THE DEVELOPMENT AND EVALUATION OF *CRYPTOPHLEBIA LEUCOTRETA* GRANULOVIRUS (CrleGV) AS A BIOLOGICAL CONTROL AGENT FOR THE MANAGEMENT OF FALSE CODLING MOTH, *CRYPTOPHLEBIA LEUCOTRETA*, ON CITRUS

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ABSTRACT

A granulovirus isolated from *Cryptophlebia leucotreta* larvae was shown through restriction endonuclease analysis to be a novel strain (CrleGV-SA). No more than one isolate could be identified from a laboratory culture of *C. leucotreta*. However, a preliminary examination of restricted DNA profiles of isolates from different geographical regions indicated some minor differences. In surface dose bioassays on artificial diet, LC_{50} and LC_{90} values with neonate larvae were estimated to be 4.095×10^3 OBs/ml and 1.185×10^5 OBs/ml respectively. LT_{50} and LT_{90} values with neonate larvae were estimated to be 4 days 22 h and 7 days 8 h, respectively. Detached fruit (navel orange) bioassays with neonate larvae indicated that virus concentrations that are likely to be effective in the field range from 1.08×10^7 to 3.819×10^{10} OBs/ml. In surface dose bioassays with fifth instar larvae LC_{50} and LC_{90} values were estimated to be 2.678×10^7 OBs/ml and 9.118×10^9 OBs/ml respectively. LT_{50} and LT_{90} values were estimated to be 7 days 17 h and 9 days 8 h, respectively. A new artificial diet for mass rearing the host was developed. Microbial contamination of diet was significantly reduced by adding nipagin and sorbic acid to the diet and by surface sterilising *C. leucotreta* eggs with Sporekill. Almost 20 % more eggs were produced from moths reared on the new diet compared to moths reared on the old diet. A further 9 % improvement in egg production and a reduction in the labour required to produce eggs, was made with the development of a new oviposition cage attached to the moth eclosion box. Virus was mass produced in fifth instar *C. leucotreta* larvae by surface inoculating diet with the LC_{90}. When 300 individuals were placed onto inoculated diet, 56 % of them were recovered six to 11 days later as infected larvae. Mean larval equivalents was 1.158×10^{11} OBs/larva. When larvae and diet were harvested together, highest yields of virus were achieved at eight days after inoculation. Microbial contamination in semi-purified preparations of CrleGV ranged from 176211 to 433594 (OB:CFU ratio). Half-life of CrleGV in the field was estimated to be less than 1 day on the northern aspect of trees and between 3 - 6 days on the southern aspect. Original activity remaining (OAR) of the virus dropped below 50 % after 5 days on the northern aspect of trees and was still at 69 % on the southern aspect of trees after 3 weeks. In field trials, CrleGV reduced *C. leucotreta* infestation of navel oranges by up to 60 % for a period of 39 days. CrleGV in combination with augmentation of the *C. leucotreta* egg parasitoid, *Trichogrammatoidea cryptophlebiae*, reduced infestation by 70 %. The integration of CrleGV into an integrated pest management (IPM) system for the management of *C. leucotreta* on citrus is proposed.

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ABBREVIATIONS

12:Ac – dodecenyl acetate

AcMNPV – *Autographa californica* multiply enveloped nucleopolyhedrovirus

AU – absorbance units

AV – ascovirus

BmNPV – *Bombyx mori* nucleopolyhedrovirus

BSA – bovine serum albumin

BV – budded virus

CFU – colony forming unit

ClCPV – *Cryptophlebia leucotreta* cypovirus

CrleGV – *Cryptophlebia leucotreta* granulovirus

CrleGV-CV – Cape Verde isolate of *Cryptophlebia leucotreta* granulovirus

CrleGV-CV3 – strain or genotype number 3 of a Cape Verde isolate of *Cryptophlebia leucotreta* granulovirus

CrleGV-SA – South African isolate of *Cryptophlebia leucotreta* granulovirus

CpGV – *Cydia pomonella* granulovirus

CpGV-*egt*$^{-}$ – *Cydia pomonella* granulovirus with the ecdysteroid-UDP glucosyl transferase gene deleted

CPV – cypovirus

ºC – degrees Celsius

D – dilution of suspension

Da – Daltons

DAT – days after treatment

DDT – dichlorodiphenyltrichloroethane

DNA – deoxyribonucleic acid

Ds DNA – double-stranded deoxyribonucleic acid

E – east

(E)-7-12:Ac – trans-7-dodecenyl acetate

(E)-8-12:Ac – trans-8-dodecenyl acetate

EDTA – ethylene diamine tetra-acetic acid

e.g. – example

egt – ecdysteroid glucosyl transferase

EM – electonmicroscopy

EPV – entomopoxvirus

et al. – *et alia* (and others)

FAO – Food and Agricultural Organisation

FCM – false codling moth

Fig. – figure

g – gram

GV – granulovirus

h – hours

ha – hectare/s

HaNPV – *Helicoverpa armigera* nucleopolyhedrovirus

HaSNPV – *Helicoverpa armigera* singly enveloped nucleopolyhedrovirus

HCl – hydrochloric acid

Hz-1 – *Helicoverpa zea*-1

HzNPV – *Helicoverpa zea* nucleopolyhedrovirus

i.e. – *id est* (that is)

IGR – insect growth regulator

IPM – integrated pest management

kb – kilobase

kbp – kilobase pair/s

kg – kilogram/s

Kr – kilorad

kV – kilovolts

ℓ – litre

LC – lethal concentration

LC_{50} – median lethal concentration

LC_{90} – 90 % lethal concentration

$LC_{99.9}$ – 99.9 % lethal concentration

LD_{50} – median lethal dosage

LT – lethal time

LT_{50} – median lethal time

LT_{90} – 90 % lethal time

LdNPV – *Lymantria dispar* nucleopolyhedrovirus

LdMNPV – *Lymantria dispar* multiply enveloped nucleopolyhedrovirus

M – molar

min – minutes

ml – millilitre

mm – millimetre

MNPV – multiply enveloped nucleopolyhedrovirus

N – north

N – number of small squares counted

Na_2CO_3 – sodium carbonate

nm - nanometre

NOB – non-occluded virus

NPV – nucleopolyhedrovirus

OB – occlusion body

OD_{260} – optical density at 260 nm

OD_{280} – optical density at 280 nm

ODV – occlusion derived virus

OpMNPV – *Orygia pseudotsugata* multiply enveloped nucleopolyhedrovirus

PbGV – *Pieris brassicae* granulovirus

PDA – potato dextrose agar

PDV – polyhedron derived virus

PE – production efficiency

pH – negative logarithm of hydrogen ion concentration

PR – productivity ratio

PrGV – *Pieris rapae* granulovirus

PV – polydnavirus

r – rad

RNA – ribonucleic acid

Rpm – revolutions per minute

RU – Rhodes University

S – south

SADCC – Southern African Development Coordination Conference

SDS – sodium dodecyl sulphate

SE – south east

SE – standard error

SeMNPV – *Spodoptera exempta* multiply enveloped nucleopolyhedrovirus

SIT – sterile insect technique

SlNPV – *Spodoptera littoralis* nucleopolyhedrovirus

SNPV – singly enveloped nucleopolyhedrovirus

sp. – species

SSW – south south west

SW – south west

TAE – tris acetate EDTA

TBE – tris borate EDTA

TE – tris-EDTA

Tris – tris (hydroxymethyl) amino methane

Tris-HCl – tris-hydrochloric acid

UV – ultra-violet

V – volts

V – volume (in millilitres)

WA – water agar

X – number of OBs counted

XcGV – *Xestia c-nigrum* granulovirus

(Z)-8-12:Ac – cis-8-dodecenyl acetate

µl – microlitre

µm – micron

º – degrees

' – minutes

% – percent

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1

PROJECT BACKGROUND AND PROPOSAL

1.1 INTRODUCTION

The false codling moth, *Cryptophlebia leucotreta*, is regarded as the most important insect pest on citrus in South Africa. A full literature survey of the insect, its biology, its pest status, and all control measures considered, has been conducted. So too, has a review on baculoviruses in general and microbial control with granuloviruses in particular, been conducted. Within the context of this information, and the pest management and marketing practicalities currently experienced by the southern African citrus industry, the objectives of this research project have been proposed.

1.2 THE HOST: *CRYPTOPHLEBIA LEUCOTRETA*

1.2.1 Taxonomy

The false codling moth, *Cryptophlebia leucotreta* (Lepidoptera: Olethreutidae), commonly referred to as FCM, was first recorded by Fuller (1901) as a pest of citrus in Natal. Fuller identified it as *Carpocapsa* sp., naming it the Natal codling moth. Simpson (1906) and Howard (1909), shortly thereafter, reported an “orange codling moth” from the old Transvaal province. Meyrick (1912) was the first to taxonomically describe the moth as *Argyroploce leucotreta* (Eucosmidae, Olethreutidae), and referred to it as the false codling moth. Clarke (1958) transferred it to the genus *Cryptophlebia*, where it has remained.

1.2.2 Distribution

C. leucotreta is endemic to Africa south of the Sahara. It has been reported from Ethiopia, Congo (Bredo, 1933), Nigeria (Thompson, 1946), Somalia (Thompson, 1946), Kenya (Gunn, 1921), Ivory Coast (Pearson, 1958), Uganda (Hargreaves, 1922), Malawi (Sweeney, 1962), Mozambique (Stofberg, 1954), Zimbabwe (Jack, 1916), Swaziland (Catling, 1969) and South Africa (Hepburn, 1947). *C. leucotreta* has also been recorded off the coast of Africa on the Cape Verde Islands (Mück, 1985), Mauritius (Meyrick, 1930), Madagascar, Reunion and St. Helena (CIBC, 1984). Most recently, the moth has been reported as an agricultural pest in Israel, probably due to an accidental introduction (Wysoki, 1986).

1.2.3 Biology and life history

Moths lay their eggs singly on the rind of the fruit of the host plant although large numbers of eggs can be laid on a single fruit. The egg is flat, oval and translucent, somewhat like an inverted saucer. It has a diameter of approximately 1 mm. The fresh egg is white, turning reddish after a few days, and then darker as the embryo develops (Plate 1.1). The lower developmental threshold of the egg is 11.9°C, and the temperature sum (days x degrees above the threshold) is 69.3 (Daiber, 1979a). This means that usually within 6 - 12 days, the larva hatches from the egg (Hepburn, 1947).

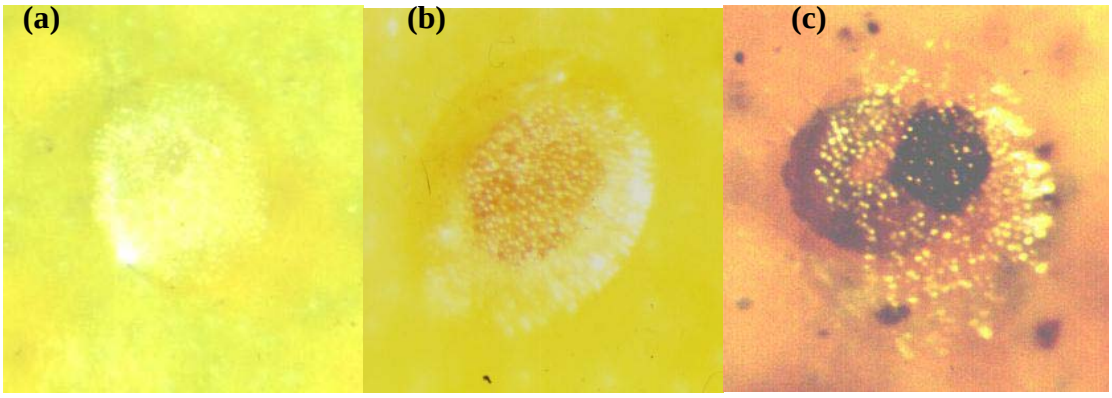

Plate 1.1 *C. leucotreta* egg, newly laid **(a)**, red coloration showing at about two days old **(b)**, neonate larva fully developed and ready to hatch **(c)**.

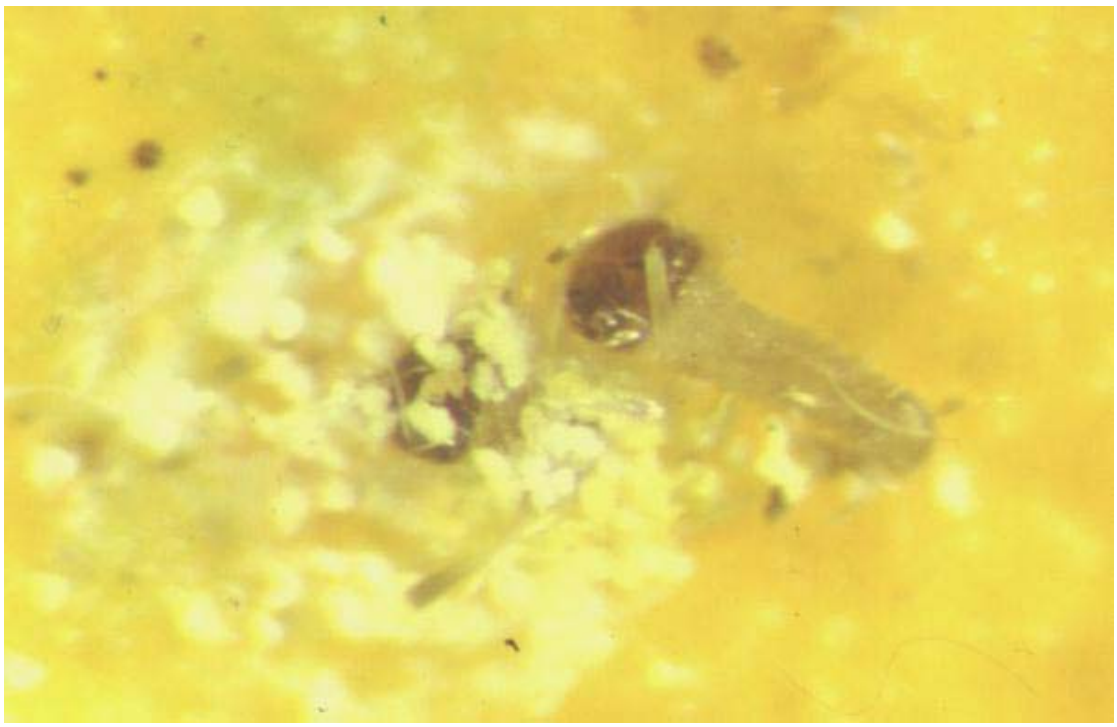

Plate 1.2 Neonate *C. leucotreta* larva boring into a citrus fruit.

Plate 1.3 Fifth instar *C. leucotreta* larva having developed inside a navel orange fruit. Note the granular black frass.

The neonate larva is white with a black head capsule and measures about 1.4 mm in length (Stofberg, 1948) (Plate 1.2). Within a short period of time the larva bores into the host fruit. Stofberg (1939) reported four larval instars, however Daiber (1979b) and Schwartz (1981) agreed that the larva has five instars. With age, the body becomes a characteristic pinkish red colour (Plate 1.3). The mature larva is 15 - 20 mm long. Larval development in the field is completed in 25 - 67 days depending on the season (Stofberg, 1954) and on food quality (Daiber, 1979b). On an artificial food source in the laboratory, larval development took 15 to 108 days, depending on temperature.

The fifth instar larva exits the fruit and drops to the ground. Often the fruit drops to the ground before the larva exits. The larva spins a cocoon from a silky body substance and soil particles (Daiber, 1979c). At this time the larva becomes a cream-coloured prepupa. It pupates in the cocoon (Plate 1.4) in loose soil, beneath surface debris, or in cracks in the soil. The pupal stage is completed within 21 - 80 days in the field, depending on the season of the year (Daiber, 1979c).

Plate 1.4 *C. leucotreta* pupae.

The adult is an inconspicuous and fairly non-descript moth (Plate 1.5). It has mottled brown gray forewings and paler more evenly coloured hind wings, which are fringed. Wingspan is 16 - 20 mm with males slightly smaller than females. Sex ratio of field populations appears to be close to 1:1. Females mate shortly after emergence and experience a pre-oviposition period of 5 - 6 days in the field (Newton, 1998). Daiber (1980) found an average fecundity of 460 eggs at a constant 25°C, but this is well above the average found by other researchers (Hepburn, 1947; Stofberg, 1939). Egg laying appears to decline after about five days under laboratory conditions.

Plate 1.5 *C. leucotreta* adult.

Total developmental period is therefore approximately 1½ - 2 months in summer and 2½ - 4 months in winter. There are five to six overlapping generations per year (Newton, 1998). *C. leucotreta* does not experience a winter diapause (Angelini & Labonne, 1970; Reed, 1974).

1.2.4 Economic importance

1.2.4.1 Host range

C. leucotreta has a fairly broad host range, being recorded from 23 species of indigenous trees and shrubs. It has effectively adopted a number of cultivated crops as hosts. These include walnuts, almonds (Annecke & Moran, 1982), macadamias (La Croix & Thindwa, 1986), cotton (Reed, 1974), maize (Schulthess *et al*., 1991), tea seeds, olives (Annecke & Moran, 1982), avocados (Erichsen & Schoeman, 1992), litchis (Newton & Crause, 1990), a few deciduous fruit species (Daiber, 1978) and citrus. In South Africa it is a major pest on macadamias, avocados, litchis and especially citrus. Acorns are also attacked (Kelly, 1914), and therefore ornamental oak trees can provide a source of inoculum for agricultural crops.

All citrus cultivars are susceptible to attack, with the exception of lemons and limes. Navels appear to be more susceptible to attack than other cultivars (Georgala, 1968).

Even within the cultivar, greater numbers of eggs were laid on certain selections than on others (Newton, 1990b). Fewer eggs were laid on Bahianinha navels than on any other selection tested (Newton, 1990b).

Any injury to a fruit can attract *C. leucotreta* to oviposit on the fruit (Newton, 1989b). *Alternaria citri* (fungal) infection, commonly known as navel end rot, or mealybug infestation of fruit could also be attractive to *C. leucotreta*. These responses may reflect a selective advantage to larvae, which can find an easier point of entry into the albedo of injured fruit (Newton, 1989b).

1.2.4.2 Pest status on citrus

C. leucotreta can be considered as one of the five major insect pests of citrus in southern Africa. There are three main reasons for its extreme importance:

1. Its infestation in the fruit causes the fruit to drop and can therefore result in a reduction in yield (Plate 1.6).
2. If infestation occurs shortly before fruit is harvested, its presence may not be detected before fruit is packed and shipped, resulting in post-harvest decay of fruit.
3. As it is endemic to sub-Saharan Africa it is considered by many of South Africa's important export markets to be a phytosanitary pest. This means that markets outside of Africa may reject entire consignments of fruit, at great loss of income to South Africa, if even the lowest level of the pest is detected in fruit.

The third factor is by far the most important. Even in situations where *C. leucotreta* infestation is at a very low level i.e. there is negligible pre-harvest fruit loss, large quantities of fruit can be rejected by foreign inspectors at the port of departure.

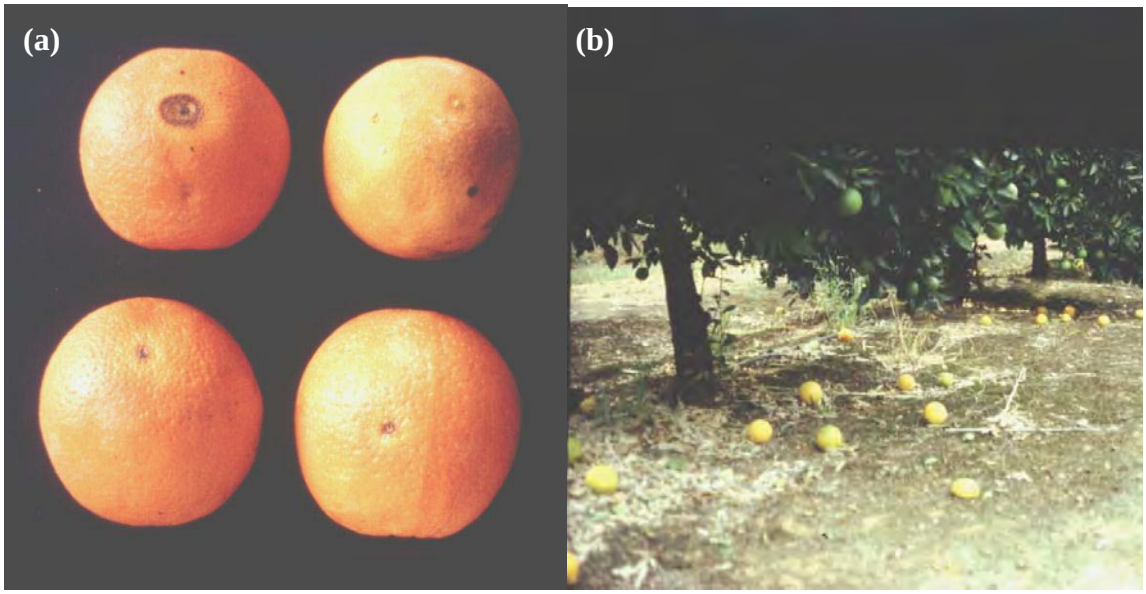

Plate 1.6 *C. leucotreta* larval entrance marks on navel oranges **(a)** and navel orange fruit drop as a result of *C. leucotreta* infestation **(b)**.

Fruit can be infested as early as November when they are no larger than 15 - 20 mm in diameter (Stofberg, 1954). However fruit drop through infestation usually reaches a peak from around February to May in the Western Cape, and December to March in the Eastern Cape. Crop loss is usually reported to be less than 2 % (Smit, 1964) but can be as high as 50 % (Gunn, 1921). In the Western Cape *C. leucotreta* has been the major cause of navel orange and soft citrus fruit drop since 1977 (Schwartz *et al.*, 1982). However, during an average season in the Eastern Cape, it will rate as the third most important factor causing fruit drop after *A. citri* navel end rot and navel end splitting (Moore, 1997).

South Africa aims to export the bulk of its citrus production. Annually, up to 60 million cartons (Shaun Brown, Capespan, personal communication) of citrus fruit are exported. This can generate an annual income of approximately R1.5 billion in foreign currency for South Africa. *C. leucotreta* annually causes a loss estimated to be in excess of R100 million, to the South African citrus industry (extracted from: Hofmeyr & Hofmeyr, 1991; 1994; Moore & Fourie, 1999; Moore & Richards, 2000). These losses are incurred through pre-harvest loss of fruit (estimated R77 million annually), post-harvest decay of fruit, and market rejection of fruit due to pest presence.

1.2.5 Pre-harvest control measures

1.2.5.1 Monitoring

In the 1930s Roux was the first to make practical application of virgin female moth attraction to male moths for monitoring activity in a citrus orchard (Myburg, 1948; Matthew, 1973). Four decades later Schwartz (1972) initiated a research programme to investigate the value of this system for determining moth abundance and seasonal activity in an orchard. The system consisted of a hollow plastic pipe (220 x 105 mm) in which a small gauze cage, containing virgin females, was hung. The floor of the pipe was smeared with adhesive. Traps were then hung in trees both bordering and within the orchards. Schwartz (1972; 1973; 1975) showed that the traps were strongly attractive to male moths and speculated that they held potential. However, he omitted to correlate his catches with egg laying, fruit infestation or fruit drop, which left their usefulness in question.

Simultaneously the sex pheromone of the female *C. leucotreta* moth was isolated and identified as trans-7-dodecenyl acetate [(E)-7-12:Ac] (Read & Warren, 1968; Read *et al*., 1974), and subsequently synthesised by Henderson & Warren (1970). Trapping experiments with synthetic sex attractant [(E)-7-12:Ac] in citrus orchards were initiated by Hofmeyr in 1969 (Hofmeyr *et al.*, 1991) and later also conducted by Daiber (1978). This formulation failed in field tests (Newton & Mastro, 1989). Persoons *et al.* (1976; 1977) subsequently showed that this was because of incorrect identification of the pheromone composition, and revealed two isomers to be present: trans-8-dodecenyl acetate [(E)-8-12:Ac] and cis-8-dodecenyl acetate [(Z)-8-12:Ac]. A third component, dodecyl acetate (12:Ac), was later discovered (Angelini *et al.*, 1981). La Croix *et al.* (1985) found that a 1:1 mixture of (E)-8-12:Ac and (Z)-8-12:Ac was adequate for field capture of *C. leucotreta*. Hofmeyr & Calitz (1991), however, found that a mixture of the two isomers plus (E)-7-12:Ac was more attractive to male moths than a two-component mixture, but that none of the evaluated synthetic attractants were competitive with virgin females. Further trials with improved pheromone dispensers demonstrated that a synthetic attractant could be as attractive or more attractive than females (Hofmeyr &

Hofmeyr, 1991). This lead to the registration (Reg. no. L4877 Act 36/1947) of the synthetic attractant and commercialisation with the trap, known as the Lorelei Trapping System.

Such a monitoring system has been regarded as essential for the development of a practical programme for any means of effective control of the pest (Schwartz, 1972). The development of effective chemical control options emphasised the need for such a practical method for predicting potentially harmful *C. leucotreta* infestations.

1.2.5.2 Chemical control

No insecticides were registered for use against *C. leucotreta* on citrus until the early 1980s. However, the first chemical trials for *C. leucotreta* control were conducted by Gunn in about 1926, but results were not satisfactory (Hepburn & Bishop, 1954). More than a quarter of a century later DDT was found to reduce fruit infestation by around two thirds or more (Hepburn, 1947; 1948; 1949; Hepburn & Bishop, 1952; Myburg, 1948). Gammexane, fixed nicotine (Myburg, 1948) and parathion (Thiophos) (Hepburn, 1948; 1949) were also found to reduce infestation, but not to the same extent as did DDT. In later trials parathion reduced infestation by 66 - 75 %, and was considered a more favourable option than was DDT as it was not as detrimental to beneficial insects (Hepburn & Bishop, 1952; 1954). However, despite all this work, none of these products were registered for use against *C. leucotreta* on citrus. Orchards, which were routinely or incidentally sprayed with organophosphates and oils, experienced egg deposition two months later than the unsprayed orchards (Schwartz, 1975).

