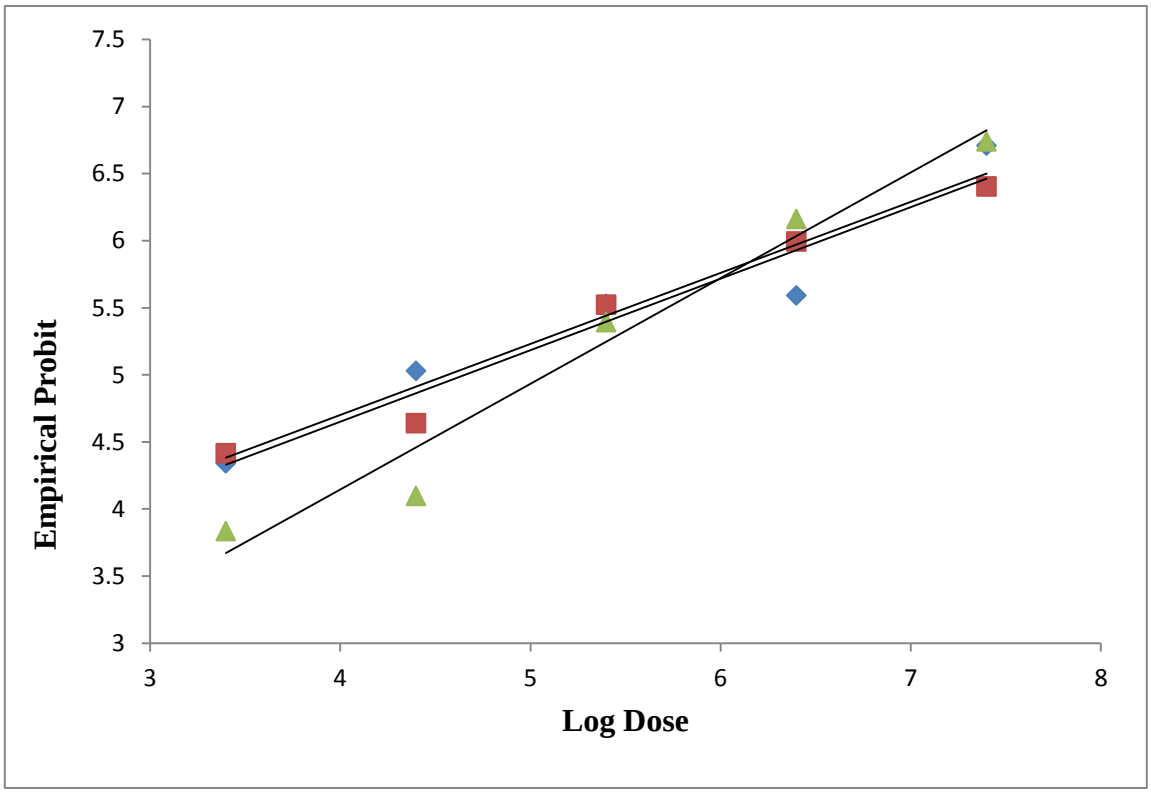


Figure 4.7 Dose-mortality probit line for CpGV-M against fifth instar *Thaumatotibia leucotreta* larvae.

4.3.3 Time-response bioassays with fourth and fifth instar *Thaumatotibia leucotreta* larvae

4.3.3.1 Time-response bioassay with fourth instar *Thaumatotibia leucotreta* larvae

No larvae mortality was observed 48 hours post inoculation and therefore all larval mortality observed was considered to be virus induced mortality. The first virus induced mortality was recorded 72 hours (3 days) post treatment (Table 4.5). At 112 hours post treatment no further mortality was recorded in any of the three replicates. Control

mortality, for all three replicates, was 7 out of a total of 150 larvae succumbing to viral infection. This resulted in 4.67% control mortality. Abbotts formula was applied to correct for control mortality. However, this negligible level of control mortality was considered to realistically reflect the conditions to which larvae would be subjected during virus production.

Table 4.5 Mortality of fourth instar *Thaumatotibia leucotreta* larvae in time-response bioassays (three replicates) with the LC_{90} concentration of CpGV.

Time after treatment		**Cumulative larval mortality (%)**		
Day	**Hours**	**Rep 1 (n=41)**	**Rep 2 (n=44)**	**Rep 3 (n=43)**
3	72	2.04	10.20	0.41
	80	8.16	48.98	48.94
	88	80.87	95.84	95.74
4	96	95.84	95.92	97.87
	104	100.00	100.00	97.87
	112			100.00

In the three replicates a total of 5 larvae (out of a total of 150) placed onto the virus inoculated diet survived to pupation. The total mortality of the treated larvae was 96.67%. For the purpose of calculating the time-response relationship, all larvae surviving to pupation were omitted. The total numbers of larvae treated in each replicate were 49, 49, and 47 respectively. This is known as biological truncation and is considered an acceptable practice (Moore *et al*., 2011). The time-mortality relationship in fourth instar *T. leucotreta* larvae (Figure 4.8) was analysed using a logit regression (Table 4.6). From this, the LT_{50} and LT_{90} for each replicate were calculated. Mean LT_{50} and LT_{90}, for the three replicates, were 81.73 h (SE = 1.65) and 88.67 h (SE = 1.08), respectively.

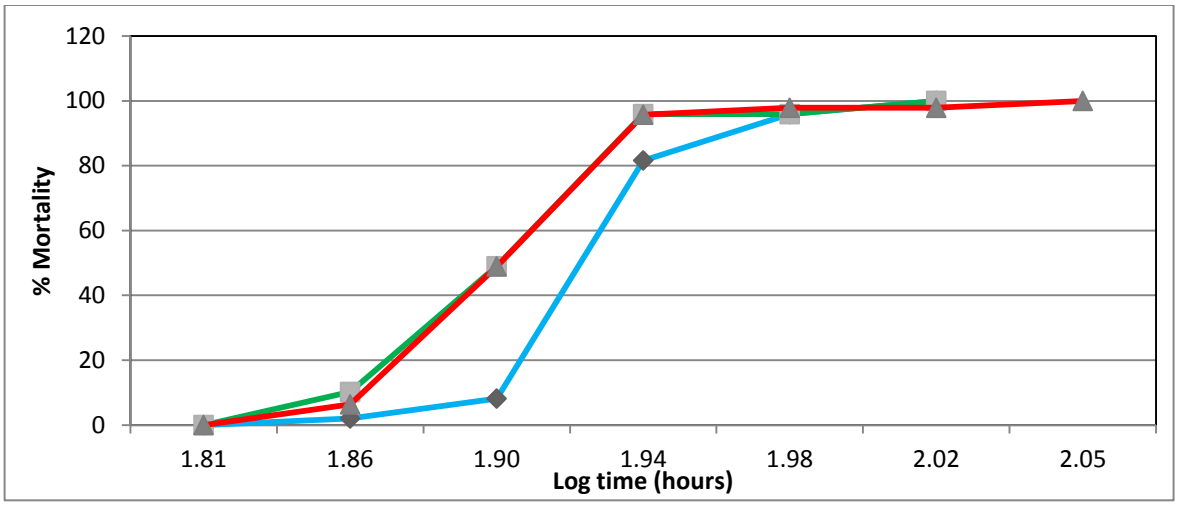


Figure 4.8 Time-mortality relationship between *Thaumatotibia leucotreta* fourth instar larvae and CpGV applied at the LC_{90} concentration in three bioassay replicates.

Table 4.6 Logistic regression data for mortality of fourth instar *Thaumatotibia leucotreta* inoculated with the LC_{90} concentration (1.64×10^5 OBs/380mm^2) of CpGV.

	Chi- square	P value	X intercept	SE	P value
Replicate 1	1433.000	< 0.0001	-31.866	3.040	< 0.0001
Replicate 2	1474.778	< 0.0001	-23.123	1.980	< 0.0001
Replicate 3	1497.185	< 0.0001	-27.018	2.515	< 0.0001

4.3.3.2 Time-response bioassay with fifth instar *Thaumatotibia leucotreta* larvae

The first virus induced mortality was observed 80 hours post treatment (Table 4.7). Two hundred hours post inoculation no further mortality was recorded in any of the replicates. Control mortality, for all five replicates, was recorded to be 4 out of a total of 250 larvae succumbing to viral infection. This resulted in 1.6% control mortality. As stated in section 4.4.1 Abbott's formula was applied to correct for control mortality.

Table 4.7 Mortality of fifth instar *Thaumatotibia leucotreta* larvae in time-response bioassays (five replicates) with the LC_{90} concentration of CpGV.

Time after treatment		Cumulative larval mortality (%)				
DAT [1]	Hours [2]	Rep 1 (n=41)	Rep 2 (n=44)	Rep 3 (n=29)	Rep 4 (n=43)	Rep 5 (n=44)
3	72	0.00	0.00	0.00	0.00	0.00
	80	2.44	2.27	3.45	0.00	0.00
	88	14.63	4.92	19.07	2.33	6.82
4	96	36.59	11.36	58.62	6.98	6.82
	104	48.78	34.09	62.07	34.88	52.27
	112	48.78	65.91	68.97	58.14	79.55
5	120	63.42	70.45	72.41	65.17	86.36
	128	65.85	83.77	82.76	76.74	88.64
	136	78.05	88.64	86.21	86.05	95.46
6	144	78.05	95.45	93.10	90.70	95.46
	152	85.07	100.00	96.55	97.67	100.00
	160	85.37		96.55	97.67	
7	168	90.24		96.55	100.00	
	176	90.24		96.55		
	184	90.24		100.00		
8	192	90.24				
	200	95.11				
	208	100.00				

[1] DAT = Days after treatment [2] Hours post inoculation of larvae

In the five replicates a total of 49 larvae (out of a total of 250) placed onto the virus inoculated diet survived to pupation. The total mortality of the treated larvae was 80.41%, less than the 90% that one would expect when inoculating with the LC_{90} concentration of virus. This figure was lower than expected due to poor infection in the third replicate. By omitting the third replicate 86% mortality was achieved, somewhat closer to what should have been expected.

For the purpose of calculating the time-response relationship, all larvae surviving to pupation were omitted. The total numbers of larvae treated in each replicate were 41, 44, 29, 43 and 44 respectively. The time-mortality relationship between CpGV and fifth instar *T. leucotreta* (Figure 4.9) was analysed using a logit regression (Table 4.8). From this, the LT_{50} and LT_{90} for each replicate were calculated. Mean LT_{50} and LT_{90} values, from the five replicates, were 111.14 h (SE = 2.25) and 136.21 h (SE = 5.26), respectively.

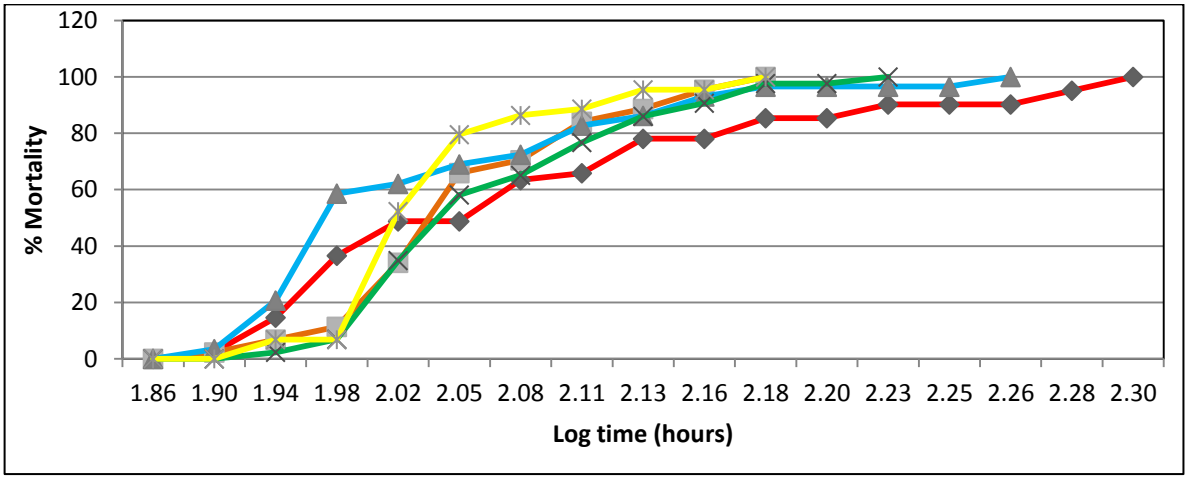


Figure 4.9 Time-mortality relationship between *Thaumatotibia leucotreta* fifth instar larvae and CpGV applied at the LC_{90} concentration in five bioassay replicates.

Table 4.8 Logistic regression data for mortality of fourth instar *Thaumatotibia leucotreta* inoculated with the LC_{90} concentration (9.78×10^6 OBs/380mm^2) of CpGV.