In laboratory trials, petroleum oils were shown to be effective *C. leucotreta* ovicides at half the concentration at which they were required to give commercial control of red scale (Myburg, 1948). However, this was not a practical option, as applications at the ideal timing for *C. leucotreta* control would have been likely to retard fruit colour development.

In field trials triazophos, a product registered for the control of citrus thrips, *Scirtothrips aurantii*, reduced *C. leucotreta* infestation of navel oranges by around 66 % (Schwartz & Weideman, 1976). In trials on macadamia nuts, prothiophos, endosulfan and cypermethrin all reduced *C. leucotreta* infestation, although only cypermethrin caused a significant (90 %) reduction (de Villiers & Wolmarans, 1980).

In laboratory trials Hofmeyr (1983a) demonstrated that synthetic pyrethroids were effective as ovicides, larvicides and oviposition inhibiters. Of six pyrethroids tested, cypermethrin proved to be the most effective. In one trial, field weathered residues of cypermethrin remained effective in preventing fruit damage for up to 143 days, after artificial infestation with eggs; 100 % of untreated fruit were damaged. In field trials two synthetic pyrethroids, cypermethrin and deltamethrin, applied two to three months before harvest, reduced fruit drop by an average of 90 % (Hofmeyr, 1983b). However, because of the potentially disruptive effect that these products would have on natural enemies of other important pests, they were never registered for control of *C. leucotreta* on citrus (Hendrik Hofmeyr, personal communication).

Two insect growth regulators (IGRs), triflumuron and teflubenzuron (benzoylated ureas), both chitin synthesis inhibitors, had little or no direct effect on *C. leucotreta* adults and larvae in laboratory trials (Hofmeyr, 1984). However, egg development was suppressed if eggs were laid on residue, but not if sprays were applied after egg laying. Residues of triflumuron, field weathered for 75 days, caused up to 85.4 % egg mortality. Triflumuron was more effective than was teflubenzuron. In field trials, *C. leucotreta*-induced fruit drop from navel orange trees was greatly reduced with a single application, either in February or in March (Hofmeyr, 1984). Newton (1987) conducted field trials in which he showed the same two IGRs to work marginally better than did the two pyrethroids, cypermethrin and decamethrin. Triflumuron reduced fruit loss by up to 86.4 %. These IGRs were the first products to be registered for the control of *C. leucotreta* on citrus.

Currently four products, Alsytin (triflumuron), Nomolt (triflubenzuron), Penncap-M (micro-encapsulated methyl parathion) and Meothrin (fenpropathrin) are registered for the control of *C. leucotreta* on citrus. Recently, *C. leucotreta* developed resistance to the

most effective of these products, Alsystin, in the Western Cape (Hofmeyr & Pringle, 1998), and possibly also in Mpumalanga. Cross-resistance has resulted in decreased efficacy of Nomolt too. Apart from the resistance factor, none of these products are entirely compatible with an integrated pest management (IPM) programme. Alsystin has been shown to be detrimental to *Trichogrammatoidea cryptophlebiae* (a hymenopteran parasitoid) (Hattingh & Tate, 1997), the most important natural enemy of *C. leucotreta*. The use of Alsystin therefore clashes with the *T. cryptophlebiae* augmentative release programmes which are currently being conducted mainly in the Western Cape and Eastern Cape Provinces (see section 1.2.5.5). Another problem is that IGR residues are not acceptable on fruit exported to the USA.

The use of Penncap-M is not practical as the pre-harvest interval exceeds the effective residual period of the product (Hendrik Hofmeyr, personal communication). The product is also extremely detrimental to natural enemies (Ware *et al.*, 1998; 1999), and therefore prone to causing secondary pest outbreaks.

Meothrin was registered in 2000 as an urgent measure, in response to the development of resistance to the IGRs. However, it is only recommended for use no earlier than five weeks before harvest. This is in an attempt to avoid secondary pest repercussions from the use of a typically broad spectrum pyrethroid. Despite this precaution, several reports of severe secondary pest outbreaks have been received (Hadlow, unpublished workshop minutes, 1999).

1.2.5.3 Cultural Control

Up until the 1980s the only method recommended for controlling *C. leucotreta* in citrus orchards was through sanitation. This meant that all infested fruit, both on the trees and on the ground, were to be collected regularly and destroyed (Hepburn, 1947). Weekly cleaning of orchards was recommended as a routine practice (Hepburn & Bishop, 1954). In this way a potential population of moths was thought to be effectively reduced. Stofberg (1954) claimed that the effectiveness of such a practice was unknown, until he conducted a series of trials from 1939 to 1953. However, similar trials were previously

conducted by Ullyett (1939), from which he concluded that weekly orchard sanitation was successful in reducing the larval population in the crop, after the progeny of the first influx of moths had been destroyed. He also showed that orchard sanitation unfortunately had an adverse effect on the level of larval parasitoids. Stofberg (1954) found that a programme of regular sanitation could save between 24 and 60 fruit per tree from *C. leucotreta* infestation.

December was originally recommended as the time of year to begin with such a sanitation programme (Georgala, 1969), but Schwartz (1974) found this to be far too late to achieve the best results. Higher numbers of pupae were recovered from orchards from late October to mid December than at any time of the season thereafter.

1.2.5.4 Natural enemies

A total of 25 natural enemies of *C. leucotreta* have been reported in the literature. This is, if one considers certain of the listings to be synonymous e.g. *Trichogramma luteum* (Ullyett, 1939), *Trichogrammoidea luteum* (Schwartz, 1977) and *Trichogrammatoidea cryptophlebiae* (Catling & Aschenborn, 1974). It is possible that the *Trichogramma* sp. recorded by Reed (1974) could also be synonymous with these. Sixteen of these species have been recorded from citrus. Of these 16 species, 12 are recorded to occur in South Africa. Of these 12 species, five are hymenopteran parasitoids, two are dipteran parasitoids (Table 1.1), two are insect predators, two are fungal entomopathogens and one a baculovirus (Table 1.2).

Ullyett (1939) was the first to investigate the natural enemies of *C. leucotreta* on citrus in South Africa. He recorded *T. luteum* to occur in high numbers at certain times of the season but believed that its value was diminished by the fact that only one larva need survive to enter and hence destroy each fruit. Ullyett (1939) suggested the movement of parasitoids from one region in South Africa to another, where they did not previously occur.

Table 1.1. Parasitoids of *C. leucotreta*.

Natural enemy		Host stage attacked	Crop	Comments	Country	Reference
Group	**Species**					
Hymenopteran parasitoid	*Trichogrammatoidea cryptophlebiae*	egg	citrus	dominant parasitoid	RSA	Catling & Aschenborn (1974)
	Trichogramma sp.	egg	cotton	>45 % parasitism	Uganda	Reed (1974)
	Chelonus curvimaculatus	egg-larval	citrus	unreliable	RSA	Searle (1964)
	Chelonus sp.	egg-larval	cotton	rare - < 15 % parasitism	Zaire, Nigeria	Bredo (1933), Pomeroy (1925)
	Apophua leucotretae	larva	citrus		Zimbabwe	Newton (1998)
	Agathis bishopi	larva	citrus		RSA	Ullyett (1939)
	Apanteles leucotretae	larva	citrus		Zimbabwe	Ullyett (1939)
	Agathis sp.	larva	citrus	common	Zimbabwe	Ullyett (1939)
	Oxycoryphae edax	larva	citrus		RSA	Newton (1998)
	Phanerotoma curvicarinata	larva	citrus		RSA	Ullyett (1939)
	Glypta leucotretae	larva	citrus	common	Zimbabwe	Ford (1934)
	Elasmus johnstoni	larva	cotton		Uganda	Le Pelley (1959)
	Ascogaster sp.	larva	cotton		Uganda	Reed (1974)
	Apanteles typhon	larva	cotton		Uganda	Reed (1974)
Tachinid fly	*Mintha* sp.	larval-pupal	citrus		RSA	Newton (1998)
	Silba virescens	larval-pupal	citrus		RSA	Moore (unpublished)
	Actia cuthbertsoni	larva	cotton		Uganda	Reed (1974)

Table 1.2. Predators and pathogens of *C. leucotreta*.

Natural enemy		Host stage attacked	Crop	Comments	Country	Reference
Group	**Species**					
Bug	*Orius* sp.	egg	citrus		RSA	Newton (1998)
	Orius insiduosus	egg	cotton		Uganda	Nyiira (1970)
	Rhynocorus albopunctatus	larva	citrus, cotton		RSA	Nyiira (1970)
Mite	*Pediculoides* sp.	egg,larva	cotton		Uganda	Nyiira (1970)
Fungus	*Aspergillus alliceus*	larva	citrus	rare	RSA	Moore *et al.* (1999)
	Beauveria bassiana	larva & pupa	citrus	too humidity-dependent to be effective	RSA	Begemann (1989)
Virus	ClCPV*	larva	laboratory			CIBC (1984)
	CrleGV	larva	laboratory, citrus		RSA	Schwartz (1981), Moore *et al.* (2000c)

* ClCPV = *C. leucotreta* cypovirus.
* CrleGV = *C. leucotreta* granulovirus.

1.2.5.5 Biological control

Ripley *et al.* (1939) were the first to consider the mass rearing and releasing of natural enemies for the control of *C. leucotreta*. This required the effective mass rearing of the host, for which Ripley *et al.* (1939) suggested a method. Theron (1948) elaborated on the method. This idea of mass rearing remained dormant for a couple of decades, until revived by Schwartz (1971; 1972), who suggested certain improvements to the previously devised method. Schwartz (1977; 1980) and Schwartz *et al.* (1982) evaluated the feasibility of mass rearing and releasing of the *C. leucotreta* egg parasitoid, *Trichogrammatoidea luteum* (previously known as *Trichogramma luteum*). Newton (1988; 1990a) and Newton & Odendaal (1990) concluded that the ideal approach to the biological control of *C. leucotreta* was to begin with high volume inundative releases early in the season, followed by pesticide applications if necessary. Newton & Odendaal (1990) showed that inundative releases of *Trichogrammatoidea cryptophlebiae* (previously known as *T. luteum*) could reduce larval population size by almost 60 % during a second consecutive release season. However, this was a result of weekly releases of an average of more than 3 million parasitoids per hectare for an average of 31 weeks. Such an intensive release programme is not likely to be economically or practically viable. However, years later Moore and Fourie (1999), Moore *et al.* (2000a) and Moore & Richards (2000; unpublished data) demonstrated that augmentative releases of the parasitoid on a monthly basis, at between 25000 and 250000 parasitoids per hectare per season, could reduce *C. leucotreta* infestation by up to 60 %.

Currently, mass rearing and releasing of *T. cryptophlebiae* is being conducted by Cederberg Insectary (previously by Goedehoop Citrus Company) in Citrusdal in the Western Cape Province (Visagie, 1997; Stephan Honiball, personal communication) and, until recently, also at Zebediela Estate in Northern Province. In the Western Cape, the parasitoid is being released monthly for four months, at a total of 100000 parasitoids per hectare.

1.2.5.6 Sterile male release technique

Myburgh (1963a), Schwartz (1979) and Du Toit (1981) investigated the lethal and sterilising effects of cobalt-60 gamma rays on *C. leucotreta*. Considerably lower exposure doses of gamma rays than those required to induce mortality, were required to achieve sterility. A dose of about 30 Kr almost completely sterilised the ova, whereas the males were not as sensitive. The sterilisation dose for sperm appeared to be between 60 and 70 Kr. Myburgh (1963a) discussed the feasibility of pest control by over flooding natural populations with insects rendered sterile by gamma irradiation. He believed that the method did not hold much promise of success due to the expansive areas in which the pest occurs. If *C. leucotreta* became established elsewhere in pockets of infestation, sterile male releases could be of value for eradication or curtailment of such outbreaks.

1.2.5.7 Mating disruption

Mating disruption has been used with great success in the deciduous fruit industry in the Western Cape Province (Blomefield & Kleinhans, 1999). During 1999, the first mating disruption product for *C. leucotreta* control (developed by BASF) was registered for use on citrus. The principle behind the system is that a high density of synthetic female moth pheromone, distributed over a fairly extensive homogenous production area, will confuse male moths to the extent that they are unable to locate female moths, and mating will be prohibited. Growers have reported limited success on citrus thus far (e.g. Ballie Wahl, Isak Bruwer, personal communication).

1.2.6 Post-harvest Control

1.2.6.1 Cold sterilisation

The only work recorded on the post-harvest cold sterilisation of *C. leucotreta* was conducted by Myburgh (1963b; 1965). He concluded that eggs were very susceptible to low temperature, larvae relatively resistant and pupae slightly more resistant (Myburgh, 1965). He showed that after 21 days exposure to a temperature of -0.5°C, no larvae

survived in artificial medium. Consequently, citrus fruit from South Africa, destined for certain markets, is sterilized in this manner while being shipped. This is however, a very expensive practice.

1.2.6.2 Gamma radiation

Myburgh (1963a) found that extremely high doses of gamma rays, ranging from nearly 200 to 400 Kr, were required to inhibit development within each respective stage of metamorphosis. Exposures between 10 and 120 Kr, at a radiation intensity of 80 r per minute, inhibited development of moths from immature stages. Gamma radiation has not been used commercially in the citrus industry.

1.3 THE PATHOGEN

1.3.1 Granuloviruses

Paillot (1926) was the first to describe a granulovirus infection, in the larva of the European cabbageworm, *Pieris brassicae*. The rod-shaped viral particle was detected with the electron microscope by Bergold (1948) in capsules obtained from infected larvae of the pine shoot roller, *Christoneura murinana*, and by Steinhaus (1949) in capsules from the variegated cutworm, *Peridroma saucia*.

1.3.1.1 Classification

More than 20 groups of viruses are known to be insect pathogens (van Regenmortel *et al.*, 2000). They have been placed in 13 viral families, but many remain unclassified (Table 1.3). Some are assigned to families of mainly vertebrate viruses, but others are in families with viruses specific for insects and related invertebrates. Viruses in the three families, Baculoviridae, Poxviridae, and Reoviridae, are unique because of the presence of occlusion bodies (OBs) in which virions, at a certain stage in their development, are

Table 1.3 Classification of virus families containing insect pathogenic viruses[a] (Hunter-Fujita *et al.*, 1998).

Family	Genera occurring in insects	OBs[b]	Examples of virus	Examples of genera occurring in vertebrates	Examples of genera occurring in plants
DNA viruses					
Ascoviridae	*Ascovirus*	-	*Trichoplusia ni* AV	None	None
Baculoviridae	*Nucleopolyhedrovirus* (NPV)	+	*Autographia californica* MNPV	None	None
	Granulovirus (GV)	+	*Cydia pomonella* GV	None	None
Iridoviridae	*Iridovirus*	-	*Chilo* iridescent virus	*Ranavirus*	None
	Chloriridovirus	-	Mosquito iridescent virus	*Lymphocystivirus*	
Parvoviridae	*Densovirus*	-	*Galleria mellonella* densovirus	*Parvovirus* *Dependovirus*	None
Polydnaviridae	*Ichnovirus*	-	*Campoletis sonorensis* PV	None	None
	Bracovirus	-	*Cotesia melananoscela* PV		
Poxviridae	*Entomopoxvirus*	+	*Amsacta moorei* EPV	*Orthopoxvirus* *Capripoxvirus* *Avipoxvirus* *Leporipoxvirus* *Parapoxvirus* *Suipoxvirus*	None
Unclassified			*Oryctes* virus Hz-1 virus Bee filamentous virus Tsetse virus Narcissus bulb fly virus		
RNA viruses					
Birnaviridae	*Birnavirus*	-	*Drosophila* X virus	*Birnavirus*	
Calciviridae		-	Chronic stunt virus	*Calicivirus*	None
Nodaviridae	*Nodavirus*	-	Black beetle virus	None	None
Picornaviridae		-	Cricket paralysis virus	*Enterovirus*	None
Reoviridae	*Cypovirus* (CPV)	+	*Bombyx mori* CPV	*Reovirus* *Orbivirus* *Rotavirus*	Phytoreovirus Fijivirus
Rhabdoviridae		-	Sigma virus	*Vessiculovirus* *Lyssavirus*	Lettuce necrotic yellows Potato yellow dwarf
Tetraviridae	*Tetravirus*	-	*Nudarelia* β virus	None	None
Unclassified		-	Various bee paralysis viruses Various *Drosophila* viruses		

[a] A large number of other vertebrate and plant viruses also occur in insects and may replicate in certain insect tissues. In general, the insect is considered to be simply a vector for transmission of these viruses although it may be that some of these viruses evolved first in the insect host. These viruses do not have significant pathogenicity for insects and therefore are not included here.
[b] Absent -, present +.

occluded at random. The OBs contribute to the stability and persistence of the viruses in the environment. The families of DNA viruses infecting insects, are Parvoviridae, Iridoviridae, Polydnaviridae, Baculoviridae, Poxviridae and Ascoviridae (Hunter-Fujita *et al.*, 1998; van Regenmortel *et al.*, 2000) (Table 1.3).

The Baculoviruses are a diverse group of large occluded DNA viruses, pathogenic predominantly for holometabolous insects (Blissard *et al.*, 1990). Baculovirus infections have been reported in over 600 species of insects (Martignoni & Iwai, 1986). Baculoviruses are primarily pathogens of insects of the order Lepidoptera, but they also infect Hymenoptera, Diptera, Coleoptera, and Trichoptera.

Taxonomically, baculoviruses used to be divided into three subgroups: A, B, and C (Blissard & Rohrmann, 1990). Subgroup A was the nuclear polyhedrosis viruses (NPVs); subgroup B was the granulosis viruses (GVs); subgroup C was the nonoccluded baculoviruses (NOBs). Francki *et al.* (1991) placed the first two subgroups in the subfamily Eubaculovirinae and the third subgroup in the subfamily Nudibaculovirinae. However, the family Baculoviridae is now considered to have only two genera: *Nucleopolyhedrovirus* (previously *Nuclear polyhedrosis virus*) (NPVs) and *Granulovirus* (previously *Granulosis virus*) (GVs) (van Regenmortel *et al.*, 2000). Previously NPVs were subdivided into multiply enveloped NPVs (MNPV) and singly enveloped NPVs (SNPV). However, despite the ongoing differentiation between MNPVs and SNPVs, this subdivision no longer formally exists, as it appears to have no taxonomic relevance (Hunter-Fujita *et al.*, 1998).

1.3.1.2 Structure

As previously mentioned, baculoviruses are protected by a proteinaceous OB. The NPVs produce large polyhedron-shaped structures called polyhedra (typically ranging from 1 - 5 μm in diameter), which contain many virions, while the GVs have smaller OBs called granules (averaging 150 nm in diameter by 400 - 600 nm in length), which normally contain a single virion (Funk *et al.*, 1997; Hunter-Fujita *et al.*, 1998) (Fig. 1.1). Both the

polyhedra and the granules consist of a protein matrix, forming a crystalline lattice, known as polyhedrin (in NPVs) or granulin (in GVs). Granules of GVs are usually ovoid or ovocylindrical, but the shapes may vary greatly. The molecular weights of granulin range from 25000 to 30000 (Tweeten *et al.*, 1981). All baculoviruses have virions of the same basic structure: an enveloped, rod-shaped nucleocapsid in which an amorphous but definite layer exists between nucleocapsid and the envelope (Frederici, 1986). The virion is also known as the nucleoprotein or DNA core, the capsid, the nucleocapsid, and the envelope (Federici, 1986) (Fig. 1.1). The virions are structurally complex and contain at least 10 - 25 polypeptides with molecular weights ranging from 10 - 160 x 10^3 Daltons (Da) (Matthews, 1982). NPVs possess several virions per occlusion, whereas GVs have only one. Within the virion is a single molecule of circular, supercoiled, double-stranded DNA of 80 to 220 kilobase pairs (kbp) (Miller, 1988).

1.3.1.3 Identification of baculoviruses

Although baculoviruses are usually named after the host species from which they were isolated, this is not considered to provide a satisfactory form of identification (Hunter-Fujita *et al.*, 1998). In several instances, quite different baculoviruses have been isolated from the same insect species while a single virus, or group of closely related strains, can occur in more than one insect species (Payne, 1986). However, in the absence of a better system of nomenclature, baculoviruses are generally still named after the host from which they were originally isolated. This is sometimes followed by a suffix, abbreviating the country in which the virus was isolated, and possibly even a further numerical suffix, if more than one isolate or genotype has been isolated. For example, a genotype of a Cape Verde isolate of *C. leucotreta* granulovirus has been referred to as CrleGV-CV3 (Jehle *et al.*, 1992a).

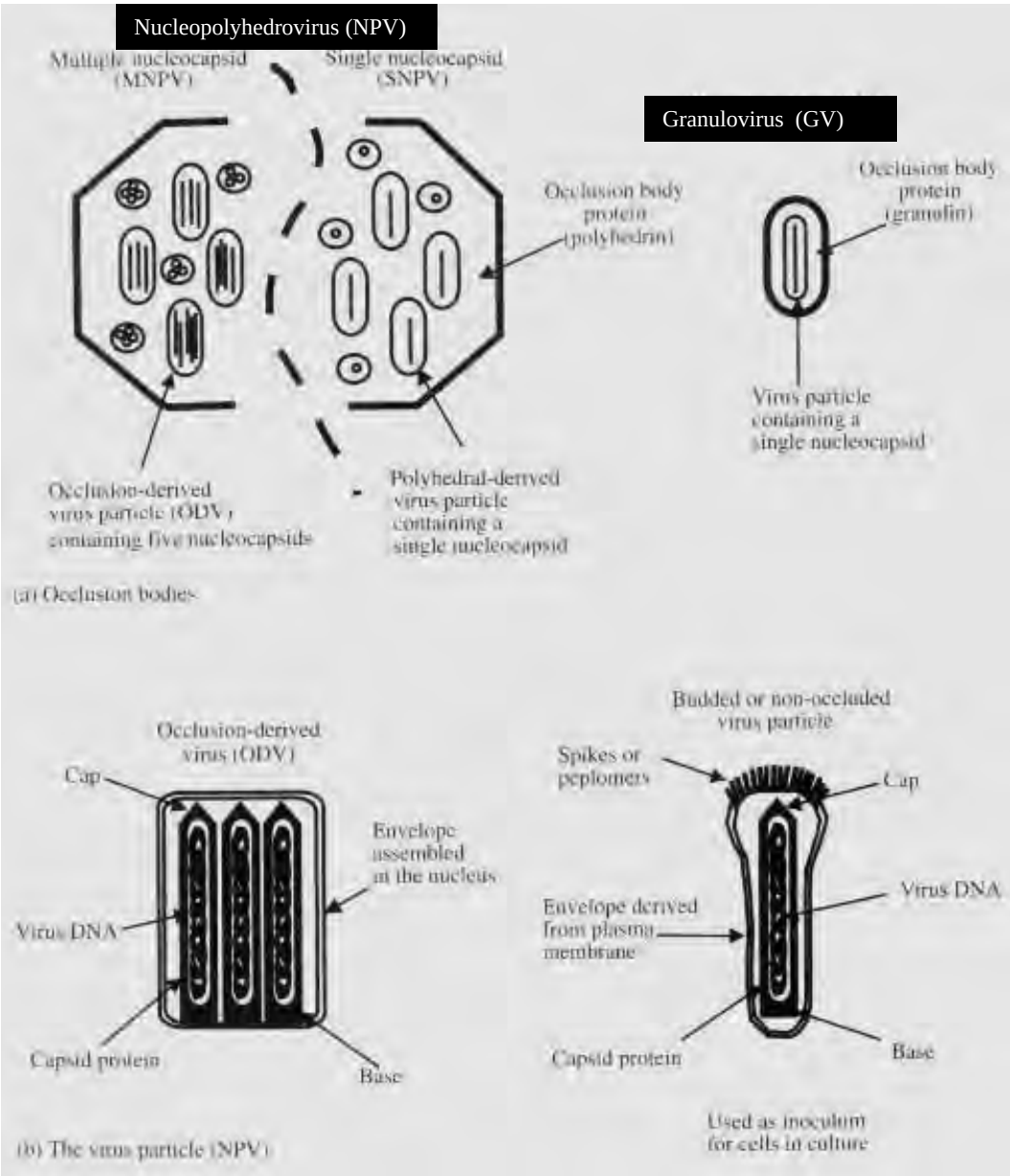

Figure 1.1 Diagrammatic representation of the morphology of members of the Baculoviridae family of insect pathogenic viruses (Hunter-Fujita *et al.*, 1998).

1.3.1.4 Host range

GVs have only been isolated from lepidopteran larvae (Crook, 1991). There is one unconfirmed case of a hymenopteran, family Pamphiliidae, being infected (Marignoni & Iwai, 1986). The number of species from which GVs have been isolated is currently around 150 (Crook, 1991). The host range of GVs appears to be restricted to a fairly

small number of species within the same family as the original host (Gröner, 1986). However, at least three GVs (from *Cydia pomonella*, *Helicoverpa armigera*, and *Scotogramma trifolii*) appear to have a moderately broad host range and cause infection in a number of species from several genera (Crook, 1991). Although also usually highly specific, there are more NPVs than GVs, which are effective against heterologous hosts. The NPVs of *Autographa californica* (AcMNPV) and *Anagrapha falcifera* (AfMNPV) were 93 times and 54 times less infectious to *C. pomonella* neonate larvae than was the homologous GV, CpGV (Lacey *et al*., 2002).

1.3.1.5 Gross pathology and symptomatology

There are three types of GV disease, each characteristic of a different type of tissue tropism encoded by the virus. In type 1 GVs, such as TnGV, the virus invades the host through the midgut epithelium, much like the typical NPV, but subsequently only infects the fat body tissue (Federici, 1993). Because other important tissues are not attacked, the larva may live as much as a week longer than a larva of the same stage infected with a similar amount of NPV (i.e. around 10 - 14 days post infection in the fourth instar) (Federici, 1997). These larvae will typically maintain their appetites and grow much larger than healthy larvae, only becoming lethargic within a day or two of death. After infection, larvae become markedly swollen, developing a creamy yellow appearance, due to the accumulation in the fat body of large numbers of infected cells packed with viral granules. Typically, there is not liquefaction of the body, as the epidermis is not infected. Larvae typically turn dark brown or black, because of the invasion of the body by gut flora, and desiccate or disintegrate (Federici, 1997).

In the type 2 GV, which includes CrleGV, the viral infection and gross pathology parallel that of the typical lepidopteran NPV disease. After invasion of the midgut, the virus attacks most of the major body tissues, including the tracheal matrix, epidermis, and fat body (Huger, 1963; Tanada & Kaya, 1993). This disease is more acute than that caused by type 1 GV, typically lasting only 5 - 10 days in larvae infected during the fourth instar

(Federici, 1997). Infected larvae swell and distend slightly, developing irregular white to yellow patches below the cuticle. After death, the body liquefies.

The third type of GV disease (type 3 GV) is unique to the HbGV, which attacks the Western grapeleaf skeletoniser, *Harrisia brillians*, and is characterised by tissue tropism restricted to the midgut epithelium, producing virions and OBs in both larvae and adults (Federici & Stern, 1990). The disease is an acute one, and larvae infected in the third or fourth instar usually die within 4 - 7 days.

1.3.1.6 Life cycle

The biology of GVs is very similar to that of NPVs. Infection with GVs normally takes place by ingestion of OBs by larvae of the host insect (Fig. 1.2), although transmission by parasites or transovarial transmission may sometimes be important (Crook, 1991). The ingested GV granules are dissociated by the highly alkaline midgut juice (Summers, 1971; Winstanley & O'Reilly, 1999), and the liberated enveloped virions attach and fuse to the plasma membrane of the microvillus of the columnar midgut cell. For many GVs, enhancin protein associated with the granules is believed to catalyse the partial disruption of the peritrophic membrane lining the midgut (Funk *et al.*, 1997). This allows the virus particles easier access to the midgut epithelial cells. The nucleocapsids enter a microvillus, migrate to the nucleus, and attach to the nuclear pores within 2 - 6 hours post infection (Tanada & Kaya, 1993). Uncoating of the nucleic acid occurs at a nucleopore (or within a nucleus in the case of an NPV) and DNA is injected into the nucleus (Summers, 1971). This is the location of the primary infection cycle (Fig. 1.2).

Virogenesis begins in a nucleus with the formation of the virogenic stroma (Tanada & Kaya, 1993). Capsids appear in 6 - 12 hours post infection and are incorporated with the viral nucleoprotein core. Progeny nucleocapsids are formed in 12 - 18 hours post infection in regions of dense aggregated material distinct from host chromatin. Shortly after the appearance of the nucleocapsids, the nuclear envelope breaks down and virogenesis continues in the nucleus and the cytoplasm. The envelopment of the nucleo-

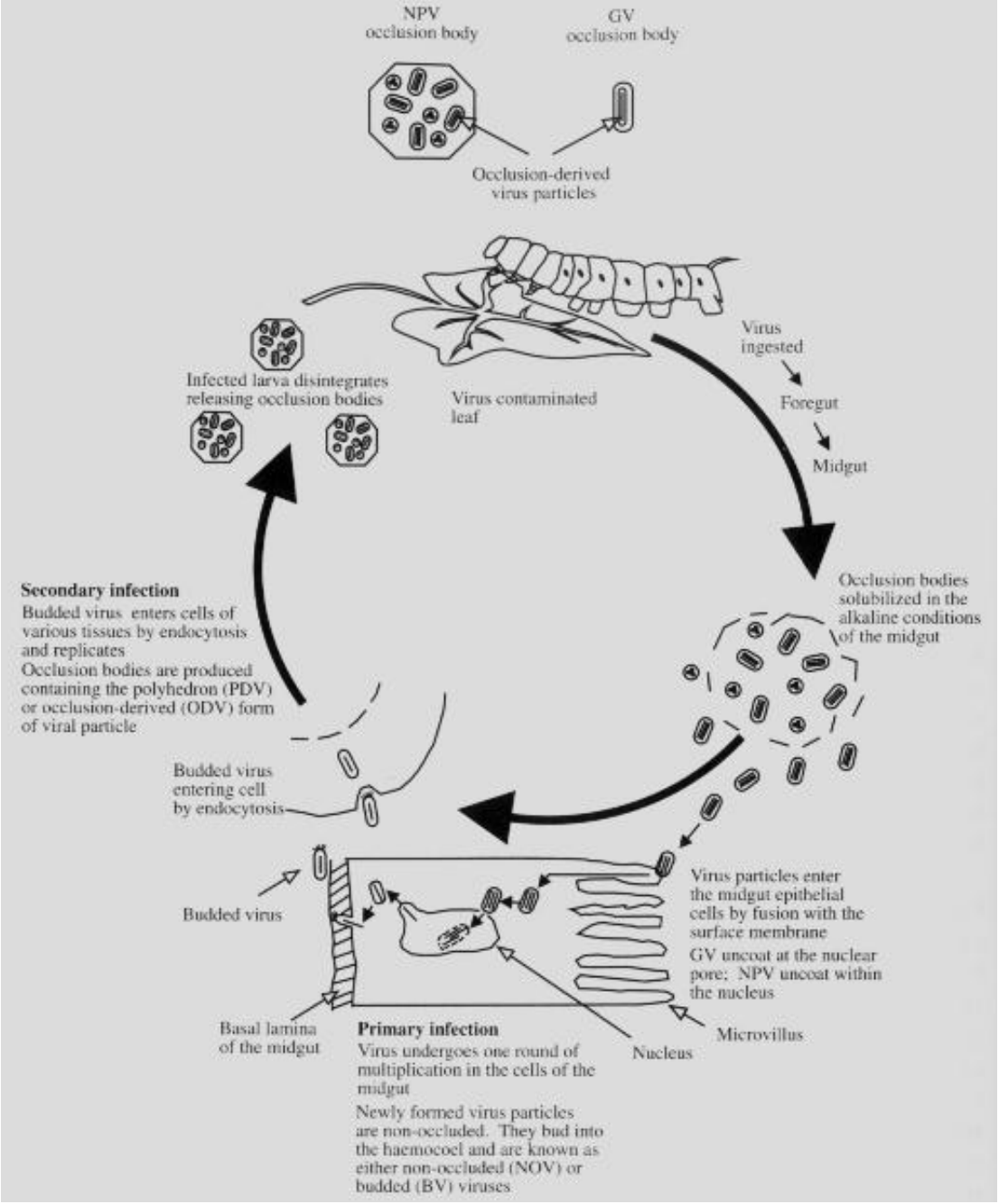

Figure 1.2 The main features of the biology of baculoviruses (Hunter-Fujita *et al.*, 1998).

capsids and their occlusion in capsules also occurs in the nucleus and the cytoplasm. About 24 hours post infection, enveloped and unenveloped nucleocapsids may occur in continuous rows in intercellular spaces between midgut cells and near the basement membrane. At a late stage, many nucleocapsids are embedded in the basement membrane or bud through the membrane and thereby acquire envelopes with peplomers.

Budded virions (Fig. 1.1) move from the midgut epithelium into the hemocoel, where secondary infection takes place (Tanada & Kaya, 1993) (Fig. 1.2). The infection of the fat body is by viropexis of nucleocapsids with peplomer envelopes. The occlusion of the virions occurs in the nuclear and cytoplasmic regions of the cell. In capsule formation, the enveloped nucleocapsids, rather than moving to the foci of granulin condensation, are occluded by granulin condensing on the viral envelope.

OBs are released into the environment when the insect dies and disintegrates. Horizontal transmission (larva to larva) of the virus can now occur, and the replication cycle will continue.

1.3.1.7 Cytopathology and histopathology

The cytopathology that occurs in the occlusion phase in secondary sites of GV infection differs markedly from that of NPVs, but parallels events that occur in GV-infected midgut cells. Regardless of the tissue tropism, the phase of replication leading to occluded virions and granules is similar in all types (Federici, 1997). After cell infection, the nucleus hypertrophies as the nucleoli enlarge and move towards the nuclear membrane along with the chromatin. As the nucleus enlarges, it ruptures and the cytoplasm and nucleoplasm mix. At this stage, numerous virogenic stromata begin to form as dense granular areas distributed throughout the cell, with the stromata interconnected by thin nucleoprotein strands. Along the edges of these, nucleocapsids begin to assemble and shortly thereafter are enveloped by membranes lying outside the stroma. In the same region, as mature virions begin to accumulate, masses of granulin appear. The virions are quickly occluded and initially form distinct masses distributed throughout the cell. As more granules form, the masses coalesce and the cell greatly enlarges. Finally the cell becomes completely filled with thousands of granules.

This cytopathology leads to a distinctive histopathology quite different from that which occurs in NPVs (Federici, 1997). After lysis of the nuclei, infected cells disassociate

from one another as the gap junctions, desmosomes, and hemidesmosomes degenerate, apparently due to the loss of nuclear functions. Almost simultaneously, the infected cells also separate from the basal lamina and, in tissue such as the fat body, move to and accumulate in the centre of the lobe. This results in the proliferation of regenerative cells situated on the internal surface of the matrix. As the regenerative cells begin to differentiate, they all develop GV infections.

1.3.1.8 Apoptosis regulation

Apoptosis is the capacity of a host cell to "commit suicide", in this case in defence against the invasion and spread of viral pathogens. In contrast to necrotic death, the plasma membrane of an apoptotic cell remains intact for an extended period of time (Clem, 1997). This prevents the leakage of intracellular components into the extracellular milieu and the inflammation that would result. There are changes in the membrane of an apoptotic cell that allow it to be rapidly recognised by other cells and phagocytised in tissues (Fadok *et al*., 1992). Late in the process of apoptosis, the DNA is digested by an endogenous endonuclease, resulting in internucleosomal cleavage (Wyllie, 1980).

It is now widely recognised that the ability of individual cells to direct their own suicide has had an important influence on the evolution of viruses and their strategies for replication (Clem, 1997). Many viruses provoke the suicide response very early in their replication process, and the premature death of the host cell can have a negative influence on the ability of the virus to replicate. Thus viruses have had to either evolve mechanisms to prevent cell suicide or find ways to cope with replicating in an apoptotic environment. In many cases the path chosen seems to have been determined by the coding capacity of the viral genome. Thus, many large DNA viruses have acquired genes that are able to directly intercede in the apoptotic pathway and prolong the life of the infected cell until after they have finished replicating. The mechanisms by which viruses induce apoptosis are in most cases not well understood, but are believed to be many and varied (Clem, 1997). In order to block apoptosis, viruses have acquired genes, such as

the ICE family protease inhibitor *p35* (Clem *et al.*, 1991) and the *iap* genes (Crook *et al.*, 1993).

1.3.1.9 Natural occurrence

Despite the large number of different baculoviruses that have now been isolated, observations on the natural incidence of baculovirus disease are limited to relatively few of these isolated viruses (Crook, 1991). There are no records whatsoever of the natural occurrence of some of these viruses since they have been isolated from laboratory insect stocks.

Attempts to determine the natural rate of spread of baculoviruses and the influential factors, have proved difficult (Dwyer & Elkinton, 1995). Rate of dispersal of the host is often not the only mechanism of dispersal, as parasitoids have been shown to play a key role in dispersing baculoviruses throughout the host population (Levin *et al.*, 1983; Gould *et al.*, 1990). Arthropod predators (Kaya, 1982) and birds (Entwistle, 1982) have been observed to fulfil a similar function.

Baculoviruses present in a host population are presumed to occur in an enzootic state. This means that the disease is constantly present in the host population at low prevalence (Tanada & Kaya, 1993). An epizootic is loosely defined as an unusually large number of disease cases in a population (Fuxa & Tanada, 1987). Natural epizootics caused by baculoviruses are not unusual. Examples are the NPV of the fall armyworm, *Spodoptera frugiperda*, in Louisiana and Texas, USA (Fuxa, 1982; 1987); the NPV of the lepidopteran *Wiseana* spp., in New Zealand (Kalmakoff & Crawford, 1982); the NPV of tent caterpillars, *Malacosoma* spp., in North America (Myers, 1993); the NPV of the gypsy moth, *Lymantria dispar*, in North America (Elkinton & Liebhold, 1990); the NPV of the African armyworm, *Spodoptera exempta*, in Kenya (Odindo, 1983); and NPVs of four species of semi-looper on soybean in Zimbabwe (Kunjeku *et al.*, 1998). High levels of pests can rapidly collapse to well below economic threshold levels. However, all of these case studies involve surface feeding pests and therefore have limited relevance to

CrleGV infections of *C. leucotreta*. Eastwell *et al.* (1999) were able to establish that 23 % of the wild population of *C. pomonella* larvae (a cryptic pest like *C. leucotreta*), surveyed at four sites in British Columbia, Canada, carried CpGV (identical or similar to the Mexican isolate, CpGV-M1). They did not identify any epizootics or speculate on the impact of the virus on the wild *C. pomonella* population.

1.3.1.10Pathogenicity

For neonate larvae, the median lethal dose (LD_{50}) of GVs can vary from less than five OBs to greater than 10^4 OBs; the median lethal time (LT_{50}) can vary from less than four days up to 25 days or even 43 days in the case of a GV infecting an alternate host (Crook, 1991). In general, the speed of kill of a GV depends on several parameters such as dose and susceptibility of the host as well as the tissue tropism. GVs have been described as "slow" or "fast" GVs (Winstanley & O'Reilly, 1999). The slow GVs, type 1 GVs, have high LD_{50} values, with death being substantially prolonged. Some of the slow noctuid GVs have much larger genomes in the region of 170 kb. The fast GVs, type 2 GVs (including CrleGV), are highly infectious, have low LD_{50} values < 5 capsules per neonate and rapid speeds of kill.

1.3.2 *Cryptophlebia leucotreta* granulovirus (CrleGV)

Angelini *et al.* (1965) were the first to describe CrleGV. This isolate was obtained from infected larvae from the Ivory Coast. Angelini & Le Rumeur (1962) stated that GV contamination, if not curtailed, was capable of causing a laboratory reared *C. leucotreta* culture to collapse. They also noted a cypovirus (previously known as a cytoplasmic polyhedrosis virus) (CPV) infection in the laboratory culture. Whitlock (1980) was interested in the virus-like rods associated with CrleGV, which he isolated from a South African laboratory culture of the insect. Another isolate was obtained from diseased larvae, which were collected on the Cape Verde Islands (Mück, 1985). A South African isolate was obtained from larvae in a laboratory culture held by the Hoechst Corporation in Germany (Jehle *et al.*, 1992a). The South African isolate, the Ivory Coast isolate and

the Cape Verde isolate can be clearly distinguished by restriction analysis (Jehle *et al.*, 1992a). Fritsch & Huber (1986) made reference to biological and biochemical characterisation of the three different isolates mentioned. This work was never published, but fragment patterns were determined by restriction enzyme analysis with *Eco*RI, *Bam*HI and *Hind*III by Fritsch (1989) in her doctoral dissertation. She thereby demonstrated that all three isolates were distinct strains. Jehle *et al.* (1992a) constructed a restriction fragment map covering almost the entire genome of the Cape Verde isolate of CrleGV. The position of the granulin gene was identified by cross-hybridisation with granulin coding fragments of *Cydia pomonella* GV (CpGV) (Jehle & Backhaus, 1994b). The size of the viral genome was determined to be 112.4 kbp (Jehle *et al.*, 1992a). Its granulin amino acid sequence was compared to that of *Autographa californica* NPV polyhedrin, and other NPVs (Jehle & Backhaus, 1994a). Jehle *et al* (1992b) examined the genetic interaction between CrleGV and CpGV co-infecting larvae of *C. leucotreta*. In so doing, the genetic interaction of unmodified GVs was examined *in vivo* in order to assess possible risks of genetic exchange of modified baculoviruses. This work was based on the discovery that CpGV is cross-infectious for larvae of *C. leucotreta*, but is about 1000 times less virulent than the specific GV (Fritsch *et al*., 1990).

Reiser *et al.* (1993) considered *C. leucotreta* as a suitable alternate host for mass production of CpGV for biological control purposes. This idea was apparently employed by Hoechst in Germany, but was unsuccessful, as CrleGV soon became the dominant virus in the culture (Cross *et al.*, 1999).

1.4 MICROBIAL CONTROL

1.4.1 Microbial control of insect pests

The term “microbial control” is most commonly used in the context of the inundative use of entomopathogenic organisms (Lacey & Goettel, 1995). Under natural conditions, entomopathogens are often responsible for significant reductions in pest populations, and

under certain circumstances obviate the need for additional interventions. However, in most cases epizootics often occur in peak populations of insect pests after economic thresholds have been surpassed (Lacey & Goettel, 1995).

Microbial control of arthropods has been investigated for over 100 years (Steinhaus, 1963), however the intensity of investigation has increased dramatically in recent years (Harper, 1987). The most widely used of all microbial control agents is *Bacillus thuringiensis* (Lacey & Goettel, 1995). Baculoviruses are also well represented among those micro-organisms which are being used successfully in microbial control programmes (Cunningham, 1988; Harper, 1987; Hunter-Fujita *et al.*, 1998; Jones *et al.*, 1993b; Lacey & Goettel, 1995).

1.4.2 Microbial control on citrus

1.4.2.1 Worldwide

Table 1.4 List of key arthropod pests of citrus and the entomopathogens utilised as microbial control agents (McCoy *et al.*, 2000).

Target host	Entomopathogen	Rate of application	References
Phyllocoptruta oleivora	*Hirsutella thompsonii*	2.2 - 4.4 kg/ha (2.8×10^8 CFU/kg)	McCoy, 1985; 1996; 1998; McCoy & Couch, 1982
Panonychus citri	Non-inclusion virus	0.1% aqueous suspension	Reed, 1981; Shaw *et al.*, 1968; van der Geest, 1985
Toxoptera citricida	Hyphomycetes fungus	$2.5 - 5.0 \times 10^{13}$ conidia/ha	Poprawski *et al.*, 1999; Tsai, 1998
Citrus root weevils (*Diaprepes, Pachnaeus, Asynonychus*	*Beauveria bassiana*	53.8 ml/m^2 (2.1×10^{10} CFUs/ml)	Quintela & McCoy, 1998
	Metharizium anisopliae	5 g/m^2 (mycelia)	Quintela & McCoy, 1998
	Entomopathogenic nematodes	4.94×10^6/ha 2×10^6/tree	Duncan *et al.*, 1996; McCoy, 1985; Morse & Lindgren, 1996

Microbial control on citrus, mainly pertaining to efforts in the USA, has been reviewed (McCoy, 1998; McCoy *et al.*, 2000). Only one virus is reported to have been isolated and used against a citrus pest: a non-inclusion virus of the citrus red mite, *Panonychus citri* (van der Geest, 1985). Six entomopathogens have been used as microbial control agents against arthropod pests of citrus (Table 1.4).

Yield of tangerines was improved by applying *Helicoverpa armigera* NPV (HaNPV) against *Helicoverpa armigera* in orchard trials in Thailand (Kunjeku *et al.*, 1998). Fritsch (1988) conducted a small scale field trial with CrleGV against *C. leucotreta* on citrus on the Cape Verde Islands. Further details on this trial are provided under the relevant section (1.4.4) later in this chapter.

1.4.2.2 South Africa

The first recording of microbial control efforts in the southern African citrus industry were as early as 1900 (Bedford, 1954). A fungus identified as *Sphaerostilbe coccophila* was used against California red scale, *Aonidiella aurantii*, but failed to induce epizootics in field trials.

Cladosporium oxysporum was isolated from citrus mealybug, *Planococcus citri*, after being found to be an effective natural control agent in the field (Samways & Grech, 1986). The fungus was grown in submerged culture and then applied, in the laboratory, to citrus mealybug; long-tailed mealybug, *Pseudococcus longispinus*; soft green scale, *Pulvinaria aethiopica*; and citrus psylla, *Trioza erytreae*. Death and hyphal growth resulted in all four species. Field applications against citrus psylla and black citrus aphid, *Toxoptera citricidus*, initially appeared promising, but the fungus proved to be too density dependent and humidity dependent to be considered as a viable economic control measure.

Begemann (1988) isolated *Beauvaria bassiana* from *C. leucotreta* pupae. He cultured the fungus and in laboratory trials achieved a 97 % mortality of larvae. Field trials were not as successful, probably due to low humidity.

A HaNPV strain, isolated by Whitlock (1974), was used in field trials against *H. armigera* (Moore, 1998; Moore *et al*., 1997; 1998; 1999a; 2000b). It was applied at rates of 1.15 x 10^6 and 7.26 x 10^5 OBs/ml, both resulting in 100% recorded mortality of *H. armigera* within 14 days after application or slightly longer. *H. armigera* damage to fruit was reduced by up to 83.7 % and rejection for export was reduced by up to 95.8 %. These results were better than those achieved with *Bacillus thuringiensis* and various standard chemical insecticides used in the same trials. As this is the only work which has been conducted with a baculovirus on citrus in southern Africa, it must be considered as a good case study to assist in directing the proposed work on CrleGV.

When one compares the results achieved in these trials with other HaNPV case studies in southern Africa on different crops (e.g. Daoust & Roome (1974) and Roome (1975) on sorghum and cotton in Botswana), the results achieved on citrus are conspicuously more impressive. There are four possible reasons for this. Firstly, on citrus there is only one generation of *H. armigera* that needs to be controlled whereas there are several on sorghum. Secondly, the shady architecture of a citrus tree probably provides more protection of the virus, against not only UV inactivation but also precipitation, than can be provided by any other crop. Thirdly, *H. armigera* is attracted to feed initially on the blossoms and flowers. Usually, only once the blossoms in the immediate proximity of a larva have been consumed, will it begin to feed on fruitlets. As most of the blossoms will eventually drop off without leading to fruit formation (Davies, 1986), a certain degree of damage can be tolerated at this stage, with negligible impact on the fruit. It is likely that only an extremely high level of infestation of *H. armigera* would have an impact on the yield of trees. Lastly, navel orange trees abscise a large proportion of their fruit as a matter of course (Davies, 1986). Not only will a number of these abscised fruit per chance be damaged fruit, but it is possible that damaged fruit could even be preferentially

abscised. Damage leads to increased ethylene production which in turn causes premature senescence and consequent abscission (Woolhouse, 1978).

B. thuringiensis var. *kurstaki* is registered for the control of citrus butterflies, *Papilio* spp.; apple leaf roller, *Tortrix capensana*; and *H. armigera* (Grout *et al.*, 1998) on citrus in South Africa.

Other pathogens recorded from citrus pests include the fungus *Sphaerostilbe aurantiicola* from California red scale, *Aoinidiella aurantii*; circular purple scale, *Chrysomphalus ficus*; and from mussel scale, *Lepidosaphes beckii* (Annecke, 1963). A *Podonectria* sp. fungus was recorded from California red scale and a *Myiophagus* sp. was identified from citrus mussel scale, *Cornuaspis beckii* (Annecke, 1963). *Fusarium coccinellum* was also noted from California red scale (Catling, 1971). Buitendag (1965) isolated a microsporidian, *Plistophora reciprocaria*, from citrus looper, *Ascotis selenaria*, larvae. Swart *et al.* (1975) reported, without quoting any evidence, that bacterial or viral diseases often kill larvae of the fruit piercing moth, *Serrodes partita*. A GV was isolated from the citrus leafroller, *Archips occidentalis* (Smith *et al.*, 1990). *Fusarium coccophilum* and *Cladosporium oxysporum* were identified from citrus psylla (van den Berg, 1996).

1.4.3 Microbial control with baculoviruses

1.4.3.1 An overview

In 1975 the first baculoviral product was registered and commercialised. This was Elcar, the NPV of *Helicoverpa zea*. Winstanley & Rovesti (1993) listed 31 viral insecticides that are registered or close to registration, for control of 24 pests. Currently 16 commercially available baculovirus formulations are listed in the Directory of Microbial Control Products and Services (Shah & Goettel, 1999). Four of these are formulations of the granulovirus CpGV, which infects the codling moth, *C. pomonella*, which belongs to the same superfamily (Tortricoidea) as does *C. leucotreta*. In a recent survey Entwistle (1998) listed all insect viruses (and associated hosts) on which research work was then in

progress or which were then employed in control practices. He listed 17 baculoviruses for Western Europe, 18 for Eastern Europe and the former Soviet Union, six for the Indian subcontinent, 10 for south-east Asia and the western Pacific, 14 for the People's Republic of China, five for Japan, nine for Africa, eight for Australasia, 14 for North America, 10 for Central America and the Caribbean, and 12 for South America.