	Chi- square	**P value**	**X intercept**	**SE**	**P value**
Replicate 1	2112.265	< 0.0001	-6.063	0.235	< 0.0001
Replicate 2	1636.362	< 0.0001	-12.387	0.677	< 0.0001
Replicate 3	2198.874	< 0.0001	-7.970	0.357	< 0.0001
Replicate 4	1974.907	< 0.0001	-11.692	0.602	< 0.0001
Replicate 5	1846.937	< 0.0001	-14.259	0.831	< 0.0001

4.3.4 Confirmation of virus production using REN analysis

REN analysis was conducted on DNA extracted from virus infected larvae recovered from the time-response bioassays to determine if the inoculated CpGV had successfully infected and replicated within the late instar *T. leucotreta* larvae. As noted in Chapter 3, section 3.3.6, the restriction digest of CpGV genomic DNA with *Hin*dIII produced only 2 prominent DNA fragments of high molecular weight as opposed to CrleGV that produced numerous prominent DNA fragments (Opoku-Debrah *et al*., 2013).

Single REN digestion of the genomic DNA with *Hin*dIII produced numerous DNA fragments in each of the samples tested with the exception of sample 3 which represents fourth instar *T. leucotreta* larvae (Figure 4.10). The restriction patterns of samples run in lane 3, 4, 6 and 7 are comparable to those described by Opoku-Debrah *et al*. (2013) and indicate the presence of a CrleGV contamination. The REN profile of the sample in lane 5 produced only 2 prominent DNA fragments of high molecular weight indicating a pure CpGV virus sample.

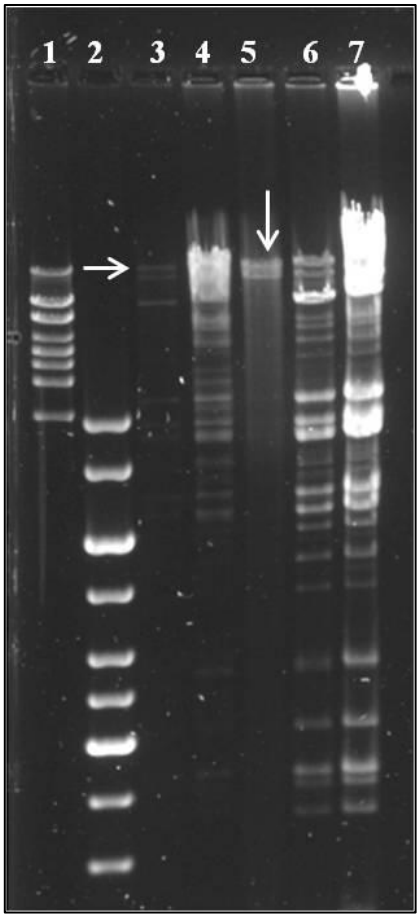


Figure 4.10 Restriction endonuclease profiles of five samples of virus, produced in *Thaumatotibia leucotreta*, digested with *Hin*dIII. Electrophoresis was conducted on 0.6% agarose gels for 16 h at 30 V. (A) Lane 1 – High range DNA marker, lane 2 – 1 Kb DNA marker, lane 3 – fourth instar larvae (sample 1), lane 4 – fourth instar larvae (sample 2), lane 5 – fourth instar larvae (sample 3), lane 6 – fifth instar larvae (sample 1), lane 7 – fifth instar larvae (sample 2). Arrows show the two prominent DNA fragments of CpGV.

4.3.5 Dose-response bioassay with CpGV inoculum produced in *Thaumatotibia leucotreta* against *Cydia pomonella* neonate larvae

Dose-response bioassays were conducted using CpGV inoculum produced in fourth instar *T. leucotreta* larvae against neonate *C. pomonella* larvae. The inoculum doses were transformed to $\log_{10}$ and the percentage mortality to empirical probits in PROBAN. The fit of the probit lines was calculated, as were the fiducial limits and the LC_{50} and LC_{90} values.

The regression lines fitted to the data in Table 4.11 for the CpGV produced in *T. leucotreta* had the equations y = 3.9040 + 0.4691x (SE of slope = 0.0645), y = 3.9303 + 0.4963x (SE of slope = 0.0657) and y = 3.6234 + 0.5273x (SE of slope = 0.0643). Deviations for the lines were homogenous for the three replicates.

G for fiducial limits was calculated to be 0.0744, 0.0674 and 0.0643 for the three replicates respectively. From the above data, the calculated LC_{50} and LC_{90} doses are presented in Table 4.10.

Table 4.9 Mortality of *Cydia pomonella* neonate larvae in dose-response bioassays with six concentration of CpGV produced in *Thaumatotibia leucotreta* larvae.

Treatment (CpGV in OBs/ml)	Replicate 1			Replicate 2			Replicate 3		
	Larval mortality (%)	Mortality corrected for control mortality (%)	Empirical probit	Larval mortality (%)	Mortality corrected for control mortality (%)	Empirical probit	Larval mortality (%)	Mortality corrected for control mortality (%)	Empirical probit
Control	8.00			6.00			8.00		
8.33×10^0	30.61	24058	4.312	34.69	30.53	4.491	28.57	22.36	4.240
5.83×10^1	44.90	40.11	4.747	42.86	39.21	4.726	44.90	40.11	4.749
4.08×10^2	61.22	57.85	5.198	63.27	60.92	5.277	48.98	44.54	4.863
2.86×10^3	71.43	68.94	5.494	71.43	69.60	5.513	57.14	53.42	5.086
2.0×10^4	79.59	77.82	5.766	85.71	84.80	6.028	83.67	82.25	5.925
1.4×10^5	93.88	93.35	6.502	95.92	95.66	6.713	97.96	97.78	7.010

Table 4.10 Comparison of the LC_{50} and LC_{90} values for both CpGV-M and CpGV produced in *Thaumatotibia leucotreta* with fiducial limits.

		Mean Dose (OBs/ml)	SE	Fiducial limits	
				Upper ± SE	Lower ± SE
CpGV-M	LC_{50}	7.21×10^2	3.54×10^2	1.99×10^3 (1.29×10^3)	5.11×10^2 (3.61×10^2)
	LC_{90}	7.33×10^4	1.77×10^4	3.97×10^5 (1.40×10^5)	3.01×10^4 (1.43×10^4)
CpGV[1]	LC_{50}	2.56×10^2	1.09×10^2	5.68×10^2 (1.63×10^2)	3.25×10^2 (2.17×10^2)
	LC_{90}	9.39×10^4	1.68×10^4	6.61×10^5 (1.83×10^5)	2.15×10^4 (6.22×10^3)

[1] CpGV inoculum produced in fourth instar *T. leucotreta* larvae.

The slope of the regression lines was compared using PROBAN to test for significant difference between the LC concentrations of each isolate. PROBAN found the residual variances of the two lines (CpGV-M and CpGV) to be homogenous as per the F test for homogeneity between two samples ($F_{4,4} = 23.16$; $P = 0.488$). The slopes of the two lines were thus comparable. Using the chi-squared test, the lines were found to be parallel so the elevations could be compared ($\chi^2 = 3.841$, df = 1; $P = 0.194$). Their elevations did not differ significantly from one another ($F_{1,9} = 5.12$; $P = 0.170$).

4.4 DISCUSSION

The objectives of this study were first to compare the CpGV yields in both fourth and fifth instar *T. leucotreta* and *C. pomonella* larvae. *Thaumatotibia leucotreta* is significantly easier, more efficient and more cost effective to rear than *C. pomonella*, which makes it a potential candidate as a heterologous host for CpGV production (Chapter 2). Secondly, in order to progress to virus production trials and establish a viable production protocol (the final aim of this thesis) it was necessary to establish both LC and LT data using probit and logit regressions, for CpGV production in *T. leucotreta*.

A droplet feeding method described by Pereira-da-Conceicoa *et al*. (2012) for the bioassay of neonate larvae was initially considered due to its accuracy and the ease in which large

numbers of larvae can be assayed. It is also possible to estimate the dosage ingested by a single larva thereby increasing the accuracy of the assay (Jones, 2000). This method, if effectively implemented, reduces a cumulative feeding rate in larvae and improves the accuracy of the data obtained. Using the surface dose method, larvae could wander on the surface, feeding intermittently on the inoculated diet, before boring into the diet. This cumulative feeding can have an effect on LC values. However, *C. pomonella* larvae could not be enticed to drink from the virus droplets in sufficient numbers and those which did became entrapped and drowned. Therefore, in this study, surface inoculation of the diet was preferred. This method was also believed to be an accurate reflection of a field application situation.

Before conducting bioassays using CpGV-M in *T. leucotreta* it was important to determine virus yield in both the homologous and heterologous host. The results of the study showed that CpGV yields in *T. leucotreta* were encouraging and comparable to those produced in the homologous host, *C. pomonella*. Reiser *et al*. (1993) showed that the highest yields of CpGV per larva were comparable for both *T. leucotreta* and *C. pomonella*, with 1.7×10^{10} OBs produced in *T. leucotreta* and 1.8×10^{10} OBs in *C. pomonella*. Similar results were obtained in this study with comparable yields produced in both hosts; however, a 4.8 X greater yield of virus was recorded in *T. leucotreta*: 8.76×10^{10} and 7.58×10^{10} OBs per larva were produced in *T. leucotreta* and *C. pomonella* respectively. Moore (2002) showed it was possible to produce yields of 1.158×10^{11} OBs/larva when inoculating *T. leucotreta* with a LC_{90} concentration of CrleGV-SA. Fritsch (1989) produced slightly less virus at 1.07×10^{10} OBs of CrleGV-CV (Cape Verde isolate of the virus) per larva.

The purpose of conducting both dose and time-response bioassays was to identify firstly, the inoculum concentration required for optimal virus production and secondly, to establish time scales pertinent to harvesting of the CpGV infected larvae. Using this information one can determine, not only how long after inoculation of the larvae harvesting should begin, but for how long the harvesting should continue. The harvesting period for fourth instar larvae was two to five days post-inoculation whereas for fifth instar larvae this increased from two to nine days post-inoculation.

The mean LC_{50} and LC_{90} values for fourth instar *T. leucotreta* were calculated at 5.96 x 10^3 OB/mm² and 1.64 x 10^4 OBs/mm² respectively. In fifth instar larvae these values were 6.88 x 10^4 and 9.78 x 10^6 OBs/mm² respectively. The slope of the regression lines was 0.4952, 0.5404 and 0.8258 for the three replicates. Moore (2002) calculated the LC_{90} for fifth instar *T. leucotreta* larvae inoculated with CrleGV (the homologous virus) to be 4.56 x 10^6 OBs/mm^2, half the concentration of what was achieved in this study. This result is thus surprising as one would have expected the LC_{90} value for CpGV production in *T. leucotreta* to be significantly higher. Reiser *et al*. (1993) conducted yield trials with CpGV in *T. leucotreta* using eight inoculum concentrations, the highest of which was 1.59 x 10^4 OBs/mm^2. They were able to show successful production of CpGV at this, and lower concentrations, but did not report percentage larval mortality. Therefore this is the first reported LC_{90} value for production of CpGV in fifth instar *T. leucotreta*. Numerous authors have conducted dose and time-response assays on late instar *C. pomonella* larvae. Sheppard & Stairs (1977) estimated the dose-mortality slope for fifth instar *C. pomonella* larvae to be 0.3478. This was significantly less steep when compared to the slope for first instar larvae which was estimated to be 1.3497. It has been reported that fifth instar *C. pomonella* larvae show an age dependent tolerance to granulovirus infection. For example, Camponovo & Benz (1984) showed the LD_{50} for 48 hour old larvae to be approximately 2300 time higher than that of 3 hour old larvae. Sheppard & Stairs (1977) observed a variable response of fifth instar larvae to CpGV and Probit analysis calculated that 1 OB could cause mortality in both fourth and fifth instar larvae. In order to obtain 70% mortality, 12 OBs were required per larva in first instar compared to 1578 OBs in fifth instar larvae. They estimated the dose-mortality slope for fifth instar *C. pomonella* larvae to be 0.3478. This was significantly less steep when compared to the slope for first instar larvae which was estimated to be 1.3497.

Logit regression analysis was used to determine the lethal time required for 50 and 90% mortality in CpGV inoculated *T. leucotreta*. For fourth instar larvae the mean LT_{50} and LT_{90} were estimated at 3 days 10 hours and 3 days 17 hours. Mean LT_{50} and LT_{90} values for fifth instar larvae were estimated at 4 days 15 hours and 5 days 16 hours. Federici (1997) stated that type 2 GV infections typically last only 5 to 10 days in larvae infected

during the fourth instar. The LT values obtained in this study confirm CpGV to be a type 2 GV. Moore *et al*. (2011) reported LT_{50} and LT_{90} values for CrleGV in fifth instar larvae of 7 days 17 h and 9 days 8 hours, respectively, a significantly longer period when compared to the results of this study. Using this information it may be possible to reduce the risk of induced homologous virus (CrleGV) production (contamination) by harvesting infected larvae 7 days post treatment. This should give the CpGV infected larvae sufficient time to succumb to the virus and possibly reduce the risk of covert CrleGV infections becoming overt as a result of sub-lethal doses of CpGV acting as a stress trigger.

Restriction analysis of the virus genomic DNA with *Hin*dIII was used to determine the purity of the virus samples recovered from fourth and fifth instar *T. leucotreta* larvae. Restriction analysis of CpGV genomic DNA with *Hin*dIII results in two DNA fragments of high molecular weight (Crook *et al*., 1985; Reiser *et al*., 1993; Eberle, 2010). Conversely, Moore (2002) and Opoku-Debrah *et al*. (2013) showed that restriction analysis of various CrleGV isolates, including CrleGV-Addo and CrleGV-Cryptogran genomic DNA with the same enzymes produced numerous prominent fragments and a number of faint sub-molar bands. Therefore it is recommended that each sample be subjected to REN analysis with *Hin*dIII as the first stage of quality control when producing CpGV in *T. leucotreta*. The results of this study indicate a CrleGV contamination in a number of the samples tested, with only a single sample shown to contain pure CpGV. This is a concern for the further development of this production technique as it is speculated that due to the nature of *in vivo* mass production of virus the observed level of CrleGV contamination could increase. In order to establish at which point in the virus production the CrleGV contamination is dominant it is recommended that a group of larvae are collected from specific time intervals to determine the level of CpGV or CrleGV production at each interval. It is possible that larvae succumbing to the CpGV virus inoculum early or late in the virus replication contain CrleGV. Increasing the sample size would also provide a more accurate indication of the potential for CpGV production in both fourth and fifth instar *T. leucotreta* larvae. It is speculated that there may have been an elevated level of covert virus in the *T. leucotreta* test population. Studies have reported the triggering effect of stresses and heterologous inoculation in the activation of homologous infections from covert to overt

lethal infection (Podgwaite & Mazzone, 1986; Burden *et al*., 2003; Il'inykh & Ul'yanova, 2005; Murillo *et al*., 2011). During the inoculation process the larvae may have been subjected to sudden changes in temperature and humidity when removed from their existing environment and placed onto the inoculated artificial diet. This change, along with the handling of the larvae may have been a sufficient stress trigger to result in the homologous virus manifesting. It is also possible that the larvae ingested a sub-lethal dosage of the CpGV inoculum resulting in a triggering of the homologous virus (Cooper *et al*., 2003). The REN analysis can provide a qualitative result regarding the purity of the CpGV product, but cannot provide a quantitative result in a mixed solution. Quantifying the amount of CpGV in a contaminated production sample is important as the level of contamination may be minimal and the production sample may contain acceptable levels for CpGV to warrant formulation into a final product. Therefore, a method for quantitative analysis such as relative qPCR is required if further investigation into the production of CpGV in *T. leucotreta* is to be considered.

It was not only important to determine if CpGV could be produced in *T. leucotreta* but also to determine whether this production methodology would have any negative effects on the virulence of the CpGV produced. A dose-response bioassay was conducted using CpGV produced in fourth instar *T. leucotreta* larvae. The lethal concentration values obtained for this sample compared favourably to those generated for CpGV-M (Chapter 3, section 3.3.11). Comparison of the regression slopes using PROBAN indicated no significant difference in the slope of the two lines. This result indicates that the virulence of CpGV produced in a heterologous host was not affected by the production technique and agrees with the findings of Reiser *et al*. (1993) who reported no loss in CpGV virulence using a similar production technique to produce CpGV in *T. leucotreta*.

4.5 CONCLUSION

Virus yields were comparable in both *T. leucotreta* and *C. pomonella*. Yields of virus in both fourth and fifth instar *T. leucotreta* larvae were encouraging and a distinct dose response was observed between treatments, which was a possible indication of CpGV

production and not CrleGV induced production. Further testing will be required to ensure stringent quality control standards can be put into place for commercial preparation of CpGV in *T. leucotreta.*

The results of this chapter indicate that CpGV could be produced in *T. leucotreta*, although the level of CrleGV contamination observed in a number of the samples tested was a cause for concern. Viral yields are comparable to the homologous host, although a higher LC dosage is required to achieve the desired mortality. The next chapter evaluates mass production of CpGV in both fourth and fifth instar *T. leucotreta* larvae when inoculated with the LC_{90} concentration of CpGV-M. Three production techniques are tested to compare virus yields and two quality control parameters, namely bacterial contamination and REN analysis of genomic DNA with *Hin*dIII are described.

5

PRODUCTION OF *CYDIA POMONELLA* GRANULOVIRUS IN A HETEROLOGOUS HOST, *THAUMATOTIBIA LEUCOTRETA*

5.1 INTRODUCTION

In Chapter 4 it was shown that *Thaumatotiba leucotreta* can be an effective heterologous host for the production of CpGV, as yields of CpGV in both *Cydia pomonella* and *T. leucotreta* were comparable. Both dose-response and time-response assays were conducted from which LC and LT data were established. For mass production trials, the LC_{90} concentration of CpGV should be applied to the surface of the diet. The lethal times for fourth and fifth instar larvae were calculated, with infected larvae likely to be ready for harvesting six to seven days post-inoculation.

Baculoviruses are regarded as highly beneficial (Miller, 1997) and are widely used in numerous applications because of their host specificity and limited impact on the environment. Approximately 60 baculovirus-based pesticides have been utilized to control various insect pests worldwide, the most successful being HearNPV, SfNPV and CpGV (Moscardi *et al*., 2011; Beas-Catena *et al*., 2014). Large scale baculovirus production can be performed *in vivo* or *in vitro*. *In vivo* production usually takes place in a homologous host while the *in vitro* production makes use of primary cell lines (Goodman & McIntosh, 1994; Goodman *et al*., 2001; Claus *et al*., 2012). Naser *et al*. (1984) were the first to report successful *in vitro* replication of CpGV in a *Cydia pomonella* primary cell line. However,

propagation by this method was considered too slow for effective virus production. Winstanley & Crook (1993) published the first report of cell cultures that were 100% susceptible to a GV with improved virus production. Their results showed that cell lines maintained at or below 21°C remained susceptible to CpGV over a period of 4 years. However, the characteristics of these cell lines and the degree of progeny virus production in them were not conducive to the development of a commercial *in vitro* production system for CpGV. Despite recent developments in cell lines with improved susceptibility and virus productivity (Davis & Granados, 1995; Granados & McKenna, 1995; Shuler *et al.*, 1995), additional advances in this technology are required for this method of virus production to be efficient and cost effective enough for consideration on a commercial scale (Lacey *et al*., 2008).

To justify commercialisation of a virus product, certain requirements need to be met. Firstly, the product should have a sufficiently large market and have a production method with which the virus is able to compete economically. Secondly, the product must demonstrate reproducible field efficiency and should have some advantages over the commonly used biological control products on the market (Beas-Catena *et al*., 2014). The basic process of *in vivo* virus production involves a number of steps including virus inoculation, virus incubation, harvesting of the infected larvae, processing the virus and, finally, storage of the virus product (Moscardi *et al*., 2011). It is important to optimise the factors that will result in improved virus production. Some of these factors include host factors (densities, age, life stage), virus inoculum (concentration, activity, purity), environmental conditions (temperature, humidity, diet) and the production scheme (Moore, 2002). In order to obtain optimal production results, maximum utilisation of larval tissue is necessary (Ignoffo, 1966). Therefore, the goal is to produce the greatest yield of active virus while ensuring that the product conforms to specific quality control standards. *In vivo* production processes invariably lead to a virus sample with both bacterial and fungal contaminants. Therefore, production should aim to limit contamination as well as ensure that no human or veterinary pathogens are present in the final formulation. For *in vivo* production in a heterologous host, good quality control standards are essential, and it is important to ensure both purity and a standard concentration of the virus inoculum.

Contaminated inoculum or a sub-lethal dosage of the virus may result in a stress trigger and the expression of the homologous virus within the host resulting in a mixed virus suspension or contaminated final product. Depending on the severity of the contamination the final product may be ineffective and unmarketable.

In order to entice growers to accept and adopt biological control measures it is essential that all products comply with a set of standards. The quality and reliability of a product is essential in ensuring acceptance and sustained usage by a grower. For many years the perception regarding biological control products has been one of inconsistency as well as reduced efficacy when compared to their chemical counterparts. Many products that have found their way into the market have been characterised as weak products with poor efficacy and questionable quality control (Harris, 1997). Quality control of the final product is not a simple exercise for baculoviruses as the production of a virus product is dependent on the virus infecting and completing a complex life cycle in its host (Jenkins & Grzywacz, 2003). Despite stringent regulatory procedures for registration of virus products, for many years there has been no widely agreed, or accepted standardised protocols for quality control (Jenkins & Grzywacz, 2003), however this has subsequently been addressed by the OECD issue paper on microbial contamination limits for microbial pest control products and is now regarded as the norm (Anonymous, 2011b).

The protocols within this study were based on those described by Moore (2002). These protocols were followed as they have been used by a commercial entity to produce CrleGV as the active ingredient of Cryptogran™ since 2005. The first aim of this study was to determine the production potential of both fourth and fifth instar *T. leucotreta* larvae using three production techniques in order to produce the maximum quality and quantity of viable CpGV in *T. leucotreta*. The first production technique involved inoculating the surface of a freshly prepared diet with the LC_{90} concentration of CpGV and introducing a specific number of larvae. Infected cadavers were harvested individually. The second production technique was the same as the first except it involved harvesting the larvae and the diet. Finally the third production technique involved removing the larvae from the rearing jar and placing them, along with the digested crumbly diet into an inoculation tray

and inoculating the LC_{90} concentration of CpGV. Both larvae and diet were harvested. The second aim was to assess the purity of CpGV produced in *T. leucotreta* by REN analysis of genomic DNA using *Hin*dIII.

5.2 MATERIALS AND METHODS

Various factors can be used to compare virus production in larval instars. Two commonly used are the production ratio and production efficiency. The productivity ratio (PR) is defined as the difference between the amount of initial virus inoculum and the per-larval yield of virus and is calculated as:

Production ratio = Yield/larva (OBs) ÷ No. of infecting OBs (OBs/mm^2)

Another measure of production is production efficiency (PE) (Shapiro *et al*., 1981) and is calculated as:

Production efficiency = OBs/larva ÷ weight of larva

5.2.1 Virus production in fourth instar *Thaumatotibia leucotreta* larvae

Twenty five cell bioassay trays (Sterilin Ltd, United Kingdom), in which the bioassays were conducted, were considered too small for mass production of CpGV. Therefore, a larger, 200 x 200 x 40 mm square glass pie dish was used for the mass production trials. The artificial diet (140 g dry ingredients with 150 ml distilled water) was prepared in the same manner as previously described in Chapter 2, section 2.2.1.2. The only change to the composition of the diet was a 33% increase of the anti-microbial agents (methyl paraben and sorbic acid) to further reduce the risk of fungal contamination, as suggested by Moore (2002).

Once the diet surface had cooled sufficiently, it was inoculated with an adjusted LC_{90} concentration of CpGV (for fourth instar *T. leucotreta* larvae). It was determined that 10

ml of inoculum was sufficient to adequately and evenly wet the diet surface in a pie dish. The LC_{90} for fourth instar larvae was 1.642 x 10^5 OBs/mm^2 (see Chapter 4, section 4.3.2). The CpGV inoculum was applied as a fine spray using a 50 ml plastic spray bottle atomizer. To ensure that the surface of the diet was inoculated with this number of OBs, it was sprayed with 10 ml of 6.56 x 10^9 OBs/ml which resulted in the correct concentration of OBs being applied evenly to the surface of the diet.

The inoculated pie dishes were placed on a laminar flow cabinet for ± 45 min, or until the surface of the diet was dry. A total of 250, 300 or 400 fourth instar larvae were placed on the diet surface (Figure 5.1A). Fourth instar larvae were selected on the basis of head capsule size as described in Chapter 2, section 2.2.2.4. Larvae were handled carefully so as to reduce any possible stress that may induce the homologous virus. The glass pie dish was covered with a plastic lid. A 100 mm diameter hole was cut in the centre and covered with a breathable membrane (PPFE 2203, BreathTech, South Africa) to allow adequate air movement and to prevent larvae from escaping.

Figure 5.1 *in Vivo* virus replication: (A) Artificial diet in a glass pie dish, inoculated with CpGV and fourth instar *Thaumatotibia leucotreta* larvae for *in vivo* production of CpGV; (B) Virus infected larvae ready for harvesting.

5.2.2 Virus production in fifth instar *Thaumatotibia leucotreta* larvae

In order to determine virus yields using mass production techniques in fifth instar *T. leucotreta* larvae the same methodology was followed as in section 5.2.1. The LC_{90} for fifth instar larvae was estimated to be 9.783×10^6 OBs/mm^2, (see Chapter 4, section 4.3.1). To ensure that the surface of the diet was inoculated with this number of OBs, it was sprayed with 9.78 ml of 4.0×10^{10} OBs/ml which resulted in the correct concentration of OBs being applied evenly to the surface of the diet. Fifth instar larvae were selected on the basis of head capsule size as described in Chapter 2, section 2.2.2.4.