Several successes have been achieved throughout the world with the use of baculoviruses, for the control of insect pests on agricultural crops (Cunningham, 1988; Harper, 1987; Hunter-Fujita *et al.*, 1998; Lacey & Goettel, 1995). It is helpful to review the successes achieved with CpGV, because of the close relation of *C. pomonella* to *C. leucotreta*, in both biology and behaviour, and the close relation between the two pathogens (CpGV and CrleGV) (Jehle *et al.*, 1992a). In the past 30 years a large number of field trials have demonstrated notable activity of CpGV against *C. pomonella* in a variety of settings across Europe, Australia, New Zealand, South America, Canada and the United States (Burgerjon, 1986; Jaques *et al.*, 1987; Lacey & Knight, 1998). Although CpGV is very effective in controlling *C. pomonella*, some of the concerns expressed by researchers and growers are: the increased number of shallow stings to the fruit, short residual activity of the virus (and the need for multiple applications), slow speed of kill, reduced effectiveness against high *C. pomonella* populations, and the expense of the virus (Lacey & Knight, 1998). CpGV is currently registered in several countries in Europe and may now also be registered in North America (Lacey *et al.*, 2000).

A number of factors have strongly influenced the level of efficacy achieved with CpGV against *C. pomonella*. In conducting field trials with CrleGV against *C. leucotreta* one can learn from the experiences with the former pest. Operational dosages of 10^{13} to 10^{14} OBs/ha are recommended for commercial CpGV products (Lacey *et al.*, 2000). In addition to dosage, amount of water in which the virus is applied is another important consideration. Best results have been achieved by spraying the trees until run off. Even when the same amount of virus/ha was used, low or ultra low volume sprays did not result in the same level of control as did spraying until run off (Payne *et al.*, 1984). The

pH of the water used should be in the range of 5 to 9. Chlorinated water can have an adverse effect on the quality of the virus. Timing of applications were found to be absolutely critical in order to achieve optimum efficacy (Huber & Dickler, 1977). As codling moth larvae feed very little on the exposed surface of apples or leaves, deposits of virus have to be present in the short time after eclosion, before the larvae enter the fruit. Huber and Dickler (1977) timed application to coincide with maximum codling moth egg laying as indicated by light and pheromone traps and visual examination of fruit. However, more recently, virus uptake was found to be independent of active feeding, and larvae became infected simply by walking or browsing on sprayed leaf sic surfaces in as little time as 3.5 min (Ballard *et al.*, 2000b). Glen and Payne (1984) made initial applications 140 - 169 accumulated day-degrees after first pheromone trap catches. In commercial operations with two or more generations of the moth per year, applications are made at 7 - 21 day intervals for up to 7 - 9 applications during the growing season. The expense of repeated application of CpGV can be reduced by tank mixing the virus with other materials requiring frequent application, such as fungicides. Frequent application assures long lasting presence of active virus in the canopy despite low persistence due to UV sensitivity. Field applications of CpGV have shown that weekly spraying using only one tenth of the normal concentration provides the same, or even better efficacy as spraying at two week intervals with the normal concentration (Dickler & Huber, 1988).

It appears that most applied work with baculoviruses has been conducted with surface feeding pests. This means that as long as the virus on the surface of the plant remains viable, the pest can potentially ingest it and become infected. *C. leucotreta*, like *C. pomonella*, is an internal (cryptic) feeder (Daiber, 1979b). There is a very brief period of time between hatching of the larva and its penetration into the fruit. Coverage of the virus on the fruit must therefore be such that the small neonate larva is likely to ingest a lethal dose of the virus before or during the time that it begins to penetrate into the fruit. Much can therefore be learned from the approaches that have lead to successful control of *C. pomonella* with CpGV. However, it is possible that *C. leucotreta* may be an even more difficult pest to control, particularly with a baculovirus, than is *C. pomonella*.

Firstly, *C. leucotreta* does not overwinter (Daiber, 1980). The moth is therefore potentially present throughout the year, and there is always an overlap of generations. Secondly, *C. leucotreta* is an active flyer. Even though control of the moth, within an orchard, may be good, high numbers of moths can rapidly reinfest an orchard. Thirdly, time between hatching of *C. leucotreta* larvae and their penetration into fruit is briefer than that for codling moth. This is due to the difference in oviposition sites. A large proportion of codling moth eggs are deposited on leaf surfaces, whereas FCM eggs are only deposited on fruit surfaces (Daiber, 1980). Codling moth larvae may even briefly feed on foliage before moving onto the fruit, whereas *C. leucotreta* only feed on the fruit. There may therefore be less opportunity for interception of the virus by *C. leucotreta*. Fourthly, the foliage of a citrus tree is denser than that of an apple tree. It is therefore more difficult to achieve adequate coverage of a citrus tree with an insecticide, than it is to achieve adequate coverage of an apple tree.

In general, virus control in Africa has been neglected (Kunjeku *et al.*, 1998). For instance, at a Southern African Development Coordination Conference (SADCC) workshop (Namponya, 1989) no mention was made of entomopathogenic viruses in Angola, Botswana, Lesotho, Malawi or Swaziland. Kunjeku *et al.* (1998) list 14 species of insect pest on which significant investigation of virus control has been conducted in Africa. In a review, Entwistle (1998) lists no virus products as being registered any where in Africa. He does, however, mention that substantial progress towards obtaining registration has been achieved with SlNPV for the control of the Egyptian cotton leaf worm, *Spodoptera littoralis,* in Egypt. Topper *et al.* (1984) showed that application of NPV (at a concentration of 5×10^{12} OBs/ha) to control *S. littoralis* in Egypt was as effective in reducing damage as either the hand-collection of egg masses or the application of chemical insecticides. More recently Jones *et al.* (1994) indicated that the virus rate can be reduced to 1×10^{12} OBs/ha. A commercial preparation of *S. littoralis* NPV called Spodopterin, was produced in France by Calliope and field tested in Egypt. Cherry *et al.* (1997) optimised *in vivo* techniques for the production of NPV in the beet armyworm, *S. exigua*, and the African armyworm, *S. exempta*, for use in Africa, however, it is not clear how much progress was made with field trials.

Kunjeku *et al.* (1998) record only three cases of significant investigation of virus control of insect pests in South Africa: on Tsetse flies, *Glossinia* spp., (an unclassified virus); wattle bagworm, *Kotochalia junodi*, (an NPV); and the African armyworm, *S. exempta* (an NPV). Subsequently, field trials were conducted with HaNPV against *H. armigera* on citrus in South Africa, with impressive results (Moore, 1998; Moore *et al.*, 1997; 1998; 1999a; 2000b). No virus has yet been registered for the control of any insect pest in South Africa.

Huber (1990) lists several advantages and disadvantages of baculoviruses as biological control agents for insect pests. Advantages are:

1. most baculoviruses are highly selective;
2. they present no residue problems;
3. they persist and multiply in pest populations;
4. they are effective at low dosage;
5. production is suited for cottage industry;
6. conventional application methods can be used.

Disadvantages are:

1. they are too selective;
2. they have short residual activity on leaf surfaces;
3. they mainly affect only larvae;
4. they are slow acting;
5. application requires careful timing and detailed knowledge of the biology of the target insect;
6. production is labour intensive;
7. preparations may require registration.

Four strategies for using baculoviruses for pest control are reviewed by Starnes *et al.* (1993). These are:

1. inoculation of virus that results in establishment and permanent regulation of the pest;

2. application of virus that results in control from epizootics, but not permanent regulation;
3. manipulation of the habitat that results in activation of the established or naturally occurring virus;
4. repeated applications of the virus to control pest insects due to little or no horizontal transmission of the virus.

For a microbial insecticide to succeed, it needs to meet with the approval of four different groups of role players (Gelernter & Trumble, 1998). These are:

1. technical (researchers);
2. farmers (insecticide users);
3. industry (biopesticide producers);
4. the public.

In evaluating several case studies the following factors were identified as instrumental in ensuring the success of a microbial insecticide (Gelernter *et al.*, 1998):

1. Microbial insecticides must not be stand-alone; they must form part of an integrated pest management (IPM) programme.
2. Conventional insecticides must be unavailable or ineffective.
3. Expectations must be realistic.
4. Benefits other than efficacy must be exploited.
5. Support is required from both private and public institutions.

Once it has been established that a virus possesses the necessary potential to be developed as a commercial biological control agent (through bioassay, study of the pest biology and a market analysis), several steps need to be successfully implemented on the road to developing a baculoviral product. These can be summarised into three categories:

1. An effective and efficient mass production system must be established.
2. The product must be appropriately formulated.
3. The product must be applied in the most suitable manner for optimisation of efficacy.

1.4.3.2 Production of baculoviruses

Even if *in vitro* systems for the propagation of baculoviruses are available, the large amount of virus needed for field use has still to be produced with *in vivo* methods (Huber, 1986b). The basis of any baculovirus production is therefore a mass rearing of the host insect, usually on artificial diet medium. Several influential factors need to be clarified to effectively rear the host, and to optimally produce the pathogen. These include host availability, host biology, environmental parameters (temperature, humidity, photoperiod and diet), design of rearing container, source of virus inoculum, purity, activity and dosage of inoculum, optimal host density, ideal age, stage and sex of host for virus production, exposure time to inoculum, virus incubation and harvesting, processing of the virus, storage of the virus, quality control, and finally, the cost of the whole process (Shapiro, 1982).

Regardless of the insect virus produced and the differences in methodology used by different workers, the basic production scheme involves virus inoculation, virus incubation, harvesting of larvae, processing of virus, and storage of virus (Shapiro 1982).

An important factor in any production system is that the host production facility must be kept strictly microbiologically isolated from all aspects of the virus production process (Hunter-Fujita *et al.*, 1998). Quality control procedures should be implemented and monitored for all steps of the production process. Ultimately, it is the quality of the virus that really matters. This pertains to activity of the virus (LC_{50}) and purity of the virus (minimisation of contaminants) (Shapiro, 1982). Before a viral preparation can be used, either as inoculum for *in vivo* production or in the final product, certain quantitative and qualitative tests must be conducted (Shapiro, 1986): 1. viral concentration must be determined, either through use of a haemocytometer counting chamber or a spectrophotometer; 2. viral activity must be determined through bioassay; 3. viral identification or integrity must be confirmed through restriction endonuclease analysis (REN) or enzyme-linked immunosorbent assay (ELISA); 4. microbial contaminants must be identified to establish that there are no human-pathogenic contaminants in the

preparation; 5. microbial contaminants must be quantified to ascertain that levels are not excessive. Acceptable levels are dictated by registration protocols.

Mass production facilities can be inexpensively developed by use of a series of interconnected temporary buildings or even mobile homes (Hunter-Fujita *et al.*, 1998), or can be technologically elaborate automated systems (Shieh, 1989).

Practical and effective systems for mass production have been described in great detail by Shapiro *et al.* (1981) for the gypsy moth, *Lymantria dispar*, NPV; by McKinley *et al.* (1989) for *S. littoralis* NPV; and by Jones *et al.* (1993a) for *H. armigera* NPV and for armyworm, *Spodoptera exigua*, NPV.

1.4.3.3 Formulation of baculoviruses

Field results have shown that formulation of microbial insecticides is not essential to their success (Jones *et al.*, 1997). However, large-scale use requires that a product is easily handled and has predictable activity. The need for formulation therefore encompasses two main areas: shelf and tank (finished spray) formulations (Hunter-Fujita *et al.*, 1998). Shelf life of a baculovirus product can be retained for a reasonable length of time by freezing, or by developing a formulation that confers stability under a reasonable range of shelf storage conditions (Hunter-Fujita *et al.*, 1998). Tank formulations are determined by the physical method of application and the rate of decay of the baculovirus once it is deposited at its target site. Tank additives may be introduced just before application, or may be part of the shelf formulation. The purpose of tank formulations would be to optimise application, enhance activity of the microbe and maximise environmental persistence (Jones *et al.*, 1997). Formulation helps to negate the inefficiency of application, and makes a product more acceptable to users.

Ballard *et al.* (2000a) found that the addition of 15 % cane molasses to CpGV substantially improved control of *C. pomonella* in both laboratory and field trials. This was achieved through a dramatic reduction in the median lethal exposure time to CpGV

for neonate larvae. Field persistence was not altered by the addition of molasses. Other additives which showed potential were sucrose, fructose, sorbitol and extracts of apple flesh and skin.

Application would be optimised through improvement of dispersion, miscibility, viscosity and particle size (Most & Quinlan, 1986). The addition of a suitable surfactant such as a spreader, wetter, sticker or emulsifier would achieve this (Hunter-Fujita *et al.*, 1998). Activity of the virus could be enhanced by potentiation of its infectivity through the addition of an optical brightener (Shapiro & Robertson, 1992). Evidence from noctuid hosts suggests that optical brighteners do not enhance the ability of midgut cells to become infected, but instead block sloughing of infected primary target cells in the midgut, thereby countering developmental resistance and increasing mortality (Engelhard & Vokman, 1995; Washburn *et al.*, 1995; 1998). For *L. dispar* NPV, the addition of stilbene brighteners reduced the LC_{50} in laboratory assays from 18000 OBs/ml to 10 - 44 OBs/ml, and the LT_{50} also fell (Shapiro & Robertson, 1992). Environmental persistence can be improved by ensuring rain fastness and UV protection (Most & Quinlan, 1986).

The final product could be produced as a dry preparation, a wet preparation or a bait preparation. Dry preparations can be air-dried infected host insects, freeze dried, spray dried, co-precipitated with lactose, micro-encapsulated or dusts and granules. Wet preparations can be flowable concentrates or concentrates which settle (Hunter-Fujita *et al.*, 1998). All of these preparations bear various advantages and disadvantages.

1.4.3.4 Application of baculoviruses

Spraying is by far the most common method of application of baculoviruses for the control of pest insects (Hunter-Fujita *et al.*, 1998). The advantage here is that for growers who are use to working with chemical insecticides, this is a familiar mode of application. However, it would be a mistake to directly equate the application of baculoviruses with that of chemicals. Viral insecticides consist of solid particles (OBs), whereas most chemical insecticides are soluble substances. The practical relevance of this point

pertains to droplet size. Whereas with a chemical, even the smallest spray droplet will contain some active ingredient, it is possible that in the case of a virus, a spray droplet could be too small to contain any OBs (depending on the concentration of the spray mix). The second important difference between chemical insecticides and baculoviruses, which will influence application, is the fact that baculoviruses need to be ingested to be effective, whereas chemicals most often at least have some form of contact action. Depending on the biology and behaviour of the pest, this may mean that coverage with a baculovirus will have to be more thorough, or at least that it will have to be targeted directly at the zone in which most feeding takes place.

One disturbing factor is the apparent loss of OBs (or any solid particles) from spray droplets in the period between the finished formulation in the spray tank and the arrival of the spray droplet at its target. Hunter-Fujita *et al*. (1998) tabulated a list of examples of this phenomenon. It is not clear how loss from spray droplets can be counteracted, but Smith & Bouse (1981) suggest the use of thickeners, rather than surfactants, in microbial spray formulations.

Smith & Bouse (1981) also emphasise the importance of accurate selection, calibration and recording of factors pertaining to spray application e.g. exact type of spray machinery, pressure of spray fluid, speed of rotation when spinning disc generators are used and flow rates of spray fluids. In combination with the details on the formulation of the product and on environmental factors, one should be able to analyse the appropriateness and success of the mode of application used.

1.4.4 Microbial control with *Cryptophlebia leucotreta* granulovirus (CrleGV)

Unlike the closely related CpGV, which has been widely tested since 1966 (Falcon *et al.*, 1968), culminating in the production of at least four commercial formulations (Lacey *et al.*, 1998), CrleGV has not been exploited for the biological control of *C. leucotreta* on agricultural crops. Only one record exists of a small-scale field trial with CrleGV, on citrus and Spanish pepper on the Cape Verde Islands (Fritsch, 1988). In the virus sprays,

concentrations of 10^8 and 10^9 OBs/ml were used, and only skimmed milk powder and a wetting agent were added to the virus suspensions. *C. leucotreta* damage was reduced by 77 % in citrus and 65 % in Spanish pepper (Fritsch, 1988). The half-life of the virus in the field was close to two days (Fritsch & Huber, 1989).

1.5 PROJECT PROPOSAL

1.5.1 Justification

C. leucotreta is regarded as the most important pest on citrus in South Africa. However, chemical control of *C. leucotreta* is problematic. Four products are registered for the control of *C. leucotreta* on citrus: Alsystin, Nomolt, Penncap-M and Meothrin. Penncap-M cannot be used, as the compulsary pre-harvest interval exceeds the period of residual efficacy. Meothrin is only recommended for use five weeks before harvest. Despite this precaution, it is still prone to causing costly outbreaks of secondary pests. Alsystin is the most effective and most suitable of all the registered products. However, resistance by *C. leucotreta* to the active ingredient has begun to develop. Due to its similarity in chemistry and activity, Nomolt is suffering the same fate. Where Alsystin and Nomolt still work effectively, there are nevertheless a number of problems associated with their use. They are detrimental to the proliferation of the highly effective egg parasitoid, *T. cryptophlebiae*, which is being mass reared and augmented to improve biological control of *C. leucotreta*. Being IGRs, they also have a detrimental effect on beneficial coccinellid beetle predators and therefore cannot be considered as being compatible with an IPM approach. Residues of Alsystin and Nomolt are not acceptable on fruit exported to the lucrative USA market either.

The answer to *C. leucotreta* management cannot lie in chemical control. Despite chemical options having been investigated for many years, no suitable chemical option exists. As fresh produce production is increasingly pressurised, by sophisticated first world markets, to move away from chemical control, bio-intensive IPM must be

considered to be the most viable line of control. As previously mentioned, a programme of parasitoid augmentation is already being pursued. However, this alone would usually not adequately reduce *C. leucotreta* levels. CrleGV, if found to be effective, would be the ideal control measure to complement parasitoid augmentation.

1.5.2 Objectives

The ultimate objective of this study is encapsulated in the thesis title, namely, to develop and evaluate CrleGV as a biological control agent, and then to integrate it into a system for the management of *C. leucotreta* on citrus.

There are several proximate objectives, which need to be met in order to be able to achieve the ultimate goal. Firstly, it will be necessary to characterise the strain of CrleGV, which has been isolated. Comparison with a previously characterised strain will enable confirmation of whether the virus is indeed an isolate of CrleGV. It will also be possible to establish whether the virus has a novel profile. Most importantly, within the context of this study, a reference point for quality control purposes will be established. It will subsequently be possible, at any stage, to establish whether work is still being conducted with the originally identified isolate, or whether another virus had taken over. This will be particularly important if the virus ever becomes commercialised.

The second objective is to establish an efficient, effective and affordable method for mass rearing the host. This will be imperative in order to be able to achieve the third objective, which is to establish a system for *in vivo* mass production of the virus.

The fourth objective, which will be pursued simultaneously with the previous two objectives, will be to establish the pathogenicity of the virus against *C. leucotreta* larvae in laboratory bioassays (dose-response and time-response studies). This will include surface dose bioassays and detached fruit (oranges) bioassays. Such bioassays will be the precursor to the fifth milestone in this study: to evaluate the efficacy and persistence of the virus in the field.

The final objective is to examine an integrated system for *C. leucotreta* control on citrus, which will be based on CrleGV sprays and augmentation of egg parasitoids, but will include other facets too.

2

VIRUS IDENTIFICATION AND CHARACTERISATION

2.1 INTRODUCTION

All isolates of baculovirus can be clearly distinguished by restriction endonuclease analysis of baculovirus genomic DNA. DNA was first identified in virus particles derived from the OBs of an NPV (Breindl & Jirovec, 1935). Further information on the base composition of NPV and GV DNAs was provided by Wyatt (1953). Electron microscopy and biochemical and biophysical studies provided the first evidence that baculovirus genomes were large circular DNAs (Harrap & Payne, 1979). Restriction endonuclease analysis was introduced into the study of baculovirus genomes in the 1970s (Miller & Dawes, 1978; Rohrmann *et al*., 1977; Smith & Summers, 1978). Initially, these analyses provided the means for more accurate estimation of total genome sizes, assessment of genomic heterogeneity and distinction between different baculoviruses. To date, several NPV genomes have been fully sequenced, namely *Autographa californica* NPV (AcMNPV) (Ayres *et al.*, 1994), *Bombyx mori* NPV (BmNPV) (Gomi *et al.*, 1999), *Orygia pseudotsugata* NPV (OpMNPV) (Ahrens *et al.*, 1997), *Spodoptera exigua* NPV (SeMNPV) (Ijkel *et al.*, 1999), *Lymantria dispar* NPV (LdMNPV) (Kuzio *et al.*, 1999) and *Helicoverpa armigera* NPV (HaSNPV) (Chen *et al.*, 2001) (the only single-nucleocapsid NPV to be sequenced). GVs have not won as much attention as have NPVs. To date only three GVs have been fully sequenced, namely *Xestia c-nigrum* GV (XcGV) (Hayakawa *et al*., 1999), *Plutella xylostella* GV (PxGV) (Hashimoto *et al.*, 2000) and *Cydia pomonella* GV (CpGV) (Luque *et al.*, 2001).

One record exists of the characterisation of a CrleGV isolate genotype (Jehle *et al.*, 1992a). The genotype of the isolate, named CrleGV-CV3, was obtained from the Cape Verde Islands. By constructing a physical map of this genotype, using hybridisation techniques with a range of restriction enzymes, a number of common sequences between CrleGV-CV3 and CpGV (*C. pomonella* GV) were shown. The size of the viral genome was determined to be 112.4 kbp. Fritsch (1989) recorded DNA profiles of two other distinct CrleGV isolates, one from Ivory Coast and one from South Africa. However, she did not document their fragment sizes. It is therefore not possible to determine the extent, of differences between these isolates and the isolate which is the subject of this study.

In this study restriction endonuclease analysis was conducted with the CrleGV isolate for three reasons. Firstly, it was necessary to confirm whether the virus was a CrleGV isolate. This could be established by determining commonality with the previously described CrleGV-CV3 (Jehle *et al.*, 1992a). Secondly, it could be established whether this virus was novel, distinct from CrleGV-CV3. Thirdly, and most importantly within the context of this work, it was necessary to establish a reference point for quality control purposes. It would then be possible, at any stage, to establish whether work was still being conducted with the originally identified isolate, or whether a contaminant virus had taken over. This would be extremely important if the virus ever became a commercialised biological control product.

Further analyses were conducted to determine whether more than one distinct isolate of CrleGV could be detected in the *C. leucotreta* laboratory culture, and to determine whether regionally distinct isolates of CrleGV existed.

2.2 MATERIALS AND METHODS

2.2.1 Virus identification

2.2.1.1 Symptomatology

A laboratory culture of *C. leucotreta* was established (see Chapter 3). Larvae 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 hanging from substrates. Larvae demonstrating such symptoms were collected either individually or were grouped together, depending on what the intended subsequent studies were, and stored at -40ºC.

2.2.1.2 Preliminary identification by light microscopy (Evans & Shapiro, 1997)

Smears on microscope slides were made of whole insect bodies which appeared to be symptomatically infected with a baculovirus. Both gut and internal organs were included in each smear. Each smear was spread thinly across a microscope slide. Slides were air dried. Buffalo Black solution (Appendix 1) was heated to 40ºC in a staining rack on a hotplate. The slide was immersed in the Buffalo Black solution for five minutes. Thereafter, the slide was washed under running tap water for 10 seconds. The slide was again left to air dry, and then examined through a light microscope at 1000X magnification under oil immersion.

2.2.1.3 Identification of infected tissue sections by electron microscopy (Glauert, 1974)

Symptomatically infected *C. leucotreta* larvae were cut in half to allow effective penetration of the solutions being used in preparation for transmission electron microscopy. They were placed into glass “Polytop” 5 ml specimen tubes (Polytop Corporation, USA) containing 2.5 % glutaraldehyde (Appendix 1), made up in 0.1 M phosphate buffer, and allowed to fix for 12 hours at 4°C. After primary fixation the glutaraldehyde was decanted off and replaced by two successive washes of phosphate buffer. Secondary fixation in 1 % osmium tetroxide (Appendix 1), also made up in phosphate buffer, followed for 90 minutes. After two further phosphate buffer washes to remove all traces of fixative, the larvae were dehydrated by being immersed for 5 - 10 minutes each, in aqueous solutions of increasing concentrations of ethanol (30 % -

100 %). After two changes of 100 % ethanol, this was replaced by two changes of a transitional solvent, propylene oxide, for 15 minutes each. In order to ensure good infiltration of the embedding medium into the larvae the propylene oxide was then replaced by successive mixtures of the unpolymerised embedding medium and propylene oxide, the first being 75 % propylene oxide and 25 % embedding medium, the second 50 % and 50 %, and the third 25 % propylene oxide and 75 % embedding medium. Infiltration was allowed to proceed for one hour in each of these mixtures. The final mixture was then replaced by pure embedding medium and final infiltration allowed to proceed for a further 12 hours. The pieces of larvae were then transferred to embedding moulds containing pure resin that was allowed to polymerise over 36 hours at 60°C.

When polymerisation of blocks of embedding medium, containing the pieces of *C. leucotreta* larva was complete, the blocks were trimmed for ultramicroscopy according to standard methods. Ultrathin (70 - 80 nm) sections were cut using an LKB UM III ultramicrotome, mounted on 300 mesh copper grids and stained with uranyl acetate (5 % aqueous) and Reynolds lead citrate (Appendix 1). The grids were viewed using a JEOL JEM 1210 transmission electron microscope operated at 100 kV. The images were recorded on Kodak 4489 electron image film.

2.2.2 Characterisation of virus isolated from multiple larvae

Multiple larvae were required in order to be able to extract sufficient viral DNA to obtain a clear profile from several different digests with different restriction enzymes.

2.2.2.1 Virus purification

Approximately 20 - 40 symptomatically infected *C. leucotreta* cadavers weighing approximately 1 - 2 g in total, were pooled. Two different protocols for virus purification were used. These were purification using a sucrose gradient, and purification using a glycerol gradient. They were found to work equally well, however, due to the shorter purification time, the second protocol was eventually preferred.