5.2.3 Virus harvesting

The pie dishes were inspected twice daily, and all virus infected cadavers were collected and stored in a freezer at -16°C (Figure 5.1B). An infected larva was identified by the characteristic features described in Chapter 3, section 3.3.2. The number of larvae harvested from each glass dish were recorded and the data analysed by ANOVA using STATISTICA (Version 12 SP2). Means for each total of larvae placed onto inoculated diet were compared using Kruskal Wallis ANOVA. Once numbers of larvae harvested were recorded, they were grouped in the freezer, regardless of which pyrex dish they were harvested from.

Two alternative methods of harvesting were also investigated. The three production methods are summarised in Table 5.1. Three pie dishes of diet were prepared and inoculated as described in section 5.2.1. Three hundred fourth instar larvae were placed into each dish. After 8 days the entire contents of one pie dish was harvested and frozen. This method was repeated for fifth instar production. A third method involved removing the hard undigested diet from 8 rearing jars and placing the larvae along with the small amounts of the remaining moist diet and frass, termed “crumbly diet”, into a sterile plastic container (150 x 200 mm) (Figure 5.2). The surface of the diet was then sprayed with the LC_{90} concentration of CpGV inoculum. It was determined that 10 ml of inoculum was sufficient to adequately and evenly wet the diet surface in the rearing container. To ensure

that the surface of the diet was inoculated with this number of OBs, it was sprayed with 10 ml of 4.92 x 10^9 OBs/ml for fourth instar inoculations and 10 ml of 2.39 x 10^{10} OBs/ml for fifth instar inoculations which resulted in the correct concentration of OBs being applied evenly to the surface of the diet. The virus inoculum was applied as a fine spray using a 50 ml plastic spray bottle atomizer. The container was sealed with a lid containing a breathable membrane. The entire contents of the container were harvested after 8 days and placed into a freezer at -16 °C until required. To estimate the approximate larval numbers inoculated per treatment, larvae from three rearing jars were removed, counted and an average obtained. The rearing jars were selected at random from the same production date used in the trial. Depending on the number of jars used in each treatment (usually 7 or 8) the number of larvae could be estimated.

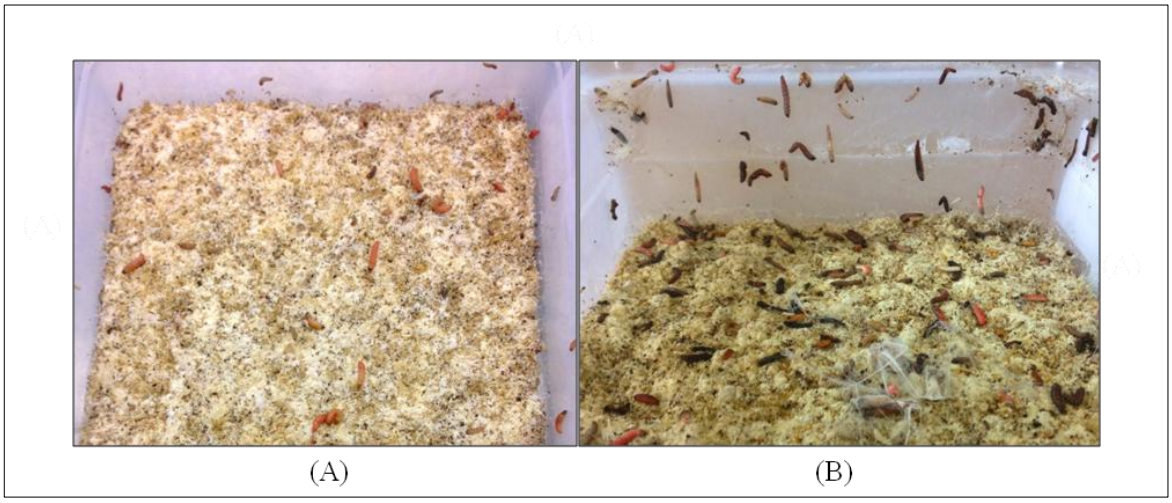


Figure 5.2 CpGV production method in *Thaumatotibia leucotreta* involving removing the larvae and digested diet into a sterilized container and inoculating the surface of the diet with the LC_{90} dose of CpGV; (A) container with freshly inoculated larvae; (B) infected larvae on the surface of the diet 7 days after treatment.

Table 5.1 Production methods used to determine CpGV production in fourth and fifth instar *Thaumatotibia leucotreta.*

Production method	Diet preparation	Number of larvae inoculated	Harvest method
1	Freshly prepared and inoculated	250, 300 and 400	Infected cadavers
2	Freshly prepared and inoculated	300	Infected cadavers and diet
3	Original rearing diet and inoculated	Various quantities	Infected cadavers and diet

5.2.4 Semi-purification of virus from diet

The semi-purification protocol described by Moore (2002) was used in this study. Large numbers of larvae (or larvae and diet) were defrosted and virus was liberated by crushing the mixture with a mortar and pestle. The product was then filtered through a layer of 100 µ nylon mesh, to produce a crude viral suspension. The suspension was diluted 1:4 with 0.1% SDS in distilled water and centrifuged at 1500 rpm (using a JA-20 Beckman rotor) for 2 min, to pellet insect debris. The supernatant was retained and the pellet resuspended in a few millilitres of 0.1% SDS. This was centrifuged again in the same manner and the resulting supernatant again retained. The process was repeated again and all retained supernatant added together. A smear was made on a microscope slide, from a small sample of the pellet, and then air dried. Buffalo Black solution was heated to 40ºC in a staining rack on a hotplate. The slide was immersed in the Buffalo Black solution for 5 min.

Thereafter, the slide was washed under running tap water for 10 seconds and left to air dry. Once dry it was examined through a light microscope at 1000 X magnification under oil immersion. Once it was established that little or no virus was present in the pellet, it was discarded. The supernatant was then centrifuged at 9000 rpm (using a JA-20 Beckman rotor) for 30 min. The resulting pellet consisted of two layers: a dark lower layer (virus)

and a pale upper layer. While the darker layer was retained at 4ºC in a few millilitres of 0.1% SDS, the lighter layer was resuspended in 0.1% SDS by vortexing. This was centrifuged at 15000 rpm in a desktop microfuge for 10 min. Again the pellet consisted of two layers. A smear was taken from the top lighter layer, stained and inspected for virus OBs in the manner described above. When it was determined that there was a negligible amount of virus in this layer, it was discarded. The bottom darker layer was added to the virus layer previously retained in 0.1% SDS and either refrigerated or frozen at –16ºC.

5.2.5 Quantification of virus

It was necessary to semi-purify the homogenate as described in section 5.2.4. It was then possible to enumerate the semi-purified virus using the Thoma counting chamber (cell depth 0.02mm, Hawksley, United Kingdom) at 400 X magnification under dark field. The same methodology was followed as described in Chapter 4, section 4.2.2.2.

Virus quantification was conducted with four batches of virus, ranging from 185 to 650 virus infected larvae, harvested over a period of time. Larval equivalents (mean OBs per larva) and mean OBs per gram of larva were calculated for each batch and the overall mean obtained. Similarly, virus production per dish, when larvae and diet were harvested together, was determined. Consequently, it could be ascertained which method of virus harvesting, produced the largest quantity of virus. Purified virus which was used for inoculation of diet for virus production was enumerated in the same manner.

5.2.6 Quality Control

5.2.6.1 Microbial contamination of infected larvae

A comparison of the level of bacterial contamination in the three virus production methods was conducted. Seven batches of production virus were assessed for microbial contamination (Table 5.2).

An initial dilution of 1:200000 was made for each treatment sample. Using this dilution, a 10 fold dilution series was made for each of the samples. A Petrifilm plate (3M^{TM}, United States of America) was used to assess bacterial contamination. The Petrifilm plate was placed onto a level surface. The top film of the plate was lifted and 1 ml of each dilution was pipetted onto centre of the bottom film. The top flap was released, allowing it to drop back into place. A spreader was placed on the top film, with the ridge side down, and pressure gently applied to distribute the inoculum over the circular area. It was important not to twist or slide the spreader. Once completed, the plate was left for 60 seconds for the gel to solidify. The inoculated plates were then incubated in a bacterial chamber (Merck, South Africa), with the clear side up, for 72 hours (3 days) at 30°C. Plates were assessed 72 hours post inoculation. The number of bacterial colony forming units (CFUs) was counted and the ratio of CFUs to OBs for each preparation was determined. Due to the small sample size and the variation between samples it was not possible to reliably determine whether differences were significant.

Table 5.2 Virus production methods assessed for microbial contamination.

	Harvested	**Purification method**
1	Larvae	Glycerol gradient centrifugation
2	Larvae (fourth instar)	Semi-purification
3	Larvae (fifth instar)	Semi-purification
4	Larvae and diet: 8 DAT[1] (4th instar)	Production methd 2
5	Larvae and diet: 8 DAT (fifth instar)	Production method 2
6	Larvae and diet: Mass production method (4th instar)	Production method 3
7	Larvae and diet: Mass production method (fifth instar)	Production method 3

[1] Days after treatment

5.2.6.2 Confirmation of virus production using REN analysis of genomic DNA

Random samples from the virus production batches were selected and subjected to the OB purification protocol as described in Chapter 3, section 3.2.6. For the analysis of CpGV produced in both fourth and fifth instar *T. leucotreta.* Only two samples were selected from the initial production technique in which 300 larvae were placed onto freshly prepared inoculated diet with only infected cadavers harvested and a further two samples were selected from the mass production technique in which the larvae along with the soft crumbly portion of the diet were inoculated with the LC_{90} concentration of CpGV. Genomic DNA was extracted from the purified OBs as described in Chapter 3, section 3.2.6. Restriction enzyme digestion using *Hin*dIII was conducted on the DNA samples as described in Chapter 3, section 3.2.9. The DNA was loaded on a 0.6% Ethbr stained agarose gel in TAE buffer. The gels were viewed using a UV trans-illuminator with an in-built camera. The gel image was captured using UVIprochemi software.

5.3 RESULTS

Both fourth and fifth instar *T. leucotreta* larvae were investigated and their potential as a host for CpGV production was assessed. A 33% higher concentration of anti-microbial agents was used in the virus production diet. Moore (2002) found this to be a necessity as fungal contamination tended to increase on the virus production diet. This was due to non-sterile larvae being introduced to the diet surface as well as the frequent opening of the production containers to inspect and remove virus infected larvae thereby resulting in a far greater probability of introducing contaminants.

5.3.1 Virus harvesting

5.3.1.1 Production of virus in fourth instar *Thaumatotibia leucotreta*

Freshly prepared *T. leucotreta* diet was inoculated with the LC_{90} concentration of CpGV (1.64×10^6 OBs/ml) and 250, 300 and 400 fourth instar larvae introduced into each dish

repressively. The highest recovery of infected larvae, relative to the numbers introduced was recorded for the 250 larvae per container (Table 5.3).

Table 5.3 Harvest of virus infected fourth instar *Thaumatotibia leucotreta* larvae, inoculated with the LC_{90} concentration of CpGV (1.64×10^6 OBs/mm²).

Larvae	Replicates	Larvae harvested Mean ± SE	Percentage harvested Mean ± SE	Period of harvest (DAT[2])
250	3	142.00 ± 7.81 ab[1]	56.80 ± 2.89	3 to 11
300	3	117.67 ± 3.12 a	39.22 ± 17.84	4 to 10
400	3	191.33 ± 8.67 b	47.83 ± 4.46	3 to 9
Mean		150.33 ± 11.44	47.95 ± 3.49	

[1] Values followed by the same letter are not significantly different ($H_{2,9} = 6.49$, $P = 0.039$; Kruskal-Wallis ANOVA). [2] DAT = days after treatment.

Increasing larval density from 250 to 300 and 400 larvae per inoculation container resulted in a decrease in the percentage recovery of infected larvae. This was possibly due to the increased probability of horizontal transmission of the virus and thus larvae may ingest greater volumes of the virus resulting in rapid infection. This may, in turn, affect the larva's ability to move out of the diet, reducing the collection rate. Secondly, initial overcrowding as larvae were introduced to the diet surface may have resulted in increased stress levels for the larvae. Larvae were observed to wander around the surface of the diet, for up to 24 hours post inoculation, resulting in periodic interactions with one another before penetrating the diet. This may trigger the covert, homologous virus to become pathogenic, resulting in larvae succumbing rapidly to the virus and dying within the diet medium. A mean of 47.95% of all larvae introduced onto the diet was harvested as virus infected larvae. The lowest recovery was recorded in the 300 larvae treatment in which only 39.22% were recovered, less than the average recovery of infected larvae. In all treatments no meaningful pupation was recorded. A total of 23 pupae out of 2850 introduced larvae were collected, resulting in 0.80% pupation rate once inoculated. The pupae were harvested along with the infected larvae in the hope that they contained a

significant virus load. The peak collection period of infected larvae, for all three treatments, was on day 4 post inoculation. This correlates with the LT_{90} (3 days 16 hours) value for fourth instar *T. leucotreta* larvae inoculated with the LC_{90} concentration of CpGV.