2.2.2.2 Purification using a sucrose gradient

The methodology reported by Jehle *et al.* (1992a) was used as the basis for this protocol. Each gram of cadavers was homogenised in 5 ml 0.05 M Tris-HCl (pH 7.6) buffer (Appendix 1) using either a DuPont Omni-mixer (speed 2 for one minute) or a mortar and pestle. The homogenate was filtered through a single layer of mira cloth into a beaker. Finely ground saccharose (50 % w/w) was dissolved into the homogenate using a magnetic stirrer. The homogenate was then centrifuged at 16000 rpm (30000 g) for 75 minutes using a Beckman JA20-108 rotor. The supernatant and the fatty layer on its surface were discarded. The pellet was resuspended in 5 ml 0.05 M Tris-HCl (pH 7.6) by vortexing. This was washed by centrifuging at 10000 rpm for 10 minutes. The supernatant was discarded and the pellet resuspended and washed again, this time for five minutes. The pellet was resuspended in 2 ml 0.05 M Tris- HCl (pH 7.6).

A 50 % - 60 % w/w sucrose gradient was prepared in a 38 ml Beckman tube, leaving space for little more than an additional 1 ml of fluid. A volume of 1 ml of homogenate (of the resuspended pellet) was placed on top of the sucrose gradient. The gradient was ultra-centrifuged at 23000 rpm (95000 g) for 120 minutes in a SW28 rotor. A distinct virus band was obtained. This was removed and retained with the used of an autopipette. The extracted virus was diluted 1:1 with 0.05 M Tris-HCl (pH 7.6) and centrifuged for five minutes at 10000 rpm. The virus pellet was resuspended in a small volume of 0.05 M Tris-HCl (pH 7.6).

2.2.2.3 Purification using a glycerol gradient

Cadavers (approximately 2 g) were homogenised in 4 ml 0.1% SDS (sodium dodecyl sulphate) (Appendix 1), using either a DuPont Omni-mixer or a mortar and pestle. A few crystals of phenylthiocarbomide were added as an anti-oxidant to prevent the formation of dark phenolic pigments. Once homogenised, the volume of homogenate was made up to 10 ml and filtered through mira cloth into a beaker. The homogenate was divided equally between two JA20-108 centrifuge tubes. This was spun for 30 minutes at 10000 rpm. The pellets obtained were resuspended in 6 ml 0.1 % SDS. Two continuous 30 -

80 % (v/v) glycerol (Appendix 1) gradients were prepared in 0.1 % SDS in 38 ml SW28 rotor tubes. This was spun for 15 minutes at 15000 rpm. Each virus band was recovered, by pipetting, into a 45 ml tube. Tubes were filled to the top with sterile distilled water and centrifuged for 14 minutes at 10000 rpm. The supernatant was discarded and the pellets resuspended in sterile distilled water. This spin was repeated twice. Finally, each pellet was resuspended in 2 ml sterile distilled water.

2.2.2.4 DNA Extraction

Regardless of the virus purification process, DNA was extracted in the same way. Suspended CrleGV was spun down into a pellet for 5 min at 10000 rpm. Each pellet was resuspended in 0.2 ml 0.05 M sodium carbonate (Appendix 1), 1 % SDS – the liquid became a clear light brown shade. Equal quantities of tris-saturated phenol (Appendix 1) and cholorform:isoamyl alcohol 24:1 (Appendix 1) were added. This mixture was shaken vigorously for 30 minutes. Thereafter it was centrifuged in a bench-top microfuge at 10000 rpm for two minutes to separate the liquid into two phases. The top aqueous layer containing the DNA was removed and retained in a new vial. The remaining phenol layer was discarded. This phenol extraction process was conducted twice. Thereafter, an equal volume of chloroform:isoamyl alcohol 24:1 was added to the extracted aqueous layer and shaken vigorously. After centrifuging the top aqueous layer was again extracted and retained. This step was repeated once.

2.2.2.5 Precipitation of DNA

DNA was then precipitated by adding sodium acetate (Appendix 1) and 95 % alcohol (Appendix 1) in the proportions 10:1:20 (DNA solution: sodium acetate: 95 % alcohol). The soft white strands of the DNA could usually be observed in the solution. This was centrifuged in a bench-top microfuge at 10000 rpm for five minutes to pellet the DNA. The supernatant was carefully pipetted from the vial, leaving the DNA pellet behind. The DNA pellet was washed by adding 1 ml 70 % alcohol (Appendix 1), without resuspending, and centrifuging at 10000 rpm for one minute. The alcohol was removed

and the lid left off the vial for 30 minutes for the DNA pellet to air dry. Each DNA pellet was resuspended in 0.02 ml 10 mM TE (pH 8.0) (Appendix 1).

2.2.2.6 Analysis of DNA concentration and purity

The concentration and purity of the DNA was determined with the aid of a spectrophotometer. An appropriate dilution of DNA in either distilled water or 10 mM TE buffer (pH 8.0) (between 1/100 and 1/300) was made. Ultra violet absorption was measured at 260 nm (OD_{260}) and at 280 nm (OD_{280}) and a scan was conducted at 220 - 320 nm. Concentration of DNA was calculated according to the fact that double stranded DNA (dsDNA) at 50 µg/ml has an absorbance of 1 AU at 260 nm (Sambrook *et al.*, 1989). Purity of DNA was determined according to the ratio of the OD_{260} to the OD_{280}. Purity of precipitated and dialysed DNA was compared.

Table 2.1 DNA digests used for CrleGV.

DNA (µl)	**Enzyme**		**Buffer**		**Distilled water (µl)**	**Total volume (µl)**
	Type	**Vol (µl) (y)**	**Type****	**Vol (µl) (z)**		
x (0.6 µg)*	*Eco*RI	⅔ x	H	2 y	20 - (x+y+z)	20
	*Bam*HI	⅔ x	B	2 y		
	*Kpn*I	⅔ x	L (+ BSA***)	2 y (+$^{3}/_{10}$ z)		
	*Nde*I	⅔ x	H	2 y		
	*Sac*I	⅔ x	A	2 y		
	*Xho*I	⅔ x	H	2 y		

*Quantities of ingredients were altered according to the concentration of DNA used, aiming to have a mass of at least 0.6 µg DNA per digest per lane of gel.
**Roche Sure-Cut buffers.
***BSA = bovine serum albumin.

2.2.2.7 DNA digestion

DNA was digested separately (single enzyme digests) with six different restriction enzymes and the appropriate buffers (Roche Sure-Cut) (Table 2.1) (Sambrook *et al.*, 1989).

A mass of 0.6 µg of DNA per lane was considered to be adequate for obtaining clear restriction fragments. However, larger quantities could be used. To ensure sufficient DNA, volumes of ingredients (Table 2.1) could be altered, depending on the concentration of the DNA. However, the total volume of the digest was always 20 µl, achieved by altering the volume of distilled water. For example, if the DNA concentration was 0.2 µg/µl, then 3 µl of DNA would be required to have 0.6 µg of DNA in the digest. To this, 2 µl of enzyme, 4 µl of the appropriate buffer and 11 µl of sterile distilled water would be added. This would make up a total volume of 20 µl. If the enzyme used was *Kpn*I, then 0.6 µl of BSA (bovine serum albumin) would also be added.

Once all the ingredients were added in the correct proportions, microtubes were flicked to mix the digests. After a very brief spin (a few seconds), at 10000 rpm in the desktop microfuge, digests were incubated at 37ºC for three hours.

2.2.2.8 Electrophoresis

A volume of 4 µl (1/6 of the resulting volume) of loading buffer (Appendix 1) without bromophenol blue was added to each digest. Microtubes were flicked to mix and then briefly centrifuged. Two gels were prepared: a 1 % agarose in TBE (tris borate) buffer (pH 8.0) gel (Appendix 1) for resolution of small fragments and a 0.7 % agarose in TAE (tris acetate) buffer (pH 8.0) gel (Appendix 1) for resolution of large fragments (Table 2.2) (Sambrook *et al.*, 1989). Either λ-DNA/*Hind*III, λ-DNA/*Bste*II or λ-Mix 19 molecular weight markers (Appendix 1) were used in the outermost lanes as a reference for measuring the sizes of restriction fragments. Occasionally more than one marker was used in a gel.

Table 2.2 Details of electrophoresis gels for resolution of CrleGV DNA fragments.

Agarose	**Buffer (Solvent)**	**Tray length**	**Digest volume/well**	**Voltage**	**Duration**
1.0 %	TBE	110 mm	20 µl	100 V	2 - 3 h
0.7 %	TAE	170 mm	20 µl	25 - 45 V	16 - 20 h

After running for the required time period, gels were stained in 20 µl ethidium bromide (10 mg/ml) in 400 ml TBE or TAE (corresponding to the buffer used in the gel) for 30 minutes. Gels were then rinsed for 15 minutes in 400 ml distilled water. Stained gels were viewed on an ultra-violet transilluminator and if bands were sufficiently distinct, gels were photographed using either a Polaroid or a digital camera. In one case the gel was photographed using an Olympus OE2 camera with a 200 ISO colour slide film.

2.2.2.9 Calculation of genome size

The size (molecular weight) of each fragment was estimated by comparison with the molecular weight markers (either λ-DNA/*Hind*III, λ-DNA/*Bste*II or λ-Mix 19). This was done by plotting the distance moved by each marker fragment against the log of its known molecular weight (in kbp). From these standard curves, the molecular weight of each fragment in a digest could be estimated from the distance it had migrated. Fragment sizes were then added to give an estimation of total CrleGV genome size.

2.2.3 Characterisation of virus isolated from individual larvae

The purpose of characterising virus from individual larvae was two-fold: to determine whether more than one isolate of CrleGV could be found in the local *C. leucotreta* laboratory culture (Chapter 3); and to determine whether there was any difference in DNA profile of virus from *C. leucotreta* larvae from different regions.

2.2.3.1 Investigation for different isolates within one laboratory culture

The existing *C. leucotreta* laboratory culture was initiated from material obtained from Goedehoop Insectary in Citrusdal, Western Cape Province. The Goedehoop culture had been running for several years, and the original source of the material from which the culture had been established was unclear. There was a low level of usually non-apparent CrleGV infection in the *C. leucotreta* mother culture, as occasionally larvae spontaneously developed signs of CrleGV infection. These larvae were removed from jars and frozen separately in microtubes. (So as to curtail any infection in the culture, jars

with infected larvae were permanently removed from the rearing room.) To check for different isolates of CrleGV within the laboratory culture, viral DNA from 12 individual infected larvae, was extracted and compared. The larvae were collected from different jars on different dates over a period of several months, to maximise the chance of detecting any different isolates. Viral DNA extracted from three larvae was compared on one agarose gel. Therefore, a total of four gels were run.

2.2.3.2 Investigation for different isolates from different regions

Virus infected larvae were also obtained from a *C. leucotreta* laboratory culture at Zebediela Estate, Northern Province. As was the case with Goedehoop Insectary, this culture was maintained for the mass production of the egg parasitoid, *T. cryptophlebiae*.

Three new (and temporary) laboratory cultures of *C. leucotreta* were established with field-collected larvae from different regions: Sundays River Valley (Eastern Cape Province), Crocodile Valley Estate (Mpumalanga Province) and Citrusdal (Western Cape Province). Larvae, which appeared to be symptomatically infected with a baculovirus, were removed from the culture, placed individually in marked microtubes, and frozen (at -40°C).

2.2.3.3 Virus purification

One infected larva was placed in each microtube and distilled water added up to 1 ml. Each larva was gently squashed with a glass pestle and vortexed for 10 seconds. The homogenate was centrifuged in a bench-top microfuge for two to three seconds to pellet the insect debris. The supernatant was then transferred to a clean microtube. This was then centrifuged for 10 minutes at 10000 rpm in a bench-top microfuge. The supernatant was removed and discarded. When possible, any dark layer of debris overlying the lighter virus pellet was loosened and removed, with the aid of a pipette tip. The pellet was resuspended in 1 ml distilled water by vortexing. This was centrifuged for 10 minutes at 10000 rpm. The supernatant was removed and the pellet resuspended in 120 µl distilled water, by vortexing. To disrupt the host enzymes and proteins, 25 µl 0.5

M EDTA (Appendix 1) and 3 µl proteinase K (20 mg/ml) (Appendix 1) were added. This mixture was incubated at 37ºC for 90 minutes. Thereafter 75 µl of 1 M sodium carbonate was added and the mixture incubated for 15 minutes at 37ºC. If the liquid did not clarify, further 15 µl volumes of sodium carbonate were added, followed by 15 minute incubation at 37ºC, until the liquid became clear. A volume of 25 µl of 10 % SDS was added and incubated for 30 minutes at 37ºC. The liquid was centrifuged for one minute at 10000 rpm to pellet undissolved OBs or any remaining debris. The supernatant was transferred to a clean microtube.

2.2.3.4 DNA extraction

An equal volume of tris-saturated phenol (Appendix 1) was added to the extracted supernatant. This was agitated by gently shaking for five minutes. The solution was then centrifuged for five minutes at 10000 rpm in the desktop microfuge. The upper layer containing DNA, clearly separated from the lower phenol layer, was removed by autopipette to a clean microtube. By cutting off the pipette tip diagonally, it was possible to remove the top layer without disturbing the interface with the bottom layer. This entire procedure was then repeated, but using an equal volume of 25:24:1 tris-saturated phenol: chloroform: isoamyl alcohol, instead of the phenol alone. When the procedure was conducted a third time, an equal volume of only 24:1 chloroform: isoamyl alcohol was used. DNA was then in solution.

2.2.3.5 Dialysis of DNA

DNA extracted from a single larva was dialysed according to an innovative protocol (Hunter-Fujita *et al.*, 1998). Dialysis was chosen above precipitation, as it was considered to achieve greater purification of DNA than would precipitation. This was considered necessary when working with the small quantities of DNA extracted from individual larvae. A microtube was prepared for dialysis of the DNA, by cutting the tube 5 mm from the top. The lower section was discarded. A 20 mm length of dialysis membrane was trimmed on both sides, to make two pieces 20 x 20 mm in size. The membrane was soaked in X1 TAE buffer (pH 8.0) for five minutes. Latex gloves, rinsed

in water to remove powder, were worn to avoid contaminating the membrane with salts. Extracted DNA (approximately 250 µl) was pipetted into the microtube cap. A single piece of dialysis membrane was placed across the cap and the tubing closed onto the cap, to seal the dialysis membrane in position. These assemblies were then placed into a large beaker containing 1 ℓ of X1 TAE buffer (pH 8.0). The cap was uppermost to ensure that the membrane remained in contact with the buffer. The beaker was placed into a refrigerator at 4ºC for 36 hours – the buffer was changed three times during this period. Thereafter, assemblies were removed from the beaker using forceps, and the membrane surface dabbed gently on a tissue to dry. The membrane was nicked with a scalpel and the DNA transferred into a microtube using a pipette.

2.2.3.6 DNA Digestion

DNA was digested with restriction enzymes and appropriate buffers in the proportions: 25 µl DNA, 3 µl buffer and 1.5 µl enzyme. Microtubes were gently flicked to mix. Digests were then incubated at 37°C for four hours. Only three enzymes were used in the digestion of viral DNA extracted from individual larvae: *Bam*HI, *Kpn*I and *Eco*RI (not necessarily all three in each gel).

2.2.3.7 Electrophoresis

A volume of 4 µl (1/6 of the resulting volume) of loading buffer without bromophenol blue was added to each digest. Microtubes were flicked to mix and briefly centrifuged. A 0.7 % agarose (in TBE buffer (pH 8.0)) gel was prepared. A λ-DNA/*Bste*II molecular weight marker was used in the outermost lanes. Gels were run at 100 V for 120 - 240 minutes. Thereafter, gels were stained in 20 µl ethidium bromide (10 mg/ml) in 400 ml TBE buffer (pH 8.0) (according to the solvent used in the gel) for 30 minutes. The stain was then carefully removed and replaced with 400 ml distilled water, in which the gels were rinsed for 15 minutes. Stained gels were viewed on an ultra-violet transilluminator and, if bands were sufficiently distinct, gels were photographed with a digital camera. It was then possible to determine whether there were any obvious differences in restriction profiles between DNA extracted from different larvae.

2.3 RESULTS AND DISCUSSION

2.3.1 Virus identification

2.3.1.1 Symptomatology

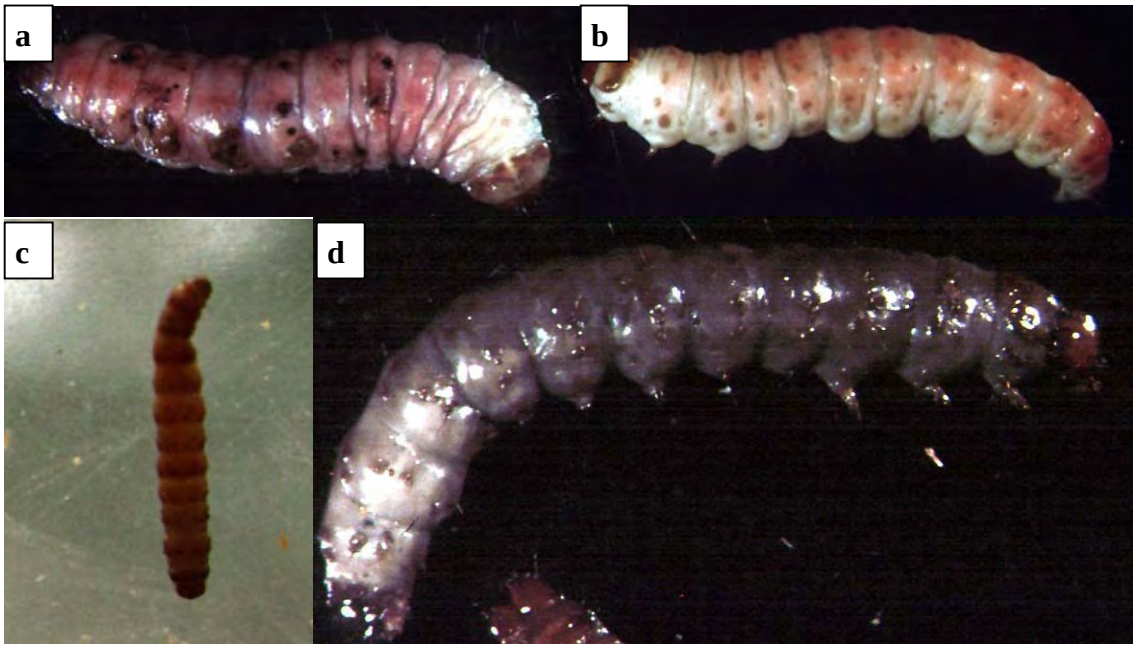

Plate 2.1 Symptomatically CrleGV infected fifth instar *C. leucotreta* larvae: with black speckling (**a**), creamy hue (**b**), inverted hanging (**c**), and brown to grey coloration (**d**).

Because of the identity of the host insect and the symptoms observed, the infection was tentatively identified as a baculovirus. The pathogen was later confirmed to be CrleGV. Most infected larvae appeared to move to the surface of the diet in which they were feeding. One of the first symptoms of infection that could be observed in larvae was a black speckling on the body (Plate 2.1a). This occurred before the larva appeared to show any more typical symptoms of infection. Thereafter, larvae would begin to take on a milky glazed appearance (Plate 2.1b), usually without losing the basis of their natural colour – pink in mature larvae and white when younger. Most larvae would then begin to climb up the inside wall of the container in which they were being reared. When death

occurred, the larva would hang in an inverted vertical position (Plate 2.1c), either from the wall or the ceiling of the container (be it cotton wool or a “Gladwrap” plastic film). Larvae could then lose the basis of their normal colour and become grey or pale brown (Plate 2.1d). If larvae were not collected shortly after they had died, they quickly became extremely soft, and would rupture easily (Plate 2.2) when attempting to remove them with a pair of forceps.

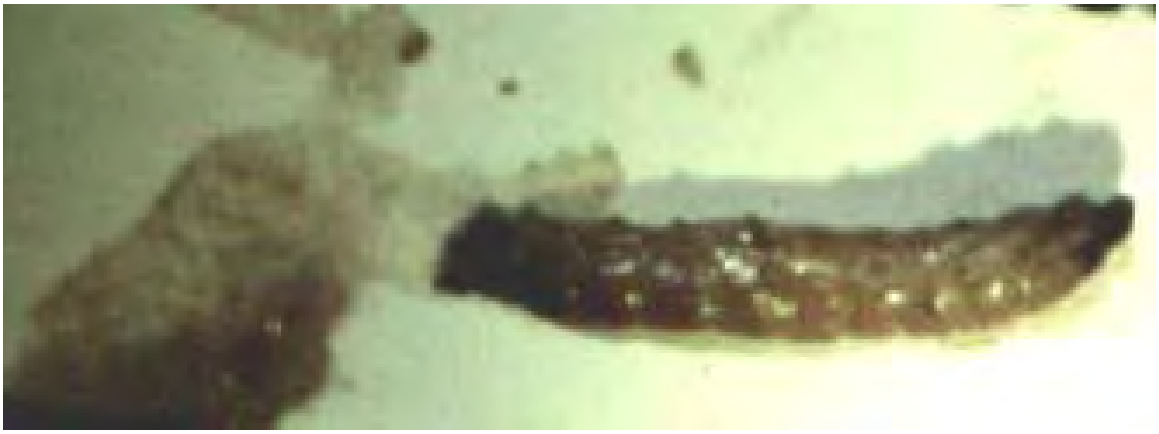

Plate 2.2 Fifth instar *C. leucotreta* larva heavily infected with CrleGV. The larva has ruptured and a milky brown liquid consisting of a high concentration of occlusion bodies has been liberated.

2.3.1.2 Preliminary identification by light microscopy

Buffalo Black stained larval smears appeared to be heavily infected with a baculovirus (Plate 2.3). Thousands upon thousands of microscopic particles, stained grey to purple in colour, were evident in high density.

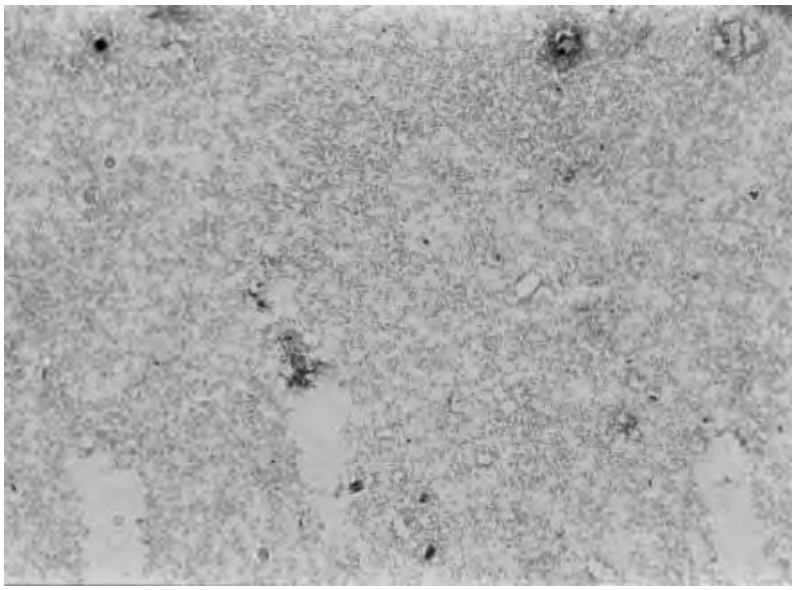

Plate 2.3 Buffalo Black stained smear of CrleGV OBs from a heavily infected *C. leucotreta* larva, viewed under oil immersion at 1000X magnification with a light microscope.

2.3.1.3 Identification by electron microscopy

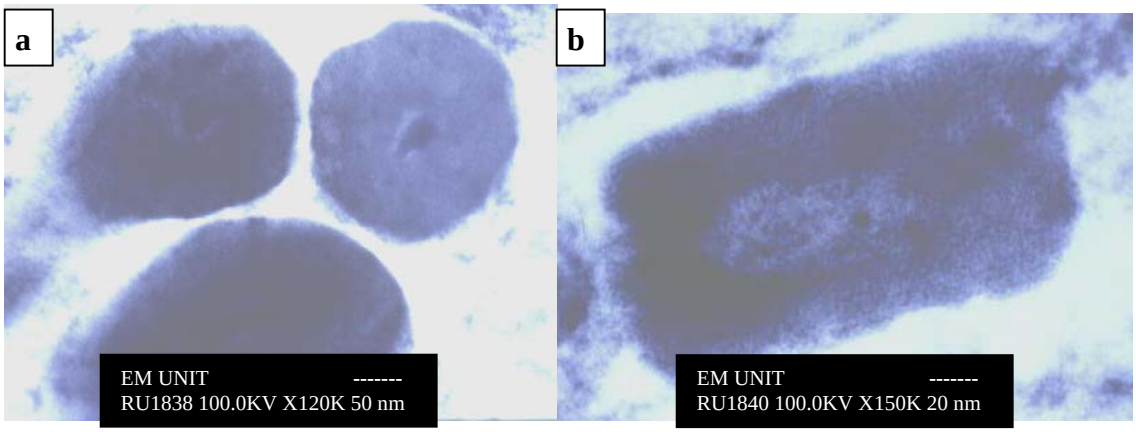

Plate 2.4 Transmission electronmicrographs of CrleGV: cross-section (**a**) and transverse section (**b**).

Transmission electronmicrographs confirmed the presence of a GV. The classical GV shape and structure was clearly evident (Plate 2.4). This included the protein granulin and inner virion or nucleocapsid. In a transverse section of an OB (Plate 2.4b) the matrix structure of the granulin was even evident. Both the diameter and the length of OBs appear to be in the region of 215 nm. This is somewhat smaller than the size recorded by Whitlock (1980), but not dissimilar to that recorded by Jehle *et al.* (1992a).