5.3.1.2 Production of virus in fifth instar *Thaumatotibia leucotreta*

As in 5.3.1.1, 250, 300 and 400 fifth instar larvae were placed into freshly prepared CpGV inoculated diet. The highest recovery of larvae, relative to the numbers introduced was recorded in the 300 larvae per container treatment (Table 5.4). However the percentage recovery was not significantly different to that achieved in the 250 larvae treatment. A decrease in recovery of infected larvae was recorded when the density of larvae within the container was increased by 33% to 400 larvae.

Table 5.4 Harvest of virus infected fifth instar *Thaumatotibia leucotreta* larvae, inoculated with the LC_{90} concentration of CpGV (9.78×10^6 OBs/mm²).

Larvae	Replicates	Larvae harvested ± SE [1]	Percentage harvested ± SE	Period of harvest (DAT[2])
250	3	111.67 ± 11.98	44.67 ± 4.79	2 to 12
300	3	136.33 ± 4.63	45.44 ± 1.54	3 to 12
400	3	132.33 ± 4.37	33.08 ± 1.09	3 to 13
Mean		126.78 ± 6.99	41.06 ± 2.47	

[1] No significant difference between treatments ($H_{2,9}$ = 2.76, P = 0.25; Kruskal-Wallis ANOVA) [2] DAT = days after treatment.

A significantly higher percentage of pupation was observed in the fifth instar treatments. A total of 97 out of 2850 larvae pupated (3.4%). It was noted that the majority of larvae pupating did so without feeding on the virus inoculated diet. Pupation may have been triggered in the fifth instar larvae due to the increased stress of having being removed from the diet, or possibly a number of the larvae within the rearing jar had developed quicker

than most and had therefore reached the stage in their development in which they were ready for pupation. Grzywacz *et al*. (1998) suggested that the penultimate instar could be selected for inoculation as the final instar may tend to be refractive to infection resulting in the larvae pupating rather than succumbing to the virus. All pupae were harvested along with the infected larvae in the hope that they contained a significant virus load.

5.3.2 Semi-purification of virus from diet

The methodology used in the semi-purification of CpGV was adequate to enable accurate enumeration of the virus using a counting chamber. If the goal of the purification was to provide virus OBs for DNA or for bioassays, this methodology would not be considered appropriate as it did not provide adequately purified OBs. In order to conduct accurate bioassays to determine the effect of virus dosage on mortality it is important to have a pure source of inoculum. If the inoculum is contaminated with bacteria or other entomopathogens, it would not be possible to accurately determine the cause of larval mortality, which may then lead to an inaccurate conclusion.

5.3.3 Quantification of virus

5.3.3.1 Yield of virus in fourth instar *Thaumatotibia leucotreta*

The yield of CpGV produced in fourth instar *T. leucotreta* larvae did not vary substantially. Virus yield ranged from 2.43×10^{10} to 3.08×10^{10} OBs per larva with the average larval weight ranging from 33 to 39 mg (Table 5.5). The highest yield of virus achieved per gram of larvae was 1.06×10^{12} OBs. The mean number of OBs per larva was 3.00×10^{10}; the CpGV inoculum was applied at 1.64×10^{6}, therefore the PR value for the production of CpGV in fourth instar *T. leucotreta* was 1.78×10^{4}. The PE for CpGV inoculated in fourth instar *T. leucotreta* larvae was 9.68×10^{8} (calculated using a mean value of 31 mg of larval mass).

Table 5.5 Yield of virus by *in vivo* production in fourth instar *Thaumatotibia leucotreta* larvae by harvesting infected larvae individually.

Number of CpGV infected larvae	Total mass of larvae (g)	Mass per larva (g)	Total OBs	OBs per larva (larval equivalent)	OBs per gram of larvae
185	5.4053	0.029	5.70×10^{12}	3.08×10^{10}	1.06×10^{12}
290	9.2941	0.032	7.04×10^{12}	2.43×10^{10}	7.58×10^{11}
193	5.8932	0.031	5.61×10^{12}	2.91×10^{10}	9.51×10^{11}
650	21.1995	0.033	8.93×10^{12}	1.37×10^{10}	4.21×10^{11}
Mean		0.031		3.00×10^{10}	9.67×10^{11}
±SE		0.001		2.43×10^{09}	7.68×10^{11}

5.3.3.2 Yield of virus produced in fifth instar *Thaumatotibia leucotreta*

The yield of CpGV produced in fifth instar *T. leucotreta* larvae did not vary substantially. Virus yield ranged from 3.50×10^{10} to 5.55×10^{10} OBs per larva with the average larval weight ranging from 39 to 45 mg (Table 5.6). The highest yield of virus achieved per gram of larvae was 1.44×10^{12} OBs.

The mean number of OBs per larva was 4.36×10^{10}. The CpGV inoculum was applied at 9.78×10^{6}, therefore the PR value for the production of CpGV in fifth instar *T. leucotreta* was 4.46×10^{3}. This value is 3.8 times lower than the value achieved for fourth instar production. The PE value for CpGV inoculated fifth instar *T. leucotreta* larvae was calculated at 1.06×10^{9} (calculated using a mean weight of 41 mg). This figure is higher than that recorded for production in fourth instar larvae indicating a higher production per larval unit. The lower PR value obtained for CpGV infected *T. leucotreta* larvae may be due to the substantially higher virus inoculum required to obtain infection. Therefore there is a poorer return on the initial inoculum concentration required to infect the heterologous host.

Table 5.6 Yield of virus by *in vivo* production in fifth instar *Thaumatotibia leucotreta* larvae by harvesting infected larvae individually.

Number of infected larvae	Total mass of larvae (g)	Mass per larva (g)	Total OBs	OBs per larva (larval equivalent)	OBs per gram of larvae
381	14.71	0.039	2.06×10^{13}	5.41×10^{10}	1.40×10^{12}
144	5.71	0.040	5.04×10^{12}	3.50×10^{10}	8.50×10^{11}
123	5.07	0.041	4.69×10^{12}	3.82×10^{10}	8.57×10^{11}
210	9.35	0.045	7.40×10^{12}	3.52×10^{10}	8.66×10^{11}
233	9.48	0.041	1.29×10^{13}	5.55×10^{10}	1.44×10^{12}
Mean		0.041		4.36×10^{10}	1.08×10^{12}
±SE		0.001		4.62×10^{09}	1.38×10^{11}

5.3.3.3 Virus yield using different harvesting techniques

Despite good yields of virus being obtained by collecting individual infected larvae, further attempts were made to improve productivity. One of the determining factors prompting further investigation was an attempt to reduce the labour component in the production process. Collecting individual infected larvae was considered too time consuming for a commercial production system. Harvesting the entire contents (larvae and diet) eight days post inoculation was assessed as well as a technique which involved inoculating a larva and diet mix.

The inoculation of 300 fourth instar *T. leucotreta* larvae (harvested with diet) resulted in a virus yield of 1.04×10^{13} OBs. The mean yield per larva was 3.48×10^{10} OBs (SE= 3.45×10^{9}). Production in fifth instar larvae, using the same protocol as fourth instar production, resulted in 1.28 times the average yield in fourth instar larva (1.34×10^{13} OBs). Mean yield per larvae was 4.45×10^{10} OBs (SE = 1.49×10^{10}) (Table 5.7).

Table 5.7 Virus yields from harvesting virus infected *Thaumatotibia leucotreta* larvae and diet 8 days post inoculation.

Instar inoculated	**Production method**	**Mean number of larvae inoculated (± SE)**	**Mean number of OBs harvested (± SE)**	**Mean number of OBs per larva (± SE)**
Fourth	Larvae and fresh diet	300	1.04×10^{13} (1.06×10^{12})	3.48×10^{10} (3.45×10^{9})
Fifth	Larvae and fresh diet	300	1.34×10^{13} (1.06×10^{12})	4.45×10^{10} (1.49×10^{10})
Fourth	Larvae and sorted diet	1141 (33.11) [1]	6.59×10^{13} (9.05×10^{12})	5.76×10^{10} (8.27×10^{9})
Fifth	Larvae and sorted diet	1763 (288.51) [1]	1.23×10^{14} (2.78×10^{13})	6.48×10^{10} (1.19×10^{10})

[1] Estimated value by averaging the numbers of larvae from 3 rearing jars from the same production date used in the trial

Pupation was not recorded during these studies. However, unlike previous fifth instar inoculations (section 5.3.1.2), it was noted that during the inoculations a large percentage of the 300 larvae began searching for a pupation site before feeding. This searching behaviour was observed up to 24 hour post inoculation after which the larvae began feeding on and penetrating into the diet. In one of the experiments, an estimated 40% of the larvae pupated without feeding. This behaviour was also observed in a small portion of the fourth instar larvae, however, within 12 hours post inoculation all the larvae had begun feeding or penetrated the diet.

Due to this observed behaviour in fifth instar larvae a third method of mass production was tested, with larvae and diet mix inoculated with the LC_{90} concentration of CpGV. Increased yields per larva were recorded in both fourth and fifth instar larvae. In fourth instar larvae, production increased by 1.66 times and fifth instar production increased by 1.46 times. The mean yield per larva in fourth instars was 5.76×10^{10} OBs (SE = 8.27×10^{9}). Production in fifth instar larvae yielded an average of 6.48×10^{10} OBs/larva (SE = 1.19×10^{10}) (Table 5.6).

5.3.4 Microbial contamination

Seven CpGV production samples, subjected to various means of purification, were tested for bacterial contamination. Contamination of the production preparations ranged from 4.40×10^{7} CFUs/ml to 5.82×10^{8} CFUs/ml, a 13-fold difference (Table 5.8). CFU:OB ratio in both the mass production samples was favourably low and surprisingly lower than the semi-purified and purified preparations. The lowest level of contamination occurred in the fifth instar mass production trial which was harvested eight days after inoculation (Table 5.4). Bacterial contamination using this production method was substantially lower than the other preparations, with the next lowest level of contamination being recorded in the fourth instar mass production preparation. Purified larvae resulted in the highest CFU:OB ratio of 1:11279. Fungal contamination was not recorded in this study.

Table 5.8 Bacterial contamination of different preparations of virus produced in fourth and fifth instar *Thaumatotibia leucotreta* larvae.

Harvested	Purification method	CFUs/ml	CFUs/Larva	CFUs:OBs
Larvae	Glycerol gradient centrifugation	1.74×10^{8}	8.25×10^{5}	1:11 279
Larvae (4^{th} instar)	Semi-purification	5.82×10^{8}	8.95×10^{5}	1:15 344
Larvae (5^{th} instar)	Semi-purification	4.78×10^{8}	2.05×10^{6}	1:26 987
Larvae and diet: 8 DAT [1] (4^{th} instar)	Semi-purification	8.80×10^{7}	2.93×10^{5}	1:118 182
Larvae and diet: 8 DAT [1] (5^{th} instar)	Semi-purification	2.04×10^{8}	6.8×10^{5}	1:65 686
Larvae and diet: Mass production method (fourth instar)	Semi-purification	1.48×10^{8}	1.3×10^{5}	1:445 270
Larvae and diet: Mass production method (fifth instar)	Semi-purification	4.40×10^{7}	2.5×10^{4}	1:2 795 455

[1] DAT days after treatment

5.3.5 Confirmation of virus production using REN analysis

Restriction endonuclease (REN) analysis was conducted on genomic DNA extracted from virus infected larvae recovered from the various mass production techniques to determine if the inoculated CpGV had successfully infected and replicated within the late instar *T. leucotreta* larvae. As noted in Chapter 4, section 4.3.4, REN analysis of viral genomic DNA with *Hin*dIII can be used to determine if the samples contained CpGV, CrleGV or a mixture of the two viruses.

5.3.5.1 Virus production in fourth instar *Thaumatotibia leucotreta*

Single REN digestion of the genomic DNA with *Hin*dIII produced numerous prominent DNA fragments in each of the samples tested. The restriction patterns of samples indicated the presence of a contaminant in the virus sample (Figure 5.3). In lanes 7 and 8 CpGV and CrleGV standards were run as a comparison for the profiles of the virus production samples. The profiles in lane 3 and 5 indicate the presence of both CpGV and CrleGV as a mixed virus suspension. The profiles for the virus samples run in lanes 4 and 6 indicate the presence of CrleGV. However, the two high molecular weight DNA fragments of CpGV are not visible in lane 4 and appear to be very faint in lane 5 indicating that there is possibly little or no CpGV present in the sample. The restriction patterns are comparable to those described by Opoku-Debrah *et al*. (2013) and indicate the presence of CrleGV contamination.