Using electronmicroscopy, Whitlock (1980) identified the existence of long virus-like rods associated with CrleGV. Granulovirus associated rods were first recorded by Smith *et al.* (1964), who theorised that these rods are produced by the virus or during the process of virus formation, and could possibly represent a method of increasing the DNA content (Smith & Brown, 1965). Smith (1976) also suggested that these rods could be formed due to a defective replicative mechanism of the virus. Such rod-like structures were not observed in the electronmicroscopic study conducted here. This is not surprising, as Whitlock (1980) reported that when centrifuged in a glycerol gradient, the rods accumulated in a band at 33 - 37 % glycerol, unlike the OBs, which formed a band in the 50 % glycerol region. In this study, no band other than the OB band, was observed when centrifuging crude virus in a 30 - 80 % glycerol gradient. An additional band was, however, often observed just above the OB band, when centrifuging in a 50 - 60 % sucrose gradient. An electronmicroscopic study was not conducted on the contents of this band.

2.3.2 Characterisation of virus isolated from multiple larvae

The precipitation and dialysis procedures each produced the same yields of DNA; however, dialysed DNA appeared to be somewhat purer (Table 2.3). At the measured concentration of 0.3 µg/µl, 2 µl of DNA would be sufficient per digest (or per lane in a gel). DNA concentrations tended to vary between 0.2 µg/µl and 2.34 µg/µl.

Table 2.3 Comparison of CrleGV DNA concentration and purity, purified either by precipitation or dialysis.

Method of DNA purification	**DNA concentration**	**DNA purity (OD_{260}:OD_{280})**
Precipitation	0.3 µg/µl	1.62
Dialysis	0.3 µg/µl	1.82

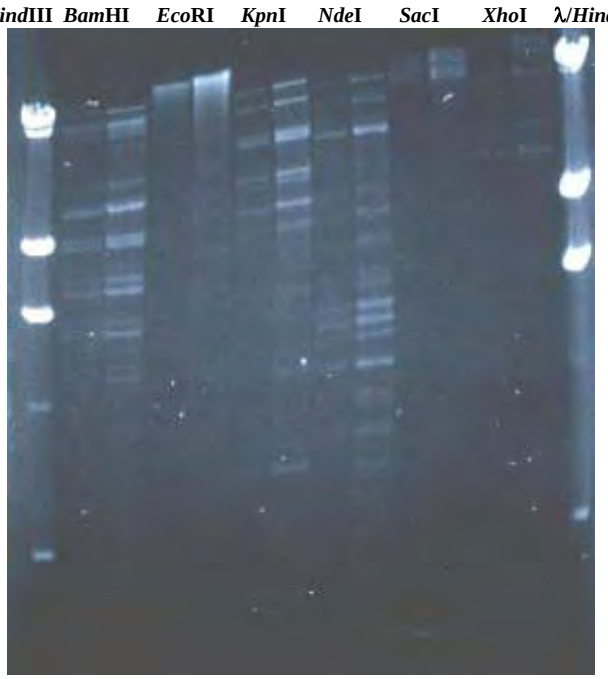

Plate 2.5 Profile of CrleGV DNA digested with various restriction enzymes and run on 0.7 % agarose gel in TAE at 25 V for 16 hours. Two lanes were run for each digest: 4 µl in the first and 20 µl in the second.

Initially 0.7 % agarose gels in TAE were run at 25 - 30 V for 16 hours (overnight), with either λ-DNA/*Hind*III (Plate 2.5) or λ-DNA/*Bste*II (Plate 2.6) molecular weight markers. This was sufficient to resolve and estimate the size of most of the fragments. However, it was necessary to run additional gels specifically for resolution and estimation of the smallest and largest fragments. In both of these first two gels, two adjacent lanes were loaded with either 4 µl or 20 µl of a specific digest, repeated for each of the six enzymes. The lower volume of digest was hopelessly insufficient to be able to observe all fragments clearly, and consequently, in subsequent gels, only one lane was loaded with 20 µl of each digest. In Plate 2.5 it can be seen that *Eco*RI did not cut the DNA. This was a problem periodically experienced with this particular restriction enzyme. It is possible that DNA was occasionally insufficiently pure, containing sticky low mol weight inhibitors that prevented *Eco*RI cutting. However, the same problem was not experienced with the other enzymes.

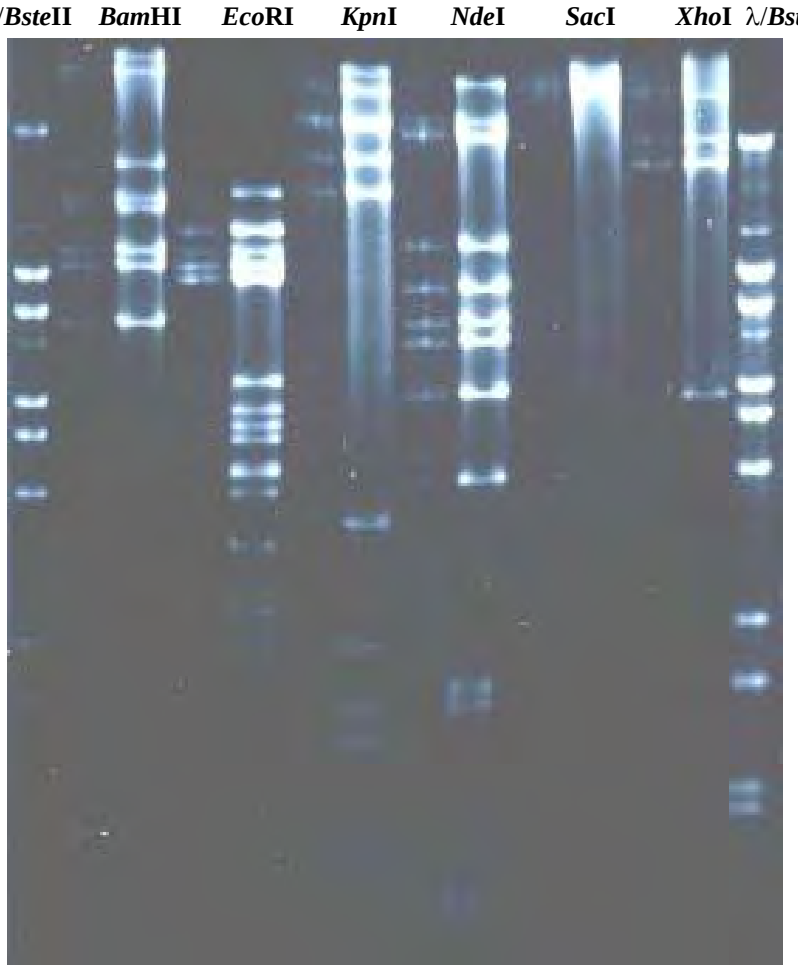

Plate 2.6 Profile of CrleGV DNA, digested with various restriction enzymes and run on a 0.7 % agarose gel in TAE at 30V for 16 hours. Two lanes were run for each digest: 4 µl in the first and 20 µl in the second.

Agarose (1.0 %) gels in TBE buffer (pH 8.0), run with either λ-DNA/*Hind*III or λ-DNA/*Bste*II markers or both, for just 90 minutes were able to resolve the smallest fragments in the digests (Plate 2.7). Fragments smaller than 1 kbp were generated by *Eco*RI, *Nde*I, *Kpn*I and *Bam*HI. The molecular weight of the smallest fragment (that of *Bam*HI) was 0.298 (Table 2.4). The size of these small fragments could be estimated with the aid of either λ-DNA/*Hind*III or λ-DNA/*Bste*II, as the smallest fragments of these DNA markers measured 125 and 117 bp respectively.

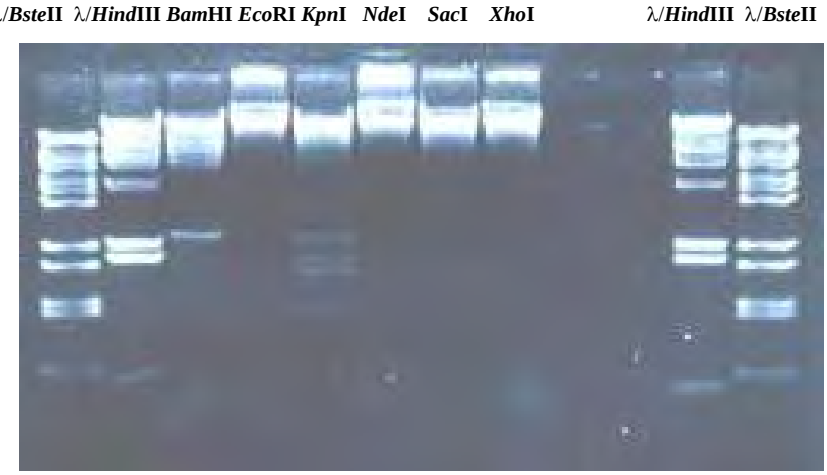

Plate 2.7 Profile of CrleGV DNA, digested with various restriction enzymes and run on a 1.0 % agarose gel in TBE at 100 V for 90 minutes.

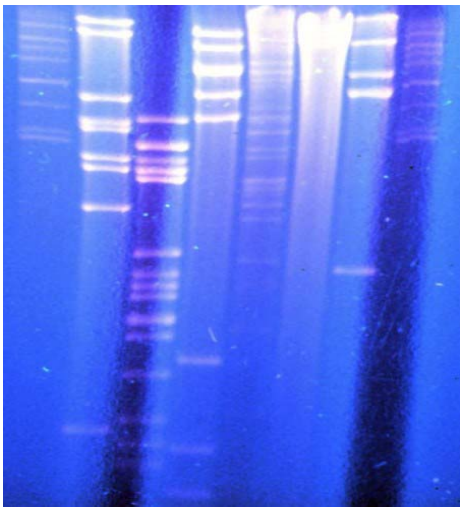

Plate 2.8 Profile of CrleGV DNA, on an ultra-violet trans-illuminator, digested with various restriction enzymes and run on a 0.7 % agarose gel in TAE at 30 V for 17 hours. (Photographed with an Olympus OE2 camera with a 200 ISO colour slide film).

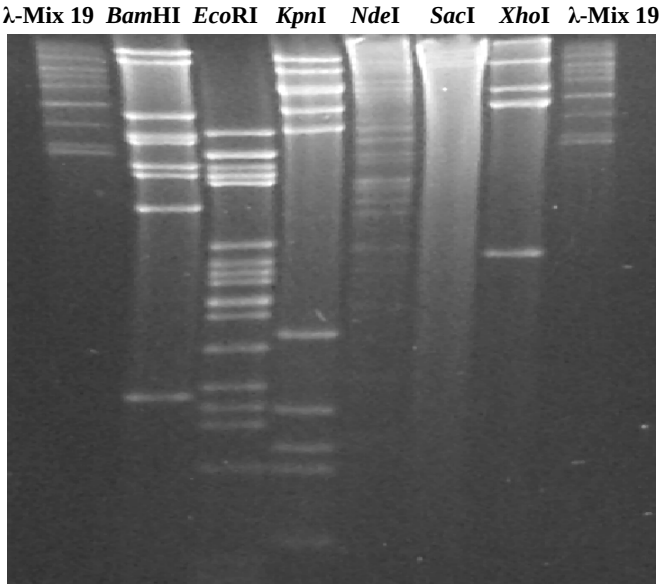

Plate 2.9 Profile of CrleGV DNA, digested with various restriction enzymes and run on a 0.7 % agarose gel in TAE at 30 V for 17 hours.

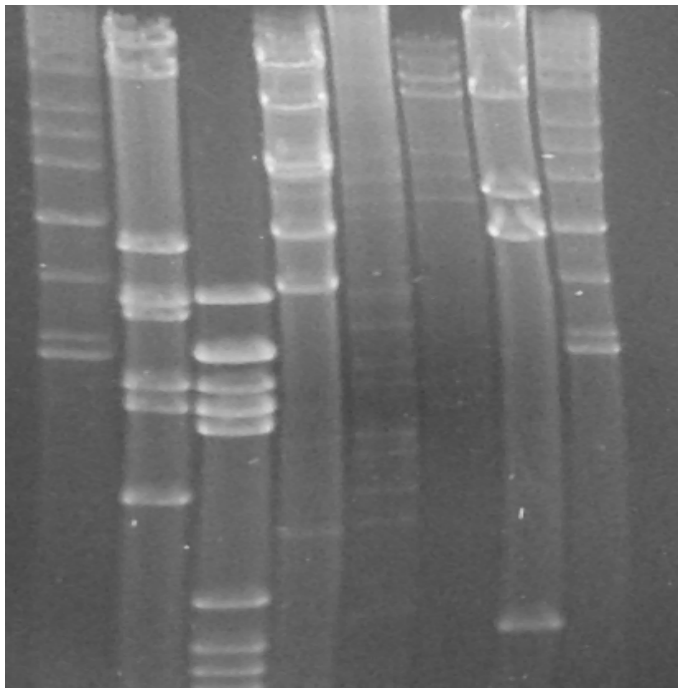

Plate 2.10 Profile of CrleGV DNA, digested with various restriction enzymes and run on a 0.7 % agarose gel in TAE at 45 V for 20 hours.

In order to estimate the sizes of the largest fragments, 1.0 % agarose in TAE gels were run for 17 hours at 30 V (Plates 2.8 & 2.9). The only marker which was appropriate for estimation of the size of the larger fragments was λ-Mix 19. The largest fragment of λ-Mix 19 was 48.502 kbp, whereas of λ-DNA/*Hind*III and λ-DNA/*Bste*II these were 23.130 and 8.454 kbp, respectively. The largest fragment generated by any of the digests was 41.495 kbp (that of *Xho*I) (Table 2.4).

As the four large *Sac*I fragments were so closely positioned, it was necessary to run the 1.0 % gel at 45 V for 20 hours in order to differentiate them from one another (Plate 2.10). The size of these four fragments were estimated to be 32.359, 27.797, 23.442 and 21.478 kbp (Table 2.4). It appears that there may have been some contamination of the DNA used in this gel, as three additional bands were noted at around 13 kbp in the *Sac*I digest. These did not appear in any of the other gels.

Through conducting a series of restriction endeonuclease analyses, a full DNA profile was formulated for CrleGV with six restriction enzymes (Plate 2.11). Fragment sizes were estimated for each gel from standard curves of distance moved by the marker fragment, against the log of its known molecular weight. Bands which, because of their intensity, were regarded as being doubles were considered to contain two fragments identical in size. Where there existed more than one size estimation for any particular fragment (due to the fragment size being estimated from more than one gel), the figures were averaged. By adding together all fragment sizes for each digest, six measurements of the total size of the CrleGV genome were estimated (Table 2.5). There was great variation in the estimations, from 104.659 kbp for *Xho*I to 122.354 kbp for *Nde*I. As the main purpose of this study was not molecular investigation of CrleGV, but rather its applied development, no attempt was made to achieve greater accuracy of estimation. This could have been done through double digestion (simultaneous digestion of DNA with two restriction enzymes instead of one), particularly for more accurate estimation of the size of the larger fragments. This exact study is currently being conducted in great detail by Singh (2002), as part of the greater project on this particular CrleGV isolate.

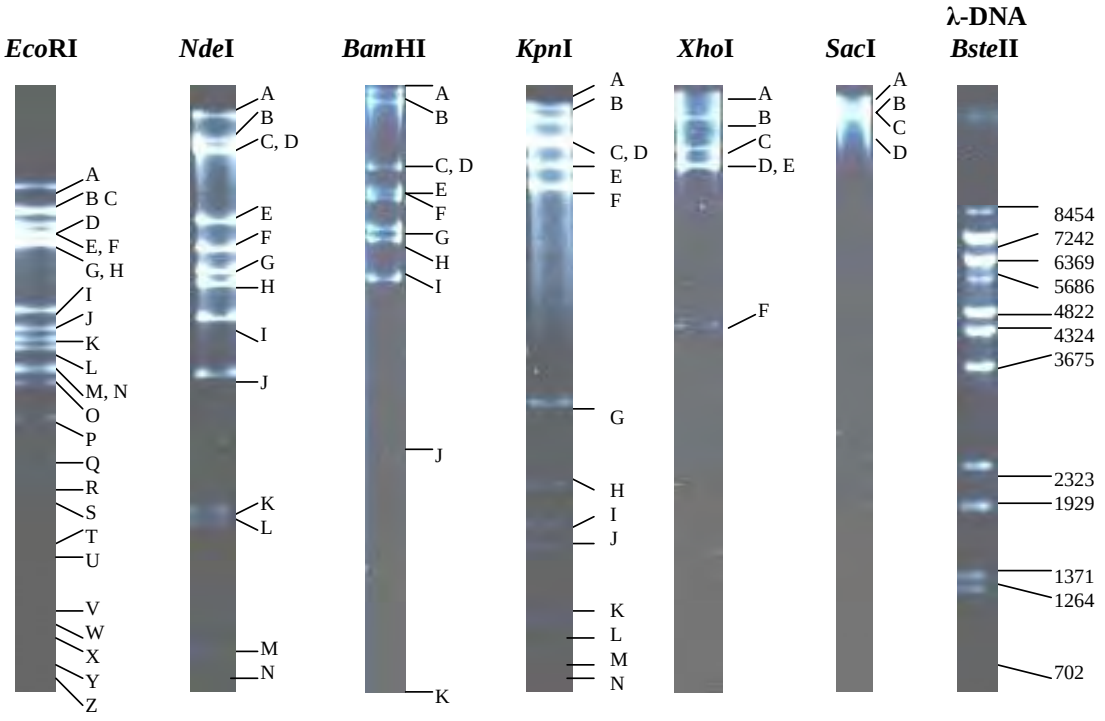

Plate 2.11 DNA profile of CrleGV-SA with six single restriction enzymes digests.

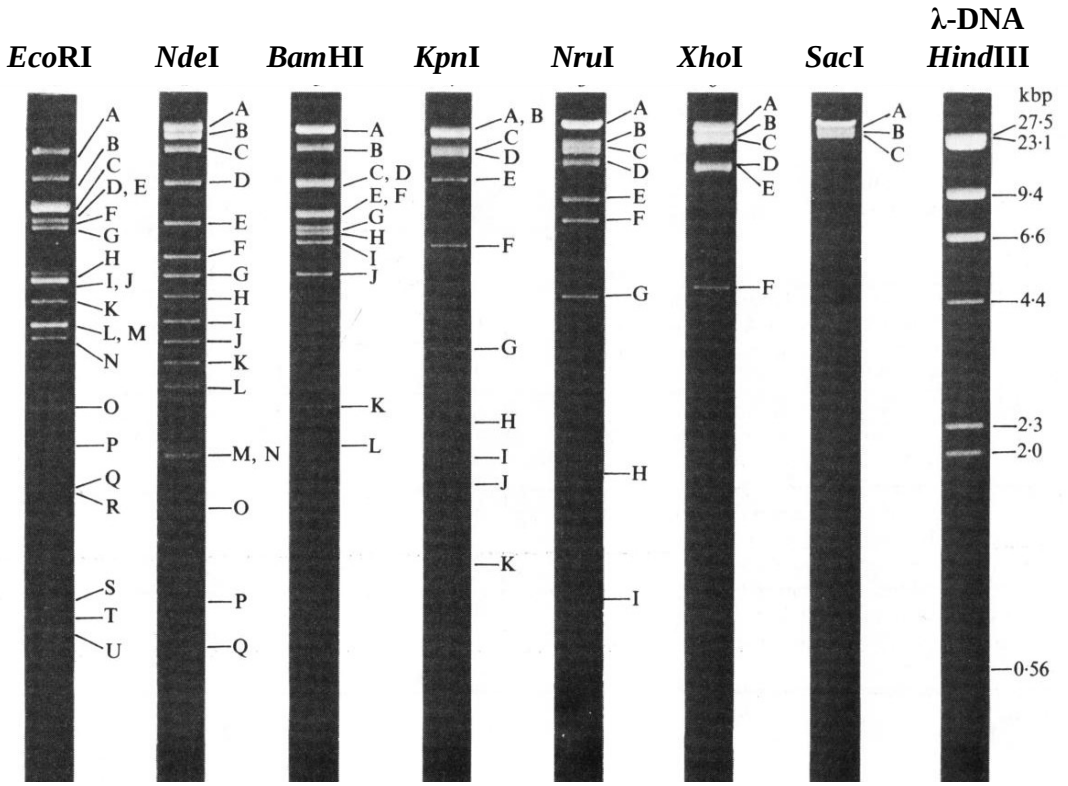

Plate 2.12 DNA profile of CrleGV-CV3 with seven single restriction enzyme digests (Jehle *et al.*, 1992a).

Despite there being great variation in the estimation of genome size with the various enzymes, if totals for each enzyme were averaged, the genome was estimated to be 112.624 kbp. This is very similar to Jehle *et al.*'s (1992a) estimation of genome size for CrleGV-CV3 of 112.350. Due to this similarity, and the obvious similarity to CrleGV-CV3 in DNA profile (Plate 2.12) (Jehle *et al.*, 1992a), it can be stated with a large degree of certainty that the virus isolated in this study is a South African isolate of CrleGV.

Table 2.4 Size* of CrleGV-SA restriction fragments.

Fragment	***Eco*RI**	***Nde*I**	***Kpn*I**	***Bam*HI**	***Xho*I**	***Sac*I**
A	8.610	23.014	26.915	27.227	41.495	32.359
B	8.279	22.594	20.045	23.067	21.528	27.797
C	8.279	18.113	18.923	9.817	13.521	23.442
D	7.852	18.113	17.989	9.817	11.534	21.478
E	7.534	8.035	14.191	9.311	11.534	
F	7.534	6.998	10.965	8.974	5.047	
G	7.128	6.266	3.234	7.709		
H	7.128	5.861	2.173	7.211		
I	5.070	4.539	1.886	5.848		
J	4.875	3.673	1.738	2.559		
K	4.416	1.888	1.167	0.298		
L	4.207	1.807	1.016			
M	3.837	1.807	0.582			
N	3.837	0.646	0.436			
O	3.715					
P	3.296					
Q	2.754					
R	2.495					
S	2.312					
T	1.811					
U	1.671					
V	1.035					
W	0.916					
X	0.809					
Y	0.676					
Z	0.484					
Total	110.560	122.354	111.838	121.26	104.659	105.076

* Expressed in kbp (kilobase pairs) relative to DNA molecular size standards.

However, not only are there sufficient similarities between this CrleGV isolate and CrleGV-CV3 to state that they are closely related, but there are several distinct

differences underlining the novelty of the new CrleGV isolate. Consequently, it is justifiable to refer to this novel isolate of CrleGV as CrleGV-SA. A direct comparison between DNA profiles of CrleGV-SA and CrleGV-CV3 with the six different restriction enzymes used, can be made (Plates 2.11 & 2.12; Tables 2.4 & 2.5). Exact sizes of fragments cannot be compared as the fragment sizes calculated for CrleGV-SA are not sufficiently accurate.

Table 2.5 Size* of CrleGV-CV3 restriction fragments (Jehle *et al.*, 1992a).

Fragment	***Eco*RI**	***Nde*I**	***Kpn*I**	***Bam*HI**	***Xho*I**	***Sac*I**
A	17.00	27.00	27.40	32.00	36.90	56.60
B	11.60	20.20	27.00	16.20	24.00	30.50
C	8.50	14.60	16.20	9.85	22.60	25.20
D	8.30	9.40	15.00	9.75	12.10	
E	8.30	6.80	10.00	7.70	12.10	
F	7.60	5.40	6.00	7.50	4.65	
G	7.30	4.75	3.30	6.85		
H	5.35	4.25	2.30	6.50		
I	5.00	3.75	1.90	6.20		
J	5.00	3.50	1.65	5.00		
K	4.50	3.05	1.10	2.60		
L	4.00	2.75	0.55	2.10		
M	4.00	1.95				
N	3.70	1.95				
O	2.60	1.45				
P	2.25	0.90				
Q	1.75	0.65				
R	1.65					
S	1.00					
T	0.85					
U	0.75					
V	0.53					
W	0.46					
X	0.44					
Total	112.43	112.35	112.40	112.25	112.35	112.30

* Expressed in kbp (kilobase pairs) relative to DNA molecular size standards.

Using *Eco*RI, 26 fragments were identified with CrleGV-SA DNA (Table 2.4) compared to 24 with CrleGV-CV3 DNA (Table 2.5). The largest CrleGV-SA fragment was 8.610 kbp whereas CrleGV-CV3 had two fragments distinctly larger than this. Several other

apparent differences exist between the *Eco*RI profiles of the two isolates, but the exact sizes of fragments cannot be compared.

Using *Nde*I, 14 fragments were identified with CrleGV-SA DNA (Table 2.4) compared to 17 with CrleGV-CV3 DNA (Table 2.5). There was little difference in the range of fragment sizes of the two profiles: 23.014 - 0.646 for CrleGV-SA and 27.000 - 0.650 for CrleGV-CV3.

Using *Kpn*I, 14 fragments were identified with CrleGV-SA DNA (Table 2.4) compared to 12 with CrleGV-CV3 DNA (Table 2.5). The most obvious difference between the two isolates was the occurrence of a fragment at 6.000 kbp on the CrleGV-CV3 profile, and the absence of any fragment between 10.965 and 3.234 kbp on the CrleGV-SA profile.

Using *Bam*HI, 11 fragments were identified with CrleGV-SA DNA (Table 2.4) compared to 12 with CrleGV-CV3 DNA (Table 2.5). Prominent differences noted were: the smallest fragment of CrleGV-SA was 0.298 kbp and that of CrleGV-CV3 was 2.100 kbp; the two largest fragments of CrleGV-SA were 27.227 and 23.067 kbp and of CrleGV-CV3 were 32.000 and 16.200 kbp. Despite the lack of accuracy in the estimation of fragment sizes of CrleGV-SA, these differences were regarded as being sufficiently large to be meaningful.