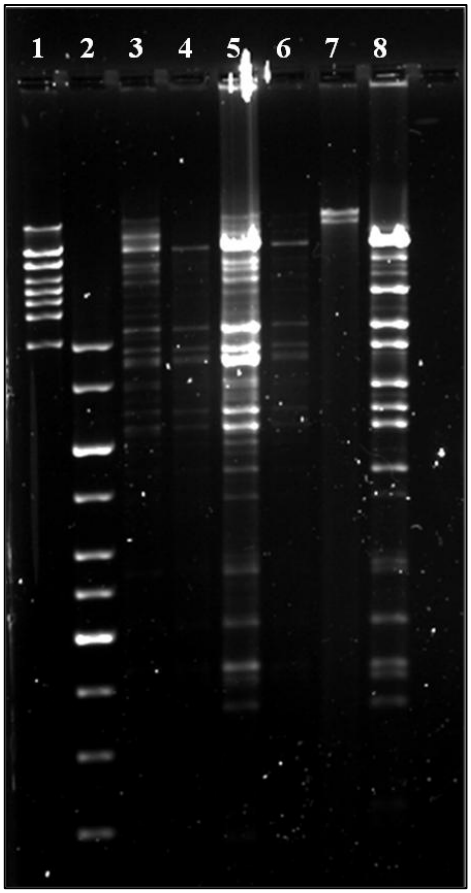


Figure 5.3 Restriction endonuclease profiles of four samples of virus, produced in *Thaumatotibia leucotreta*, digested with *Hin*dIII. Electrophoresis was conducted on 0.6% agarose gels for 16 h at 30 V. (A) Lane 1 – High range DNA marker, lane 2 – 1 Kb DNA marker, lane 3 – fourth instar larvae (sample 1), lane 4 – fourth instar larvae (sample 2), lane 5 – fourth instar, mass production technique (sample 3), lane 6 – fourth instar mass production technique (sample 4), lane 7 – CpGV standard, lane 7 – CrleGV standard.

5.3.5.2 Virus production in fifth instar *Thaumatotibia leucotreta*

Single REN digestion of the genomic DNA with *Hin*dIII produced numerous prominent DNA fragments in each of the samples tested with the exception of the sample in lane 6. The restriction patterns of all the samples in lane 3, 4 and 5 indicated the presence of a contaminant in the virus sample (Figure 5.3). As in Chapter 4 the restriction patterns are comparable to those described by Opoku-Debrah *et al*. (2013) and indicate the presence of CrleGV contamination. The virus production sample in lane 6 has a single high molecular

weight band. However, the second band is not visible. It is suspected that the sample is pure CpGV, however due to the large amount of genomic DNA present digestion is possibly incomplete. Therefore the second band may be present although not visible on the gel (Figure 5.4).

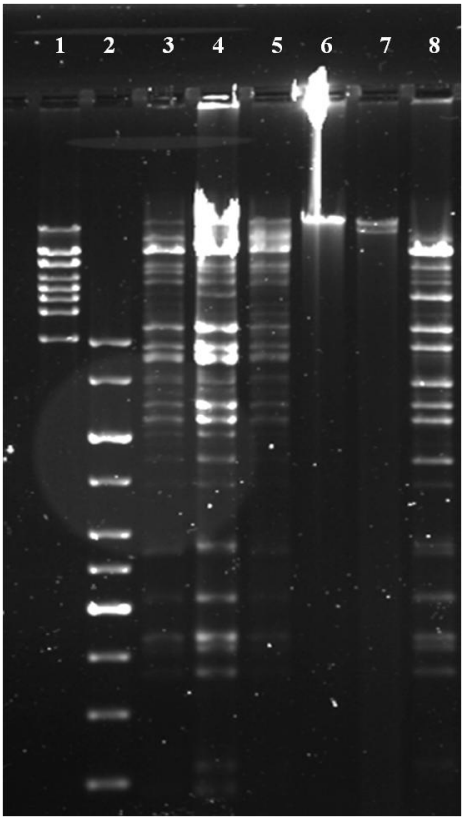


Figure 5.4 Restriction endonuclease profiles of five samples of virus, produced in *Thaumatotibia leucotreta*, digested with *Hin*dIII. Electrophoresis was conducted on 0.6% agarose gels for 16 h at 30 V. (A) Lane 1 – High range DNA marker, lane 2 – 1 Kb DNA marker, lane 3 – fifth instar larvae (sample 1), lane 4 – fifth instar larvae (sample 2), lane 5 – fifth instar, mass production technique (sample 3), lane 6 – fifth instar, mass production technique (sample 4), lane 7 – CpGV standard, lane 8 – CrleGV standard.

5.4 DISCUSSION

The main priority in virus production is the efficient utilisation of host tissue for the production of viable virus and as many as 10^9 OBs can be produced per larva (Moscardi *et al*., 2011; Rodriguez *et al*., 2012). Historically late instar larval stages have been used in commercial virus production systems. This is typically because more virus can be produced per individual in the older larval instars (Moore, 2002; Grzywacz *et al*., 1998).

It was therefore decided to assess both fourth and fifth instar, *T. leucotreta*, larvae as potential life stages for CpGV production. Third instar larvae were not used as they were considered too small and delicate for a commercial production process. *Thaumatotibia leucotreta* larvae are cryptic feeders, therefore inoculation of the larvae requires that they be physically removed from the rearing diet. The removal of larvae may result in an increased risk of injury or stress to the larvae, resulting in increased mortality and the possibility of triggering the covert homologous virus. Consideration would also have had to be given to a lower inoculum dosage. With inoculation of the third instar life stage, it would be important for the larvae to continue growing during the infection period to ensure, at death, the larva is of a substantial size and contains a high virus load. Applying low doses of the virus could increase the risk of the covert virus becoming the dominant isolate in the final product.

The average recovery of virus infected larvae for fourth and fifth instar *T. leucotreta* inoculations was 47.95% and 41.06% respectively. The maximum percentage larval recovery from all production trials was 56.80%. These percentage recoveries were considered to be unacceptably low. The low percentage recovery of infected larvae may have been influenced by a number of factors. Some infected larvae that exited the diet ruptured when attempts were made to collect them. These larvae could therefore not be included in the final percentage of larvae harvested. Although no record was kept of these failed collection attempts, this was not the norm and was considered an insignificant loss. The main reason for the low percentage recovery of infected larvae appeared to be larval behaviour. *Thaumatotibia leucotreta* larvae are cryptic feeders therefore collection is

dependent on the larvae exiting the diet once infected. It is common for baculovirus infected larvae to move out of a food source, disperse or climb to an elevated position once infected (Tanada & Kaya, 1993; Moscardi, 1999). However, although it is reported as common for larvae to do so, this is not always the case. During this study, a significant number of the larvae remained within the diet and were therefore not collected. This behaviour was also noted in CrleGV infected *T. leucotreta* larvae by Moore (2002) and it was suggested that the reason for the infected larvae not exiting the diet may have been a conflict in behavioural cues. The larvae seek out a suitable pupation site beneath the orchard and but also respond to the virus infection that forces them upwards. The cue to find a suitable pupation site may have been stronger as this may relate to their natural behaviour in the environment in which they would pupate within a soil substrate. During the rearing of *C. pomonella* in this study, it was noted that infected larvae did not display the behavioural characteristic of exiting the diet as observed in *T. leucotreta* infected larvae. The majority of the larvae that succumbed to the virus infection remained within the diet.

It was not only important to quantify the percentage recovery of infected larvae but also to establish the quantity of virus being produced per larval unit. A low percentage recovery with a high larval yield may be sufficient to warrant further investigation into production on a commercial scale. The average production of OBs per larva for fourth and fifth instar was 2.80×10^{10} and 4.36×10^{10} respectively. CpGV yields under mass production conditions were lower than those achieved when larvae were inoculated and harvested from single rearing cells (section 4.3.2). In single cell inoculations the average yield of OBs per larva was 5.78×10^{10} and 7.64×10^{10} for fourth and fifth instars respectively (Section 4.3.2). Mean CpGV yields from fifth instar *C. pomonella* larvae have been reported in the range of 2×10^{9} to 1×10^{11} OBs/larva (Falcon *et al*., 1968; Benz & Wäger, 1971; Glen & Payne, 1984). Glen & Payne (1984) obtained CpGV yields of 9.0×10^{9} OBs in *C. pomonella* larvae. They also found that the yield could be increased to 1.6×10^{10} OBs per larva by adding a juvenile hormone analogue namely, methoprene, to the diet. Reiser *et al*. (1993) achieved a maximum CpGV yield of 1.8×10^{10} OBs in fifth instar *T. leucotreta* larvae. Their study also concluded that the yield of virus per larval unit decreases in mass

production systems. Fifth instar larvae produced the highest amount of OBs per larval unit which is in agreement with this study. Moore (2002) showed yields of 1.158×10^{11} OBs per larva in fifth instar *T. leucotreta* larvae which is significantly higher than any of the yields achieved in this study.

Occlusion body production per larva and OB production per milligram of host weight may be considered as measures of success in virus production. These may be influenced by initial virus inoculum dosage, duration of infection, larval growth rate and the total weight gain during the infection period (Shapiro, 1986). The production ratio value (PR) for GV's should be higher than those of NPVs, as generally larval equivalents for GV are higher than those of NPVs (Crook, 1991). OB production per milligram of body weight for GV's in lepidopteran hosts has been reported to be approximately 2.0×10^{7} (Entwislte & Evans, 1985). In this study the PR values were found to be higher than this. For both fourth and fifth instar larvae the PR values were 1.71×10^{4} and 4.46×10^{3}, respectively. PE values were calculated to be 9.03×10^{8} and 1.064×10^{9}. Moore (2002) produced PR and PE values of 1.268×10^{4} and 2.363×10^{9} respectively for production of CrleGV in fifth instar *T. leucotreta* larvae. These values compare favourably to the results achieved in this study. Rios-Velasco *et al*. (2012) observed that the OB production per milligram of larval weight of *Spodoptera frugiperda* ranged from 3.13×10^{6} to 1.3×10^{7} in late instar larvae. Vasquez *et al*. (2002) noted a similar level of virus production per larva, reporting OB production levels per larva of 5.4×10^{8} and 7.3×10^{8} for fifth and sixth instar larvae.

Although good yields were obtained from collecting individually infected larvae, the need for an efficient commercial production system required further experimentation in developing methods of virus production that could reduce both the labour and time. This was done by harvesting the infected larvae along with the entire contents of the inoculation dish. This method may also have the added benefit of UV protection for the virus in the final product formulation, as it has been shown that a crude extract of virus containing insect particles and a dietary component provided significant UV protection for the virus particles (David, 1969; Arthurs *et al.*, 2006).

It is important to compare yield per container when considering the efficiency of a mass production system. The average yield of CpGV for fourth instar *T .leucotreta* was 3.48 x 10^{10} OBs/larva and 4.45 x 10^{10} OBs/larva for fifth instar larvae. This resulted in virus yields per dish of 1.04 x 10^{13} and 1.34 x 10^{13} OBs for fourth and fifth instar larvae respectively. The yield per larva was comparable to that achieved by collecting larvae individually. Considering that the average percentage collection of fourth and fifth instar larvae was 47.95 and 41.06%, the virus yields per larva would have to exceed 7.34 x 10^{10} OBs/larva or 1.09 x 10^{11} OBs/larva, to result in a better yield of virus than that achieved by harvesting the entire contents on the production dish.

Three production techniques were performed during this study. It was noted that not all fourth and fifth instar *T. leucotreta* larvae fed on, and penetrated, the virus inoculated diet. A number of larvae (and in some instances a meaningful proportion of them) sought out a pupation site and successfully pupated without consuming a lethal dosage of CpGV. This was most evident in fifth instar inoculations and therefore suggests that the production data of OBs per larval unit may be an underestimation of what is possible. Grzywacz *et al*. (1998) suggested that in some instances it is preferable to use younger instars for virus production as the final instar may be refractive to infection resulting in pupation rather than the larva succumbing to viral infection. It is also pertinent to acknowledge that in most commercial rearing programmes, the larval development may not be synchronous and late instar virus treated larvae may pupate and influence the yield of virus. There are however a number of disadvantages to using late instars for virus production. These include (i) increased time for larval growth as well as the time from inoculation to harvest of the virus infected larvae, (ii) increased risk of stress induced pupation and (iii) increased risk of bacterial contamination during larval development as well as during the virus production period. In an attempt to reduce stress induced pupation, a technique involving liberating the larvae from the diet by removing the hard undigested pieces but retaining the digested "crumbly" portion of the diet was used. It was observed that all larvae moved into the soft crumbly pieces of diet within 20 minutes post inoculation. Inoculation of freshly prepared diet resulted in the first signs of larval infection 2 to 3 days and 3 to 4 days post inoculation in fourth and fifth instar *T. leucotreta* respectively. Using the above mentioned method,

peak infection was observed 4 to 5 days post inoculation in fourth instar production. This was closer to that of the LT_{90} for fourth instars (3 days 16 hours). In fifth instar production peak infection was observed 5 to 6 days post inoculation (LT_{90} = 5 days 16 hours). This technique resulted in the highest viral yield. The average yield of CpGV for fourth instar *T. leucotreta* was 5.76 x 10^{10} OBs/larva and 6.48 x 10^{10} OBs/larva for fifth instar larvae. This resulted in virus yields per dish of 6.59 x 10^{13} OBs and 1.23 x 10^{14} OBs for fourth and fifth instar larvae respectively. This method not only provided the greatest yields of virus but also required the least amount of labour and time per inoculation.