There was little difference between the DNA profiles of CrleGV-SA (Table 2.4) and CrleGV-CV3 (Table 2.5) with *Xho*I.

*Sac*I generated four fragments from CrleGV-SA DNA (Table 2.4) but only three from CrleGV-CV3 DNA (Table 2.5).

Detailed comparison of the profile of this CrleGV-SA isolate with that referred to by Fritsch (1989) as ClGV-SA was not possible. Although Fritsch produced a DNA profile for the isolate (Plate 2.13), she did not characterise it i.e. fragment sizes for the digests used (*Eco*RI, *Bam*HI and *Hind*III) were not recorded. However, Fritsch (1989) detected

two submolar bands at around 10 kbp, produced by the *Bam*HI digest (Plate 2.13), which did not appear in the *Bam*HI digest of CrleGV-SA DNA in this study. This would seem to indicate that the two South African isolates are different. DNA profiles of two other isolates compared by Fritsch (1989) (Plate 2.13) were distinctly different from CrleGV-SA. One of them was the Cape Verde isolate characterised by Jehle *et al*. (1992a). The other was an isolate from the Ivory Coast. There were several obvious differences in the DNA profiles of this isolate and CrleGV-SA (Plate 2.11) produced by both the *Eco*RI and *Bam*HI digests.

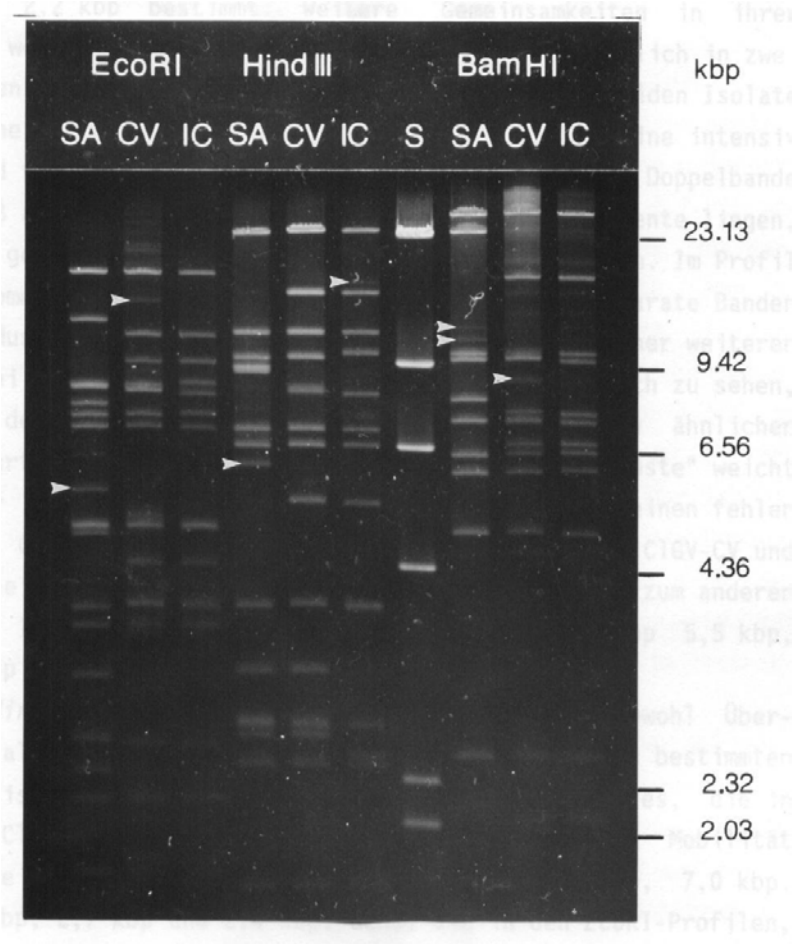

Plate 2.13 DNA profiles of three different CrleGV isolates (Fritsch, 1989).

2.3.3 Characterisation of virus isolated from individual larvae

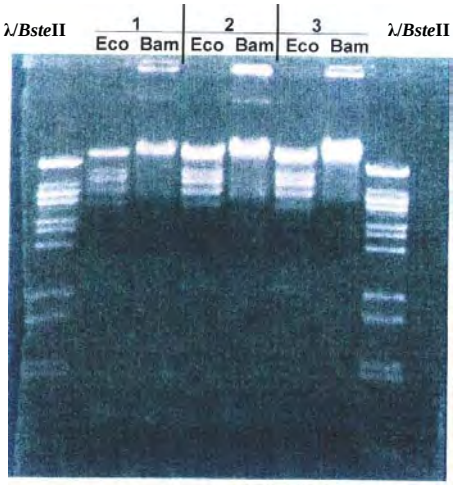

Plate 2.14 DNA profiles (generated by gel electrophoresis after digestion with two different restriction enzymes) of CrleGV extracted from three individual larvae from one laboratory culture of *C. leucotreta*. This 1.0 % agarose gel in TBE was run at 100 V for 90 mintes with λ-DNA/*Bste*II molecular weight markers.

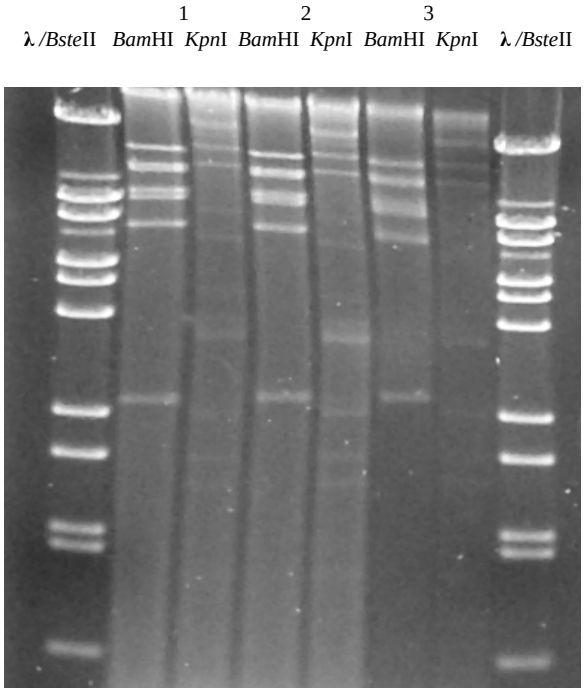

Plate 2.15 DNA profiles (generated by gel electrophoresis after digestion with two different restriction enzymes) of CrleGV extracted from three individual larvae from one laboratory culture of *C. leucotreta*. This 1.0 % agarose gel in TAE was run at 100 V for 4 hours.

2.3.3.1 Investigation for different isolates within one laboratory culture

No differences could be detected in the profiles of DNA extracted from individual larvae, digested with either *Bam*HI or *Eco*RI, run at 100V for 90 minutes (Plate 2.14). Viral DNA from three larvae was examined per gel. Another three gels were run for four hours at 100 V, with viral DNA digested with *Bam*HI and *Kpn*I (viral DNA from three larvae per gel) (Plate 2.15). Once again, no difference could be detected between profiles. It therefore appeared that no more than one dominant isolate of CrleGV occurred in the *C. leucotreta* laboratory culture.

2.3.3.2 Investigation for different isolates from different regions

Very few larvae, which appeared to be infected with virus, were collected from the Sundays River Valley and Mpumalanga cultures. So as not to sacrifice precious virus, no attempt to purify virus from these larvae was made. Once a CrleGV-free laboratory culture of *C. leucotreta* has been established (attempts are currently being made in this regard), any virus extracted from these larvae will be bulked up *in vivo*, without any danger of contamination with a resident non-apparent virus. It was not possible to bulk up any virus in larvae of the Sundays River Valley and Mpumalanga mother cultures themselves, as these both died out, after a few generations in the laboratory. This was attributed to the often, normal difficulty of getting field collected insects to adapt to laboratory conditions.

Several larvae showing symptoms of baculoviral infection were identified and removed from the Citrusdal culture. Of the three new regional cultures, virus was therefore isolated only from the Citrusdal culture. The DNA profile of this virus was compared with that of virus extracted from the Zebediela culture and of confirmed CrleGV-SA.

DNA was digested with *Bam*HI, *Eco*RI or *Kpn*I. There appears to be some difference between the profiles of all three isolates. The Zebediela and Citrusdal *Eco*RI profiles lacked the largest band, which was present in the CrleGV-SA profile (Plate 2.16). The

Zebediela *Kpn*I profile also lacked the large band, which was present in the profiles of both the Citrusdal and CrleGV-SA isolates (Plate 2.16). However, a confusing factor was the presence of a band in the *Kpn*I profiles of all three isolates, between4.8 and 5.7 kbp (indicated by arrows in Plate 2.16). This band was not observed in *Kpn*I profiles in previous gels with CrleGV-SA. There may therefore, have been some DNA contamination in the *Kpn*I digests. The DNA profiles of these three CrleGV isolates should for that reason be compared again in another gel. This ought to confirm whether the viruses from the three different sources represent different isolates.

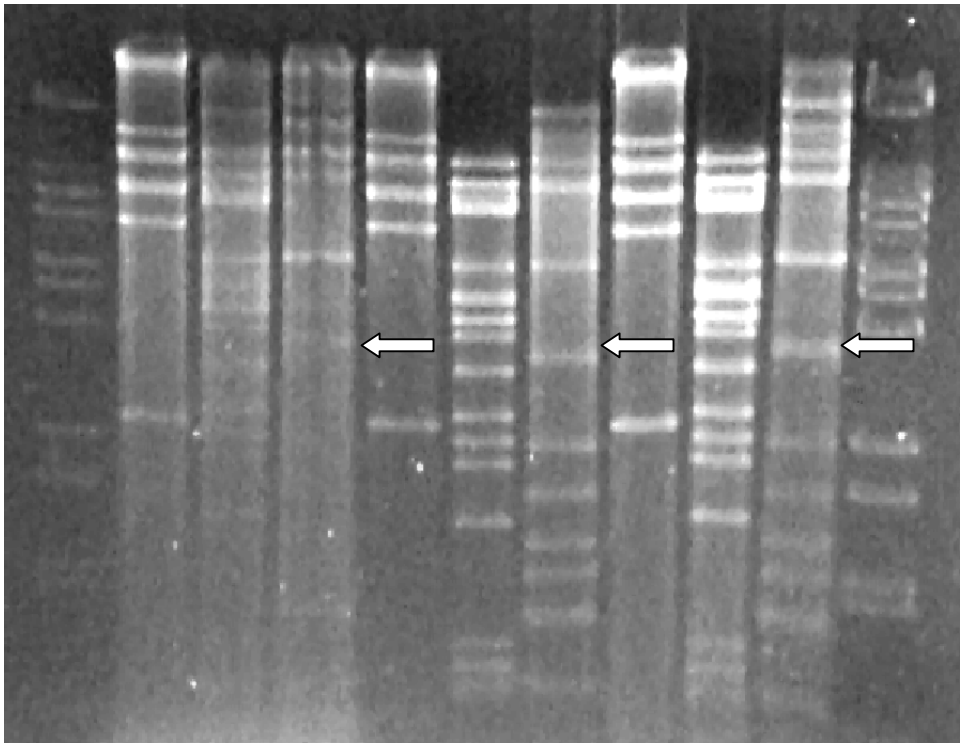

Figure 2.16 DNA profiles (generated by gel electrophoresis after digestion with three different restriction enzymes) of CrleGV extracted from three individual *C. leucotreta* larvae, each from a different source (confirmed CrleGV-SA, Zebediela Insectary and field-collected from Citrusdal). This 0.7 % agarose gel in TBE was run at 100 V for 2 hours. (Arrows indicate the position of a band in *Kpn*I profiles, not observed in previous gels with CrleGV-SA).

2.4 CONCLUSION

The virus isolated from *C. leucotreta* larvae was characterised by restriction endonuclease analysis and shown to be a previously uncharacterised isolate of CrleGV, which is now referred to as CrleGV-SA. The OB has a diameter estimated to be 215 nm and the size of the genome is in the region of 112 kbp. Both distinct similarities and distinct differences were noted between CrleGV-SA and a previously characterised genotype from Cape Verde Islands, CrleGV-CV3. In a limited survey, it appeared that there may be more than one South African isolate of CrleGV. This should be confirmed in future work.

This information on CrleGV-SA places the project in good stead to proceed. It is now possible to establish, at any stage, whether the virus being used is the originally characterised isolate, or if a contaminant virus has taken over. This will be extremely important if the virus ever reaches a stage of commercial viability as a biological control agent.

If any other strains of CrleGV are isolated in future, and differences in potency or productivity between strains are identified, characterisation will enable distinction between the strains.

3

BIOASSAYS

3.1 INTRODUCTION

Bioassays are used to measure dose-response relationships or time-response relationships. However, dose-responses with viruses are unlike those with chemical insecticides. It is assumed that each individual virus particle is capable of inducing infection, independently of any others that might be present (Ridout *et al*., 1993). Therefore increases in responses to increasing dosage, do not result from additive effects within the organism, but from increased probability of one or more virus particles successfully passing through the various natural barriers to infection. Increases in dosages will only have the desired effect if the dosages (virus particles) are evenly distributed between droplets.

Jones (2000) differentiates between mass dosing bioassays and individual dosing bioassays for determining dose-response relationship. A number of different techniques can be employed in conducting individual dosing bioassays, most of which are based on allowing a larva to consume the whole of a substrate on which the virus has been placed. Therefore, an accurate assessment of virus dosage consumed can be made, and median lethal dose (LD_{50}) (the dose required to kill 50% of the test insects), as opposed to median lethal concentration (LC_{50}) (the concentration required to kill 50% of the test insects), can be estimated. For the purpose of this study it is considered more important to determine LC_{50} than LD_{50}, as in field application (the ultimate objective of this study) it is impossible to determine exactly what dose of virus will be consumed by a larva.

The pathogenicity of baculoviruses has been characterised mostly by their median lethal dose (an estimate of the infectivity of the virus to its host) (van Beek & Hughes, 1998). Virulence, which in the case of baculoviruses is best determined by the speed by which a given virus elicits the desired response (median lethal time), is another important measure of the effectiveness of the virus as a biopesticidal agent and has received considerably less attention in the past. However, this has changed, not only as a means of identifying the most virulent isolates for field use (Hughes *et al.*, 1983), but due to the reduction in killing time by genetic modification of baculoviruses (van Beek & Hughes, 1998).

It is essential that dose-response and time-response tests are conducted in the laboratory before any field trials are executed. Obviously, the life stage used in the laboratory should be the same life stage that will be targeted in the field i.e. neonate larvae. Firstly, to justify further investigation, 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. Secondly, the concentrations and application rates that should be tested in the field, must be established. Despite there often being great disparity between laboratory and field conditions, and differences in the biology of both the host and the pathogen in these different environments (Evans, 1994), it is important to establish a starting point. Thirdly, a norm for pathogenicity must be established. This can be used as a quality control standard for the testing of all further batches of virus. Once an acceptable bioassay protocol has been established, bioassays can also be used to test the impact of formulation additives on virus potency and to test samples of virus recovered from the field in order to further understand activity.

It is also important to conduct bioassays with the life stage that will be used for virus production i.e. fifth instar larvae. By so doing, it will be possible to determine the concentration of virus with which the diet should be inoculated and the incubation time after which virus infected-larvae should be harvested.

3.2 MATERIALS AND METHODS

3.2.1 Surface dose bioassays with neonate larvae

3.2.1.1 General methodology

Firstly, it was necessary to establish a suitable methodology for conducting the bioassays. Survival of neonate larvae was compared on the standard artificial diet (described in sections 4.3.5 and 4.4) in three different containers. These were 25 cell bioassay trays (100 mm x 100 mm x 17 mm deep, each cell being 20 mm x 20 mm x 17 mm) (Sterilin, UK), plastic pots (30 ml capacity) (Evron, South Africa) and plastic Petri dishes (88 mm in diameter and 12 mm deep) (Hepro Cape, South Africa). As the diet was autoclaved and therefore not fluid, it could not be poured. It was therefore not possible to prepare the diet within any of the three plastic containers. Diet had to be prepared in heat resistant containers and transferred into the bioassay containers. The standard artificial diet was prepared in round glass pie dishes measuring 220 mm in diameter and 40 mm deep. In each dish 94 g of dry ingredients, in their standard proportions, were mixed with 100 ml of distilled water. This made a mixture, sufficiently moist to settle in a thin layer over the bottom of the dish. Each dish was sealed with a layer of silver foil and either autoclaved at 121ºC for 20 minutes or baked in an oven at 180ºC for 25 minutes. Baking was sometimes preferred to autoclaving for practical reasons – more dishes of diet could be prepared in the oven at one time than in the autoclave. The cooked diet was placed in a laminar flow cabinet to dry.

Once dry, sterile 25 cell bioassay trays (Plate 3.1) were impressed, like biscuit cutters, inverted into the diet. After sliding the trays slightly, while still impressed, to loosen the diet from the dish surface, the trays were lifted. Diet remained inserted within the cells. As the thickness of the diet was less than the depth of the tray, it was necessary to press the diet down to the bottom of the tray. After larvae had been placed into cells, trays were closed and sealed with multiple layers of paper towelling, followed by a layer of thick transparent plastic and the tray lid. These were firmly held in place with the aid of four Bulldog clips – one on each side of the tray.

Plate 3.1 Bioassay tray (with 25 cells) filled with artificial diet, with paper towelling, plastic sheet, plastic lid (underneath) and Bulldog clips, used for closing and sealing the tray. These trays were tested and used for dose-response surface dose bioassays with CrleGV-SA against neonate and fifth instar *C. leucotreta* larvae.

Diet for the 30 ml pots (Plate 3.2) was prepared in a similar way, except that 188 g of diet and 200 g of distilled water were used in each dish. Once dry, round plugs were cut from the diet, using a custom-made 23.5 mm diameter biscuit cutter. They were tightly inserted into the pots. Once larvae were placed into the pots they were sealed with plastic lids, which were plugged with cotton wool tightly inserted into a central hole (± 15 mm in diameter). It was found that without the cotton wool plug, moisture accumulated in the pot.

Diet for bioassays conducted in Petri dishes was prepared in the same way as described for both the bioassay trays and the pots. Smaller square diet plugs, measuring 7 mm x 7 mm x 5 mm were cut from the diet prepared as for the bioassay trays. From diet prepared as for the pots, larger diet plugs, measuring, 20 mm x 15 mm x 5 mm were cut. These were placed individually into the plastic Petri dishes.

Therefore, in one trial, survival of neonate larvae in 25 cell bioassays trays, pots and Petri dishes was compared (Table 3.1).

Plate 3.2 Plastic pots (30 ml capacity) containing artificial diet. These pots were tested and used for time-response surface dose bioassays with CrleGV-SA against neonate larvae (with a layer of filter paper under the lid instead of a cotton wool plug in the lid) and fifth instar *C. leucotreta* larvae.

Table 3.1 Containers compared and numbers of neonate *C. leucotreta* larvae used in a survival assessment trial (trial was not replicated).

Container		**Number of larvae per cell/pot/dish**	**Total number of larvae**
25 cell bioassay tray		1	25
		1	25
Pot		5	15
		10	30
Petri dish	Small plug*	1	10
	Large plug*	1	10

*Dimensions of large diet plugs were 20 mm x 15 mm x 5 mm; dimensions of small diet plugs were 7 mm x 7 mm x 5 mm.

Survival of larvae was determined after 8 days. After 24 hours, larvae from one bioassay tray were moved to fresh diet in another bioassay tray. After a further seven days, survival of larvae was again checked. This was done to simulate the most appropriate methodology for determining dose-response and time-response curves i.e. to keep larvae on virus inoculated diet for only 24 hours and then to move larvae to untreated diet.

As survival was generally unsatisfactory, it was considered necessary to change the diet to make it more suitable for assaying neonate larvae. A diet containing agar, which could be poured into containers while still hot, and settle in a smooth even layer, was considered to be more suitable. Different quantities of diet and agar were tested. The protocol settled on was:

1. Autoclave 6 g agar in 167 ml distilled water at 121°C for 15 min.

2. Briefly homogenise 50 g dry ingredients (normal proportions of artificial diet; see section 4.3.5) in 167 ml distilled water heated to 70°C.
3. Add autoclaved agar while still liquid (well above 50ºC) and homogenise briefly.

Diet was then poured into the container (bioassay trays or pots) as a thin layer of 5 - 10 mm. The thinner the diet, the easier it was to detect the larvae, although if there was less than 5 mm of diet, it would desiccate too rapidly. Depending on the amount of diet required, the quantities of ingredients could be doubled or tripled. Diet was allowed to cool in the laminar flow cabinet in which the preparation took place. Larvae were then placed onto the diet, one larva per cell (of the bioassay trays) or 10 larvae per pot. This was done with the aid of a size 000 paint brush, which had been sterilised in 2 % sodium hypochlorite and rinsed in sterile distilled water. To ensure that larvae were from the same batch and had emerged at the same time, egg sheets, removed after 24 h exposure to moths, were placed into sealed honey jars. Only larvae from jars in which emergence began that morning, were used that day. Also, before placing larvae onto the diet, all larvae were cleared from the inside of the honey jar lid. The lid was then replaced and only larvae moving onto the lid within the next few minutes were used. This was done to ensure that only sufficiently fit larvae were used. Bioassay trays were closed and sealed in the same manner as before. Pots were sealed with a single layer of filter paper (to absorb excess moisture) inserted underneath the plastic lid. Containers were kept at 27°C. After eight days, survival of larvae was recorded by carefully dissecting the diet. This was done with the aid of a 5X magnification “Optivisor” head-loop (Donegan Optical Co., USA).

3.2.1.2 Dose-response bioassays

Surface dose bioassays were conducted in the 25 cell bioassay trays (Plate 3.1). Each cell was filled with a layer of 5 - 10 mm of diet with agar. Five five-fold dilutions of purified CrleGV in sterile distilled water (described in sections 2.2.2.1 - 2.2.2.3) and a sterile distilled water control were used (Fig. 3.1). 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. It was previously ascertained that 50 µl was sufficient to fully cover the surface of each square of diet. Inoculated bioassay trays were left within the laminar flow cabinet for ± 30 minutes, until the diet had dried adequately. One neonate larva was then placed into each cell. All larvae were from the same batch of eggs, having all hatched on the morning that the assay was initiated. Trays were sealed in the manner described above, marked and kept at 27ºC.

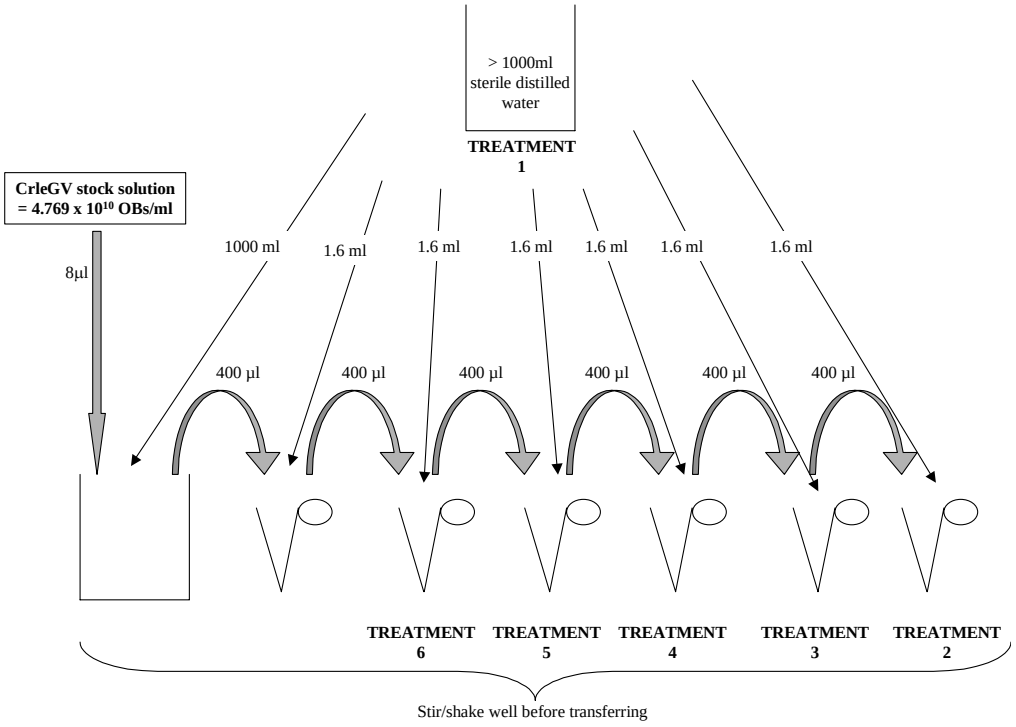

Figure 3.1 Five-fold dilution series of CrleGV-SA for surface treatment dosage-mortality bioassays with neonate *C. leucotreta* larvae. Each treatment was thoroughly mixed (stirred or shaken) prior to transferral of the 400 µl aliquot to the next lower dose. A clean pipette tip was used for each transferral.

The dosages used in the five CrleGV-SA treatments were determined by conducting several trial and error bioassays with one tray (25 cells) of larvae per treatment (dilution). Treatments were prepared in three-, four- and five-fold dilution series. Determination of the correct dilution rates and concentrations was therefore a fairly

lengthy process.

After 10 days, 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 90 % of larvae in a sample) were calculated for each assay. Overall values were obtained by calculating the means from the three assays.