Bacterial contamination varied from 4.40 x 10^{7} CFU/ml to 5.82 x 10^{8} CFU/ml. This variation was lower than expected with a 13-fold difference recorded in this study. This was substantially lower than what was recorded by Moore (2002) who noted a 273-fold difference in contamination between several semi-purified samples of CrleGV. The 13-fold range of bacterial contamination observed in the production samples is not regarded as a high level of variation. Grzywacz *et al.* (1997) observed up to a 1000 fold difference in bacterial contamination between production batches of the same virus. However bacterial contamination in the production samples was unacceptably high for most of the CpGV samples. It has been recommended that the viable bacterial count should not exceed 1 x 10^{8} CFU/ml for formulated products with an activity of 1 x 10^{9} OBs ml^{-1} or 5 x 10^{8} CFU/g for formulated dry products (Jenkins & Grzywacz, 2003; Anonymous, 2011b). The high level of bacteria recorded in this study may have been due to two reasons. Firstly, the length of time from production of the CpGV samples to the testing of bacterial contamination was approximately 4 weeks. During this period samples were left at 4ºC before the bacterial contamination trials could be conducted. This may have been sufficient time for the contamination to develop within the sample. No microbial suppressants, such as glycerin, were added to the production samples during this period. Jones *et al*. (1993) noted that bacterial contamination was found to be lower in larvae harvested before death. Harvesting the infected larvae 1 to 2 days earlier may have reduced the contamination levels to below the threshold and should be considered for future production. It is however pertinent to establish the effect that this may have on the virus as Ignoffo & Shapiro (1978) identified that harvesting HzSNPV from infected larvae prior to death resulted in lower yields of

virus as well as a reduced potency of OBs. Therefore with an application rate of 5 x 10^{15} OBs/ha (registered rate of CrleGV on citrus), the number of contaminant microbes would be equivalent to 1.79 x 10^{9} microbes/ha. Moore (2002) stated that this figure should not be considered excessive considering the top 15 cm of fertile soil may contain up to 4000 kg of bacteria and fungi per hectare.

REN analysis of viral genomic DNA with *Hin*dIII indicated that, with the exception of one CpGV production sample, all samples were contaminated with CrleGV as the homologous virus. A detectable level of CrleGV was expected. However, the level of CrleGV contamination in each sample was a cause for concern. In the previous chapter, production of CpGV in fourth and fifth instar *T. leucotreta* larvae was confirmed in laboratory bioassays. However, even under bioassay conditions only a single sample had no detectible CrleGV contamination. In progressing from single larva to mass inoculations, a breakdown in the inoculation technique or an increase stress triggers resulted in substantial CrleGV contamination of the final product. The results of this study indicated that there was little or no correlation between the production processes and the purity of CpGV obtained in the final production samples. This is however an important aspect to consider and warrants further investigation. Studies have reported the triggering effect of stresses and heterologous inoculation in the activation of homologous infections (Podgwaite & Mazzone, 1986; Burden *et al*., 2003; Il'inykh & Ul'yanova, 2005; Murillo *et al*., 2011). It is speculated that the CrleGV contamination was a result of CpGV inoculation serving as a stress trigger inducing manifestation and replication of the homologous virus within the *T. leucotreta* larvae, resulting in larval mortality as a result of CrleGV infection as opposed to the desired CpGV production.

The semi-purification method may be preferable in producing virus for field application as small particles present in the suspension could offer protection against harmful ultra-violet (UV) rays as it has been shown that a crude suspension of virus has a better persistence than that of a purified formulation (David, 1969; Arthurs *et al*., 2006). Grzywacz *et al.* (1997) concluded that the use of centrifugation procedures used in their study were not effective in reducing contamination. This observation, together with the costs of

centrifugation casts serious doubts on the value of this particular purification procedure. Possible benefit of insect and dietary particles in the final preparation may outweigh the necessity to attempt to reduce contamination to a minimum by centrifugation.

5.5 CONCLUSION

Acceptable yields of virus were obtained using all three production methods tested in this study. Collecting infected larvae was considered time consuming and not practical for *in vivo* virus production on a commercial scale. Harvesting larvae along with the diet not only produced good virus yields but also reduced the labour and time component.

It is recommended that a complete set of quality control parameters be developed and implemented on each virus production batch. Restriction analysis with *Hin*dIII was sufficient to confirm the presence of a pure CpGV, or a mixed suspension with a CrleGV contamination in a production sample. This technique was simply a qualitative assessment and no quantitative data were obtained. Preliminary data (not presented in this study) using a qPCR technique has showed it is possible to determine the proportion of each virus in a mixed or contaminated production sample. This study has been initiated and is currently in progress in conjunction with the Nelson Mandela Metropolitan University, Port Elizabeth, South Africa. Production samples of CpGV produced in *T. leucotreta* have been evaluated using this methodology with initial data indicating that this technique, will be a valuable tool in the assessment of CpGV purity from a production sample in *T. leucotreta* (B. Dhladhla, Nelson Mandela Metropolitan University, personal communication). The quality control protocols established in this study were able to determine, the total amount of virus present (enumeration), the microbial contamination as well as the virulence (neonate bioassays) of virus produced in a heterologous host. Further investigation into the qPCR technique is required before the quality control protocol is completed and the ratio of CpGV:CrleGV in a mixed virus suspension can be accurately determined.

6

GENERAL DISCUSSION

6.1 INTRODUCTION

Cydia pomonella is a significant economic pest of apples and pears worldwide. South Africa is no exception, with up to 80% crop losses recorded if control measures are not implemented by the grower (Myburg, 1980; Pringle *et al*., 2003). Historically, control of this pest has been achieved with the use of broad spectrum insecticides. Due to growing awareness of the potential negative effects of harsh chemical applications, concerns over employee safety and the environmental impact of certain farming practices, serious consideration has been given to developing softer control measures for implementation into an IPM strategy. Biological control measures, specifically baculoviruses, have in the past been considered inferior to chemical control practices and have usually been more costly (Schumacher, 2002). However, research results are slowly changing this perception and more affordable (Lacey & Shapiro-Ilan, 2008), good quality products are now available in the market place as alternatives to chemical control. Three examples of such products are Vivus Max (AgBiTech, Australia) for the control of *H. armigera* and *H. punctigera*, Cryptogran™ (River Bioscience Pty Ltd, South Africa) for the control of *T. leucotreta* and Madex for control of *C. pomonella*. All products have been widely accepted and are used as an important tool in IPM programmes. It is therefore imperative that research continues into isolating novel baculovirus isolates, improving products and improving production systems. This will not only reduce the cost of biological control products to growers but will also help to manage the development of resistance to the currently available virus isolates.

This study aimed to identify novel CpGV isolates from South Africa and to test a different means of CpGV production that, if effective, would reduce production costs significantly. The main objectives of this study were threefold. Firstly, to bioprospect for novel isolates of CpGV and compare these genetically to the Mexican strain of the virus, CpGV-M, found in the registered CpGV based products in South Africa. This involved a number of sub-objectives including bioprospecting for CpGV isolates from various deciduous fruit growing regions of South Africa, characterisation of the virus isolates using REN analysis of genomic DNA and sequencing of selected viral genes and evaluation of biological activity of novel isolates in comparison to CpGV-M. The second main objective was to determine if CpGV could be efficiently produced in a heterologous host, *T. leucotreta*. This again involved a number of steps which included comparing the rearing efficiency of each potential host, comparing viral yields produced in both fourth and fifth instar *C. pomonella* and *T. leucotreta* larvae, and establishing dose- and time-response relationships for CpGV production in both fourth and fifth instar *T. leucotreta* larvae. The final objective was to develop a set of quality control parameters in order to evaluate the quality of the virus product produced in *T. leucotreta*.

6.2 BIOPROSPECTING FOR NOVEL CpGV ISOLATES AND EVALUATING BIOLOGICAL ACTIVITY

Several studies have indicated that virus isolates collected from the field and from laboratory cultures may exhibit high levels of genetic variation with implications for virulence against the host (Weitzman *et al*., 1992; Eastwell *et al*., 1999; Rezapanah *et al*., 2008; Opoku-Debrah *et al*., 2013). Four isolates of CpGV were successfully isolated from field collected larvae and a fifth from a lab culture. With the exception of CpGV-SA, all isolates were genetically identical to the Mexican strain of the virus found in Madex® and Carpovirusine™. This is a first report of a genotypically distinct CpGV isolate identified in South Africa. Importantly, CpGV-SA now serves as a reference point for quality control comparison. It will be important to ensure that the genetic integrity of the isolate is maintained during any future work. It is possible that over time a mutation in the isolate or contamination of an inoculum source could result in a genotypically different virus being

used for experimentation. The genetic novelty of CpGV-SA is currently being further investigated at Rhodes University, Grahamstown, South Africa, and a full genome sequence has been obtained for comparison with that of the Mexican strain. DNA profiles obtained by REN digestion of genomic DNA differ significantly between CpGV-SA and the Mexican strain. CpGV-SA appears to be more closely related to the Canadian stain of CpGV than to other strains (B. Motsoeneng, Rhodes University, personal communication). It is speculated that the Canadian strain of the virus might have been introduced into South Africa on contaminated egg sheets imported from the Canadian SIR programme in British Colombia, Canada. How CpGV-SA appeared on an isolated farm with no previous records of granulovirus applications or sterile moth releases and its origin remains unknown. The discovery of a genetically different isolate in South Africa is exciting from a resistance management perspective. Resistance to the Mexican strain of CpGV has been documented in over 35 countries in Europe (Jehle, 2008; Jehle *et al*., 2010; Schmitt *et al*., 2013). For the South African grower, CpGV-SA may be used as a tool to reduce the risk of resistance development in South African field populations of *C. pomonella* to the Mexican strain of CpGV. Opoku-Debrah *et al*. (2013) suggested that the conventional norm of applying single genetic isolates of baculoviruses in commercial applications be reconsidered in order to safeguard the integrity of baculovirus based products. The application of a product containing a mixture of isolates, or alternating specific isolates, may help to manage the development of resistance to a frequently applied isolate.

CpGV-SA was tested against neonate *C. pomonella* larvae using a surface dose methodology and its virulence compared to the Mexican strain. The results of this study confirm that CpGV-SA is sufficiently virulent to warrant further investigation and development as a microbial control agent. This study has established the norm for virulence for CpGV-SA which can be used as a point of reference. Significant deviations from the norm would indicate a potential problem with a production batch. These deviations may be as a result of reduced isolate virulence, incorrect enumeration of the virus or potential contamination in the product. Having a reference point would allow for an informed decision by the producer regarding the formulation and distribution of each production batch. Considering the promising biological activity and potential marketability

of CpGV-SA, it is recommended that the isolate be formulated either as a single isolate for alternation with the CpGV-M based products or as a mixed isolate formulation and tested in field trials against the standard control measure currently used by growers.

6.3 POTENTIAL OF *CYDIA POMONELLA* AND *THAUMATOTIBIA LEUCOTRETA* AS HOSTS FOR CpGV PRODUCTION

After identifying and testing the biological activity CpGV-SA, it was important to justify the use of *T. leucotreta* as a heterologous host for CpGV production. Risks may include reduced virus yields, CrleGV contaminated virus suspensions and possible negative effects on the biological activity of the virus if produced in a heterologous host (Gröner *et al*., 1990). By comparison, production of CpGV in *C. pomonella*, the homologous host, would be relatively risk free. If a methodology could be developed to reliably produce CpGV in *T. leucotreta* it would potentially mean that CpGV could be produced at a significantly reduced cost. For a product to enter into and compete in an already competitive CpGV market it would require the following: (i) a superior efficacy over the commercially used isolates, (ii) a novel isolate of CpGV with comparable efficacy to the commercially used isolates for use in resistance management or (iii) it should compete on price. Production in CpGV in *T. leucotreta* favours the latter.

The ability to rear insects under laboratory conditions, as opposed to wild-types, has allowed for accurate studies on their biology and behavior (Huho *et al*., 2007). Rearing of each individual host was considered. There are many published diets for both *C. pomonella* (Brinton *et al*., 1969; Howell, 1972b; Toba & Howell, 1991; Bloem *et al*., 2000; Botto, 2006; Hansen & Anderson, 2006; Stenekamp, 2011) and *T. leucotreta* (Ripley *et al*., 1939; Theron, 1948; Bot, 1965; Moore *et al*., 2014) that have been shown to produce excellent numbers of individuals per gram of diet. However, many of these diets are intricate and costly to produce. The aim of a commercial venture should be to produce the best quality product at the lowest possible cost in order to not only improve profit but also provide the growers with a product that conforms to the highest possible standards. With this in mind, only diets which have been used on a mass production scale were considered in this study.

The Guennelon *et al*. (1981) diet was preferred due to the availability of dietary ingredients and ease of use and it has been used by a commercial company (ENTOMON Technologies Pty Ltd) in South Africa for the mass production of *C. pomonella* for the past four years. The *T. leucotreta* diet developed by Moore *et al*. (2014) has been used successfully by River Bioscience Pty Ltd, South Africa, for the commercial production of CrleGV for 10 years with excellent results. Both cultures were maintained as small lab cultures for the purpose of this study for a period of four years using these diets with limited losses to contamination by virus outbreaks. However, low levels of virus contamination were occasionally observed in the *T. leucotreta* culture in 2013 and 2014. It was suspected that the level of covert virus in the population had increased over time, resulting in the occasional epizootic (covert to overt). It would be difficult to determine the effect of the latent (internal) and exogenous (inoculated) virus during the infection process without a reliable technique for the detection of latent infection. The insect's response to latent viral infection is largely determined by the physiological status of the virus and by stress factors (Il'inykh & Ul'yanova, 2005). If further work is to continue on production of CpGV in *T. leucotreta*, the level of covert virus in the cultures needs to be quantified and monitored with the goal of reducing this to undetectable levels. A number of techniques for the detection of covert virus infections have been developed (Il'inykh *et al.*, 1995; Lin *et al.*, 1999; Cooper *et al.*, 2003; Il'inykh *et al.*, 2004).

In order to compare the rearing efficiency of each host, biological parameters such as fecundity, time to hatch and larval developmental times were compared. Rearing of *T. leucotreta* was not only significantly easier than *C. pomonella*, with higher fecundity, shorter developmental times and greater production per gram of diet, but could be done at a significantly reduced dietary cost. *Thaumatotibia leucotreta* was significantly more fecund than *C. pomonella*. Larval developmental time to reach fifth instar was 59.9% shorter for *T. leucotreta* and importantly it was noted that 68.8% more *T. leucotreta* larvae could be produced per gram of diet in comparison to *C. pomonella*. Faster development equates to more generations per year and thus the potential to produce larger volumes of virus *in vivo*. The cost of diet production was 60.4% lower for *T. leucotreta* diet than for *C. pomonella*. Production of CpGV in *T. leucotreta* required less insectary space, reduced sanitation costs

and, importantly, was less labour intensive. Considering these points, the cost benefit of producing CpGV in *T. leucotreta* is potentially significant.

6.4 PROPAGATION OF VIRUS IN *THAUMATOTIBIA LEUCOTRETA* AND *CYDIA POMONELLA* AND BIOASSAYS

Various concentrations of CpGV were tested against both fourth and fifth instar *C. pomonella* and *T. leucotreta* larvae to determine and compare virus yield and mortality at each dose. Virus yield per larva was comparable in the two hosts, validating the concept of producing CpGV in *T. leucotreta*. Yield per larva was also comparable to reported yields in the literature (Reiser *et al*., 1993; Moore, 2002).

The results of the surface dose bioassays confirmed that CpGV can infect and replicate within fourth and fifth instar *T. leucotreta* larvae. The LC_{90} doses were calculated to be 1.64×10^6 and 9.78×10^6 OBs/mm^2 for fourth and fifth instar *T. leucotreta* respectively. LC_{90} dose for fifth instar was approximately double the concentration reported by Moore (2002) for CrleGV. The LT_{90} values were 3 days 10 hours and 5 days 16 hours for fourth and fifth instar *T. leucotreta* respectively. The median lethal times were significantly shorter than reported by Moore *et al*. (2011) who reported a LT_{90} value of 9 days 8 hours for fifth instar *T. leucotreta* larvae when inoculated with the LC_{90} dose of CrleGV. The calculation of both the LC_{90} dose and the LT_{90} time are requirements for the development of a successful CpGV production process in *T. leucotreta*.

It was important to determine that the virus produced during the dose- and time-response assays was in fact CpGV and not the homologous virus, or a mixture of the two. Single restriction analysis of genomic DNA extracted from CpGV infected larvae obtained from the time-response study using *Hin*dIII confirmed the production of CpGV in *T. leucotreta*. This confirmed the observation by Reiser *et al*. (1993) that CpGV can be produced in *T. leucotreta* using an individual inoculation technique. However, unlike Reiser *et al*. (1993) who reported that CpGV produced in both *C. pomonella* and *T. leucotreta* was identical in the DNA restriction patterns, only a single pure CpGV sample was obtained from the five

samples of CpGV obtained from virus infected larvae from the time-response bioassay in this study. This was a major concern as the method used was the most likely scenario for success, as the conditions under which the experiment was conducted were designed to limit the stress on the larvae. Larvae were gently placed onto freshly prepared virus-inoculated diet in single cells, therefore reducing stress factors such as larval interaction. The CrleGV contamination observed in the samples indicated that mass inoculation or a commercial scale inoculation method would result in a number of new challenges that could adversely affect CpGV production.

6.5 MASS PRODUCTION OF CpGV IN *THAUMATOTIBIA LEUCOTRETA* AND QUALITY CONTROL

Mass production trials were conducted in such a way as to try and reduce stress triggers. Virus yields produced in *T. leucotreta* were satisfactory and similar to the levels of CrleGV production reported by (Moore, 2002). However, quality control analysis of the product indicated unacceptably high levels of CrleGV contamination in both the fourth and fifth instar production samples. This was a not unexpected as the *Hin*dIII analysis of the infected larvae from the time-response bioassay had indicated CrleGV contamination.

The main obstacle in the production of a virus in a heterologous host would be the effect of stress factors during inoculation and virus replication. These stress factors could trigger the covert virus within the host to manifest, resulting in the CrleGV contamination. For this production technique to succeed, levels of covert virus in the *T. leucotreta* culture and external stress triggers need to be kept at a minimum. Rapid changes in environmental conditions, sub-optimal rearing conditions for the host, an impure inoculum source and elevated levels of covert virus in the host may act as stress triggers for the homologous virus to manifest. Studies have reported the triggering effect of stresses and heterologous inoculation in the activation of homologous infections (Podgwaite & Mazzone, 1986; Hughes *et al*., 1993, 1994; Burden *et al*., 2003; Il'inykh & Ul'yanova, 2005; Murillo *et al*., 2011). In order to reduce these risks the following is suggested:

Firstly, to significantly reduce the risk of CrleGV contamination, a virus-free *T. leucotreta* culture should be used for CpGV inoculations. Observations during this study indicated that the level of covert virus manifesting overtly in the *T. leucotreta* culture increased from levels of approximately 0.2% to 1.5%. Attempts to rid a *T. leucotreta* culture of low levels of covert virus have thus far been unsuccessful with detectable levels of the virus remaining within the population (Ludewig, 2003). Singh (2002) reported that a suspected virus-free field population of *T. leucotreta* has been identified in the Sundays River Valley, Eastern Cape, South Africa. If a virus-free laboratory culture, or one containing almost undetectable levels of covert virus could be established, production of CpGV in *T. leucotreta* would carry significantly less risk of the homologous virus contaminating the end product. It was noted that during the latter part of this study, the level of covert virus (CrleGV) that existed in the laboratory culture of *T. leucotreta* increased. Although almost undetectable at the start of this study, levels of up to 1.5% loss in rearing jars were recorded early in 2014 which was a meaningful increase. Secondly it is important to manage the environmental conditions accurately as well as to ensure the purity of the inoculum. If environmental conditions are not maintained at optimal levels during inoculation or a small amount of CrleGV is present in the virus inoculum the natural resistance of the larva may be compromised, allowing the homologous isolate to become virulent. Lastly, larvae placed onto a freshly prepared inoculated diet which has a crumbly consistency would enable the larvae to penetrate the diet quickly and easily, reducing stress levels and avoiding larval interactions, thereby reducing the risk of the covert virus manifesting.

It is speculated that the virus inoculation process resulted in considerable stress on the larvae as a result of having to manually remove larvae from the rearing diet and introduce them into a new environment. It is therefore recommended that the production method be reconsidered if production of CpGV in *T. leucotreta* is the ultimate objective. Ideally, inoculation of the larvae should take place without having to handle or remove the larvae from the diet. It is suggested that larvae be reared in plastic rearing trays, containing smaller cells sufficient for 3 to 4 larvae each. A high dose of purified OBs can be injected onto the surface of the diet when the larvae reach the intended instar, thereby limiting

stresses that may have triggered the homologous virus to manifest. By controlling humidity levels during rearing ensuring that the diet is partially desiccated when the larvae reach the fourth instar life stage. The injecting of virus inoculum onto the surface could re-hydrate the diet without substantially elevating humidity levels and could possibly entice the larvae to drink the virus suspension, increasing the chances of infection.

Improving virus yields per larva should also be considered to increase profitability by reducing the number of larvae required in production. Increases in virus yield could be achieved by the incorporation of a juvenile hormone mimic into the diet before inoculation of the virus (Glen & Payne, 1984). This would increase larval size and therefore increase the potential for virus production per larval unit.

6.5.1 Quality control of virus production

At the onset of this study it was considered imperative that a quality control protocol be developed to determine the quality and quantity of CpGV produced in *T. leucotreta* due to the associated risks of producing virus in a heterologous host. *Hin*dIII analysis proved to be effective in determining the purity of the virus produced in *T. leucotreta* because DNA profiles generated by this enzyme differ between CpGV and CrleGV. Therefore, this method is recommended as the first step in quality control procedures as it proved a quick and decisive technique for identifying CrleGV contamination. However, this method only allowed for a qualitative rather than a quantitative analysis of viral products. An accurate method of evaluating the degree of contamination with CrleGV is real-time qPCR which can identify CpGV or CrleGV in a production sample and also give an indication of the amount of each virus present. The preliminary qPCR data obtained in this study is not presented although significant strides have been made towards the development of an assay for quantification of each virus in a mixed virus product. For example, preliminary data have shown that PCR amplification of genomic DNA in samples using CpGV or CrleGV *granulin* primers produces two distinct melt peaks at 91ºC and 88ºC for CpGV and CrleGV respectively, which can be used to estimate the relative quantities of each virus present. In addition, standard curve data has successfully been generated for each set of primers by

amplifying CpGV and CrleGV mixtures of known concentration. This work is currently continuing at the Nelson Mandela Metropolitan University, South Africa and will be an important part of quality control procedures if the production methodology is adopted by a commercial entity.

Bacterial contamination in the CpGV production preparations was generally acceptable. These trials were conducted a number of weeks after the production samples were produced. Even though the samples were stored at 4ºC, a build-up of bacterial contamination may have adversely affected the results. In order to reduce the risk of bacterial contamination it is recommended that 40% glycerine be added to the virus directly after a quality control sample has been taken. Glycerine acts as a microbial suppressant and would reduce the risk of elevated microbial levels in the production sample over time (Litsky *et al*., 1971; Moore, 2002; Saegeman *et al*., 2008). In this study only basic determination of bacterial contamination was conducted. With the possible outcome of the study being a commercial CpGV based product, it is important to quantify fugal contamination and identify the species of contaminants within the production sample to ensure that no unacceptable or mammalian pathogens are present. This will also be a requirement for the registration of a CpGV-SA based microbial product.

6.6 CONCLUSION

The results from this study confirm that it is possible to produce CpGV in *T. leucotreta* and support the findings of Reiser *et al*. (1993) who showed it was possible to produce CpGV in *T. leucotreta* in laboratory scale Petri dish experiments. However, this study has highlighted some of the significant risks involved in the techniques used for producing CpGV in *T. leucotreta*. Therefore, serious consideration needs to be given to establishing virus-free host material (*T. leucotreta*) for the production of CpGV, as this would negate the risk of covert virus being expressed and hence contaminating the production sample of CpGV. In order to limit the risk of CrleGV contamination, several essential points must be taken into account: (i) by reducing the level of covert virus in the *T. leucotreta* culture, the risk of CrleGV production can be lowered; (ii) stress factors during the virus inoculation

process must be kept to a minimum; (iii) a diet into which the larvae can quickly and easily penetrate to avoid larval interactions during inoculation must be available; (iv) a freshly prepared diet should be free of bacterial, fungal and viral contamination, therefore reducing the risk of a contaminant in the production sample; and (v) incorporation the virus into the diet and not relying on surface sterilisation as an inoculation method may be advantageous. The above mentioned points are aimed at ensuring the correct virus uptake by the larva as well as reducing stresses that may trigger the homologous virus, CrleGV, resulting in poor yields of CpGV in the production process.

A drawback with a number of biological products is their expense in relation to chemical alternatives. CpGV production in *T. leucotreta*, if effective, will reduce production cost, making the product more affordable to the grower. The results of this study suggest that this technology has potential. However, in order for progress to be made in developing a mass production technique for CpGV production in *T. leucotreta*, significant developments are required in reducing the amount of covert virus within the host population and diet development will be essential in limiting stress factors. A better understanding of the role that temperature and humidity fluctuations play in adding to larval stress may also help to reduce the risk of CrleGV contamination in the final product. For a commercial entity, production of CpGV in *T. leucotreta* may pose a higher risk at this point in time. Significant advances in the diet and inoculation process need to be made to warrant further investigation into this method of CpGV production.

Insect control is currently in a transition phase, with growers being compelled to move away from harsh chemical applications and towards an IPM approach to insect control. Challenges for the future include the production and formulation of effective biological control agents that can not only compete with the chemical standards in efficacy and product quality, but are also affordable. Continuation of this research and the development of a mass production technique may allow for reliable production of CpGV in *T. leucotreta*, which should significantly reduce the cost of CpGV production, resulting in CpGV-based products that can be made available to growers at an affordable price.

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