3.2.1.3 Time-response bioassays

Surface dose bioassays were conducted in the 30 ml plastic pots. The diet containing agar, described above, was poured into 10 pots to a level of no more than 10 mm. Once the diet had cooled within a laminar flow cabinet, five pots were inoculated with 276 µl of a LC_{90} suspension of purified CrleGV-SA (see sections 2.2.2.1 – 2.2.2.3 for purification protocol) and five pots were treated with 276 µl of sterile distilled water as a control. Within the laminar flow cabinet, diet surfaces dried satisfactorily within ± 30 min. Ten neonate larvae were then placed into each pot, onto the diet surface. This was done with the aid of a 000 paint brush, first placing larvae onto the control diet and then those onto the virus treated diet. Pots were sealed with filter paper and plastic lids, marked and kept at 27ºC. There were therefore 50 larvae per treatment, and bioassays were replicated three times.

After 16 hours, pots were opened one by one, starting with the control pots, and inspected for any dead larvae. Thereafter, pots were checked every 8 h (three times a day): at 07h00, 15h00 and 23h00. When dead larvae were observed, they were immediately and gently (so as not to rupture them) removed from the pots, using a 000 paint brush. Inspections were continued at the stated time intervals until virus induced mortality appeared to have ceased i.e. when no mortality was observed during 48 consecutive hours. Diet was then dissected so that surviving larvae could be recorded. Time-response relationships were determined using a logit version of a

probit model (Bliss, 1937), suitable for multiple observations over time. This appears to be one of the most common and acceptable techniques for the analysis of time-response data (Jones, 2000; Lacey *et al.*, 2002; Throne *et al.*, 1995). The analyses was conducted using the computer programme, GenStat 6.1 (Lawes Agricultural Trust, 2002). From these, LT_{50} (time to kill 50 % of larvae in a sample) and LT_{90} (time to kill 90 % of larvae in a sample) were calculated.

3.2.2 Detached fruit bioassays

To improve coverage of treatments on fruit, a suitable wetter was selected by dipping five fruit (navel oranges) into one of eight different adjuvant treatments, each with red food colorant added (Moir's) (1 ml/400 ml water) so that spreading could be more easily observed. The pH of the water used was neutral (7.0). The adjuvants used were Orchex mineral oil (Sunwood Chemicals, South Africa), Kynobuff (active ingredients (not specifically disclosed): wetting agents, spreading agents, acidifier, pH indicator, penetrating agents; Kynoch, South Africa), Bladbuff 5 (active ingredients (not specifically disclosed): wetting agents, spreading agents, acidifier, pH indicator, penetrating agents; Plaaskem, South Africa), Citowett (active ingredient: octylphenoxy-polyethoxyethanol; Dupont, Canada), Agral 90 (active ingredient: nonylphenoxy-polyethoxyethanol; Syngenta, Switzerland), Nu-Film 17 (active ingredient: di-1-p Menthene) and Nu-Film P (active ingredient: poly-1-p Menthene) (both Hygrotech International, South Africa). Spreading of treatments was then assessed by visual impression. Various concentrations of the most impressive treatment were compared. The appropriate rate of the selected adjuvant was added to all treatments in each bioassay.

One hundred and eighty Lina navel oranges, which had begun colouring, were used in the first detached fruit assay. Oranges, with stems attached (± 50 mm long), were placed in a large cage with a high density of newly emerged *C. leucotreta* moths, which oviposited on the fruit. Fruit were removed from the cage after 24 h and number of eggs per fruit counted. Fruit were randomly divided into six groups of 30, one group for each treatment. Fruit were marked (using an indelible felt tip marker) with the number representing the treatment which was to be applied to that group of

fruit. Once eggs had turned pink i.e. about two days after oviposition and one day before hatching (at 27°C), treatments were prepared. Five CrleGV-SA concentrations were prepared as a series of five-fold dilutions of a sonicated (for 60 s) stock solution of virus of 5.4 x 10^{11} OBs/ml (Fig. 3.2). The range of treatments included the LC_{90} estimated from the surface dose bioassays. Thirty fruit (one group of randomly selected fruit) were dipped into each treatment, including a distilled water control. Fruit were placed on a wire mesh grid to dry. Once dry, fruit were kept at 27°C. Two weeks post treatment, fruit were dissected and inspected for *C. leucotreta* larvae.

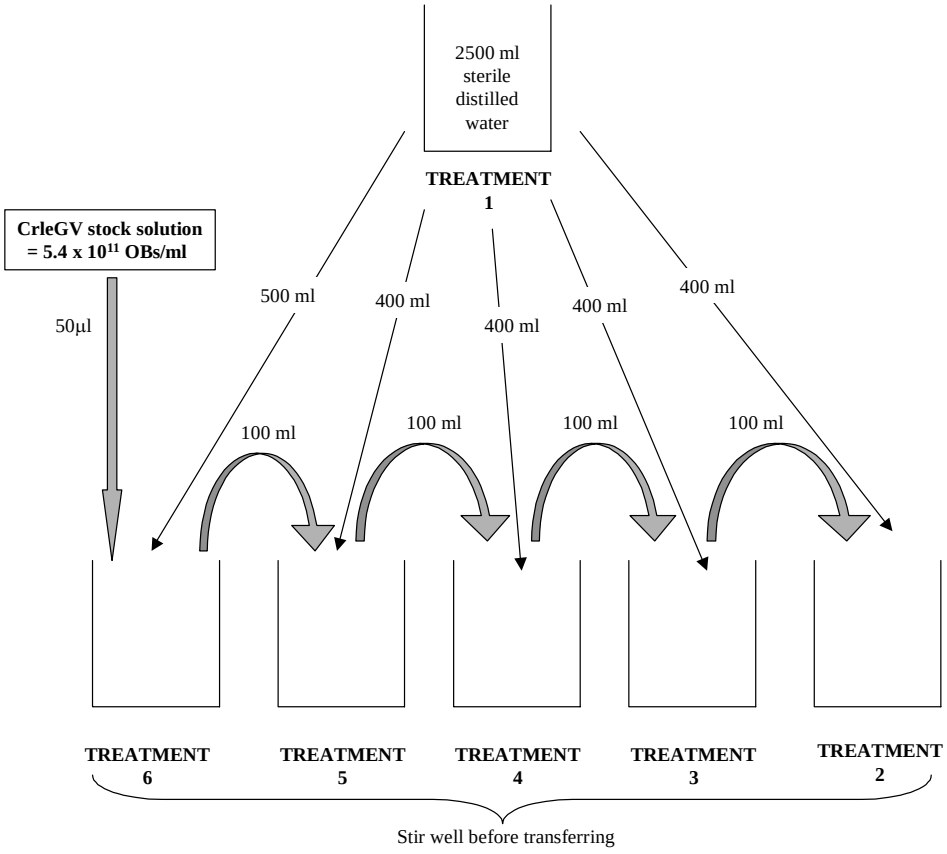

Fig. 3.2 Five-fold dilution series of CrleGV-SA for detached fruit dip dosage-mortality bioassays with neonate *C. leucotreta* larvae. Each treatment was thoroughly mixed with its own sterilised stainless steel spatula prior to transferral of the 100 ml aliquot to the next lower dose. A clean measuring cylinder was used for each transferral. The wetter, Bladbuff 5, was added to each treatment as 300 µl in 400 ml.

In a second detached fruit bioassay (replicated three times), Rustenburg navel oranges, which were beginning to colour up, were used. In this bioassay, instead of allowing moths to oviposit on fruit, larvae were placed onto fruit (once fruit had been

treated and treatments had dried). Two neonate larvae were placed onto each fruit, using a different brush for each treatment, starting from fruit treated with the most dilute to the most concentrated virus treatment. It was determined that higher concentrations of CrleGV-SA (prepared in a two-fold dilution series) were required in this assay (Fig. 3.3).

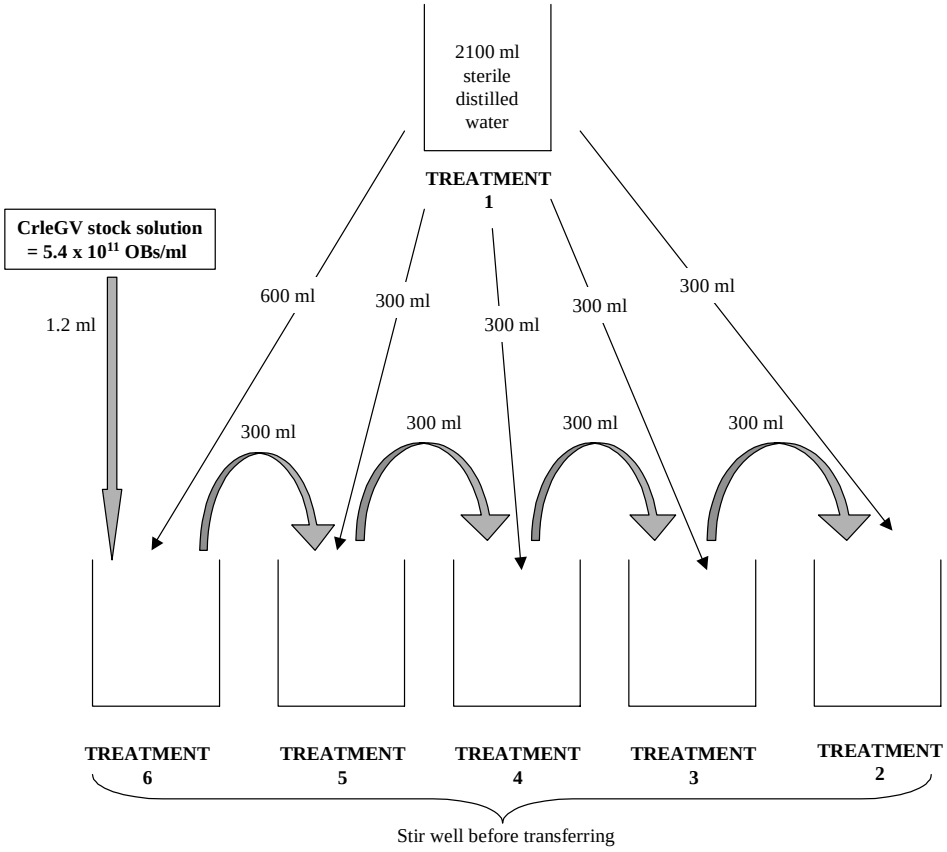

Fig. 3.3 Two-fold dilution series of CrleGV-SA for detached fruit dip dosage-mortality bioassays with neonate *C. leucotreta* larvae. Each treatment was thoroughly mixed with its own sterilised stainless steel spatula prior to transferral of the 300 ml aliquot to the next lower dose. A clean measuring cylinder was used for each transferral. The wetter, Bladbuff 5, was added to each treatment as 225 µl in 400 ml.

Dose-response curves (using PROBAN (Van Ark, 1995)) were calculated according to the reduction in fruit infested relative to the control, and the reduction in total number of larvae infesting fruit relative to the control. As infestation in the control was considered to be the baseline, no correction for control mortality was included. From the probit lines, LC_{50} and LC_{90} were estimated. Mean numbers of larvae

infesting the fruit from each treatment were calculated and subjected to an ANOVA (analysis of variance) test, where after the means were compared using the Bonferonni LSD (least significant difference) test. The computer package used to conduct the calculations was Statgraphics Plus for Windows Version 2.0 (Statistical Graphics Corporation, 1996).

3.2.3 Surface dose bioassays with fifth instar larvae

3.2.3.1 General methodology

Fifth instar larvae were used for CrleGV-SA production as this is the stage that has been used for CpGV production, using surface dosing. Surface dose bioassays were therefore conducted to determine the virus concentration for inoculation of diet.

Firstly, an acceptable bioassay methodology was sought - the most important factor being high survival of control larvae. It was accepted that the virus production diet would be the same as that used for rearing the host culture. It was therefore necessary to conduct the bioassays on this diet. Agar was not considered to be a necessary ingredient in the diet. Its cost also served to preclude its use. It was crucial to find a suitable container in which to conduct the bioassays. The same three containers that were tested for neonate bioassays were compared here: 25 cell bioassay trays plastic Petri dishes and plastic pots (see section 3.2.1 for specifications).

Diet for the bioassay trays and plastic pots was prepared, cut and inserted in the containers in exactly the same way as described for the neonate bioassays (see section 3.2.1).

Diet for bioassays conducted in Petri dishes was also prepared in the same way as described for neonate larvae (see section 3.2.1). However, small diet plugs measured 10 mm x 8 mm x 8 mm with an average mass of 0.61 and large diet plugs measured 22 mm x 32 mm x 4 mm with an average mass of 2.73 g. These were placed individually into the plastic Petri dishes.

Fifth instar larvae were selected according to head capsule size in accordance with

Dyar's rule (Dyar, 1890). Dyar's rule states that there is a reasonably precise relationship between larval instar and head capsule width when the measurements are plotted on a logarithmic scale. A sample of 10 individuals were removed from a jar of diet which appeared to have larvae at the correct stage – this being early fifth instar, identified by the large size and dark pink colour. Only jars in which pupation had not yet begun were selected (at 27°C this was ± 14 days after eggs were introduced onto the diet). Larvae were placed into a freezer (± -10ºC) 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 - 1.49 mm for fifth instar *C. leucotreta* larvae (Daiber, 1979b) then larvae from that particular batch were used for the bioassay. Larvae were then placed onto diet: singly and in twos in bioassay tray cells; singly, in twos and in threes, into 30 ml pots (25 replicates of each of these five treatments); singly onto each diet plug in each Petri dish (10 replicates for each plug size). After introduction, containers were kept at 27ºC for 14 days, before survival was determined.

Therefore, in one trial, survival of fifth instar larvae in 25 cell bioassay trays, pots and Petri dishes was compared (Table 3.2).

Table 3.2 Containers compared and numbers of fifth instar *C. leucotreta* larvae used in a survival assessment trial (trial was not replicated).

Container		**Number of larvae per cell/pot/dish**	**Total number of larvae**
25 cell bioassay tray		1	25
		2	50
Pot		1	25
		2	50
		3	75
Petri dish	Small plug	1	10
	Large plug	1	10

3.2.3.2 Dose-response bioassays

Surface dose bioassays were conducted in 25 cell bioassay trays (Plate 3.1). Six glass pie dishes of diet were required to fill 12 bioassay trays: two trays were impressed into each dish. Purified CrleGV-SA (see section 2.2.2.1 - 2.2.2.3 for purification protocol) was diluted in sterile distilled water in a five-fold series of five treatments

(Fig. 3.4) and a sterile distilled water control was used as a sixth treatment. 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 minutes, until the diet had adequately dried. One fifth instar larva was then placed into each cell. Trays were sealed in the manner previously described (section 3.2.1.1), marked and kept at 27ºC.

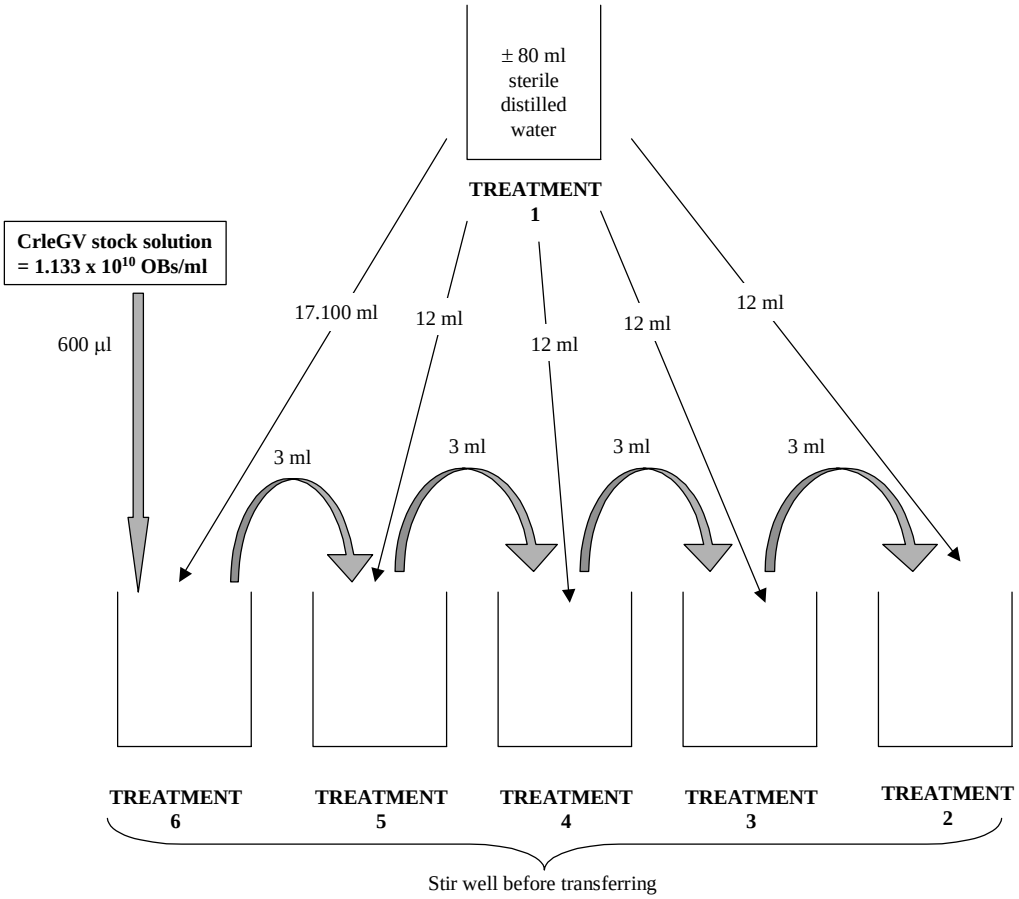

Figure 3.4 Five-fold dilution series of CrleGV-SA for surface treatment dose-response bioassays with fifth instar *C. leucotreta* larvae. Each treatment was thoroughly mixed with its own sterilised stainless steel spatula prior to transferral of the 3 ml aliquot to the next lower dose. A clean pipette tip was used for each transferral.

The dosages used in the five CrleGV-SA treatments were determined by conducting several trial and error bioassays with one tray (25 cells) of larvae per treatment. Treatments were always prepared in a five-fold dilution series. This eventually proved to be the appropriate dilution factor.

After 14 days, trays were opened and inspected. Larvae (or pupae, as many of the survivors had become) were recorded as alive or dead. The dose-response curve was calculated by probit analysis (Finney, 1971) using PROBAN (Van Ark, 1995). 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} were calculated.

3.2.3.3 Time-response bioassays

Surface dose bioassays were conducted in 30 ml plastic pots (Plate 3.2) rather than bioassay trays. As it was necessary to frequently open the containers for inspection of larvae, pots were found to be easier and safer (larvae could not easily escape) to open and close than bioassay trays. One hundred round plugs were cut from four pie dishes of diet. Fifty plugs were dipped into sterile distilled water and the other 50 plugs were dipped into a LC_{90} (calculated from the dose-response bioassays) solution of purified CrleGV-SA. Plugs were placed on a thin wire mesh drying rack, in the laminar flow cabinet until sufficiently dry (± 30 min). They were then inserted into the pots. One fifth instar larva was placed into each pot and pots were sealed with cotton wool-plugged plastic lids. Bioassays were replicated three times.

After 16 hours, pots were opened (starting with the control pots) one by one, and inspected for any dead larvae. Thereafter, pots were inspected every eight hours (three times a day): at 07h00, 15h00 and 23h00, until all larvae had either died or pupated. Time-response relationships were determined using a logit version (logit transformation) of a probit analysis, using the statistical package, GenStat 6.1 (Lawes Agricultural Trust, 2002). From these, LT_{50} and LT_{90} were calculated.

3.3 RESULTS AND DISCUSSION

3.3.1 Surface dose bioassays with neonate larvae

3.3.1.1 General methodology

Table 3.3 A comparison of survival of *C. leucotreta* neonate larvae on artificial diet in three different containers.

Container		**Number of larvae per cell/pot/dish**	**Total number of larvae**	**% Larvae surviving**	
				After 24 h	**After 8 days**
25 cell bioassay tray		1	25	52.0	38.0
		1	25	-	46.0
Polypot		5	15	-	40.0
		10	30	-	43.3
Petri-dish	Small plug*	1	10	-	0
	Large plug*	1	10	-	80.0

Survival (either real or apparent) was poor in all containers except on the large diet plugs in Petri dishes (Table 3.3). The problem was considered to be as much an inability to detect the small larvae in the diet (whether alive or dead) as actual mortality. The diet was coarse and granular and of a coloration that could fairly easily camouflage the larvae. It was therefore difficult to detect a small first to second instar larva in a relatively large container. There was no survival on small plugs in Petri dishes as the diet desiccated too rapidly. Recorded survival was highest on large plugs (in Petri dishes) (Table 3.3), possibly because these were smaller volumes of diet than those in the other two containers and were therefore easier to inspect thoroughly. Transferral of larvae from one bioassay tray to another after 24 hours appeared to increase mortality (Table 3.3). However, mortality of non-transferred larvae was also unacceptably high, and as the trial was not replicated it could not be concluded for certain whether transferring the larvae did significantly increase mortality. Nevertheless, it was decided that in bioassays, larvae would not be transferred after 24 hours from inoculated diet onto fresh diet. As *C. leucotreta* is a cryptic pest, remaining within the diet once it has penetrated, it will not normally re-emerge from the diet, unless sick or ready to pupate and will not be re-exposed to the

virus inoculated surface. There was therefore considered to be little justification for transferring the larvae from the surface inoculated diet to fresh diet.

Diet containing a gelling agent (agar) substantially improved the ability to detect small larvae on or within the diet. This diet surface was smooth and regular. It was easy to track any burrowing within this diet and therefore not difficult to find small larvae.

Table 3.4 Survival of neonate larvae on agar based diets in 25 cell bioassay trays.

Treatment	**Larvae recovered**
3 ml agar + 100 g diet + 333 ml distilled water	0 (0 %)
6 ml agar + 100 g diet + 333 ml distilled water	2 (8 %)
9 ml agar + 100 g diet + 333 ml distilled water	7 (28 %)
3 ml agar + 50 g diet + 333 ml distilled water	16 (64 %)
6 ml agar + 50 g diet + 333 ml distilled water	20 (80 %)

Survival of neonate larvae in bioassay trays was highest on the diet made with 6 ml agar, 50 g dry ingredients of the standard diet and 333 ml distilled water (Table 3.4). Ideally, one would want a method which provided 100 % survival of control larvae. However, after repeated performance of this assay technique, the highest survival rate attained was 85 %. It was therefore decided that this methodology would be used for dose-response assays and that if control mortality exceeded 20 % then the results of that particular replicate would not be used.

The plastic pots were considered to be more suitable for use in the time-response bioassays. Not only could more than one larva be placed into each pot, but individual pots could easily and safely be opened for frequent checking of mortality – unlike the risk of larvae escaping when bioassay trays were opened. Ten larvae were placed into each pot. The chance of horizontal transmission of virus from one infected larva to an uninfected or less infected larva occurring, was considered to be acceptably low. Relative to the size of the neonate larvae, the volume of diet was large. The probability of larvae not encountering one another during the time period of the trial (eight days) was considered to be sufficiently high. This probability was increased by the fact that the larvae are cryptic feeders and not surface feeders. This supposition was confirmed when larvae were dissected out of the diet. Once the most suitable proportions of diet ingredients, water and agar were determined in the bioassay trays,

survival of larvae on that diet in pots, was determined. After eight days, larvae out of 10 surviving in each of five pots was: nine, nine, seven, six and six. This demonstrated an average mortality of 26 %, but a potential survival of 90 %. As with the dose-response bioassays, it was decided that only the results from bioassays in which control mortality did not exceed 20 % would be considered.

3.3.1.2 Dose-response bioassays

PROBAN (Van Ark, 1995) transformed the doses to $\log_{10}$ and the percentage response to empirical probits. Using this information, the fit of the probit lines was calculated, as were the fiducial limits and LC_{50} to $LC_{99.9}$ (Appendix 2).

The regression (probit) lines fitted to the corrected data in Table 3.5 (Fig. 3.5) had the equations y = 1.931 + 0.841x (SE of slope = 0.1318), y = 2.176 + 0.789x (SE of slope = 0.1119) and y = 0.673 + 1.200x (SE of slope = 0.1667). Deviations from two of the three lines were estimated to be homogenous (Appendix 2), making the chi-squared test more applicable than would have been the case if deviations were heterogenous (Van Ark, 1995). Deviations from the line from the other assay were considered homogenous by combining doses. The slopes of two of the three lines were within the range of 1 - 2 expected for virus assays (Jones, 2000).

G for fiducial limits were calculated to be 0.0943, 0.0773 and 0.0741 for the three lines, respectively (Appendix 2). According to Van Ark (1995) values greater than approximately 0.025, mean that the variation of mortalities is rather large. However, experimental procedures or the value of the probit line should only come into question if G exceeds 0.25.

By using Bartlett's test for homogeneity of residual variances, the residual variances of the three lines were determined to be homogenous (Appendix 2). The slopes of the lines were thus comparable. The chi-squared test showed the lines to be parallel and their elevations to be comparable. The elevations of the three lines did not differ significantly from one another.

Table 3.5 Mortality of neonate *C. leucotreta* larvae in dose response bioassays with five concentrations of CrleGV-SA, replicated three times. Fifty individuals were tested per treatment per replicate.

Treatment (CrleGV 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**
Distilled water control	16.00	-	-	8.00	-	-	12.00	-	-
1.221×10^2	36.00	23.81	4.288	24.00	17.39	4.061	20.00	9.09	3.665
6.104×10^2	32.00	19.05	4.124	40.00	34.78	4.609	20.00	9.09	3.665
3.052×10^3	44.00	33.33	4.569	32.00	26.09	4.359	52.00	45.45	4.886
1.526×10^4	72.00	66.67	5.431	72.00	69.57	5.512	84.00	81.82	5.909
7.630×10^4	92.00	90.48	6.309	92.00	91.30	6.359	92.00	90.91	6.335

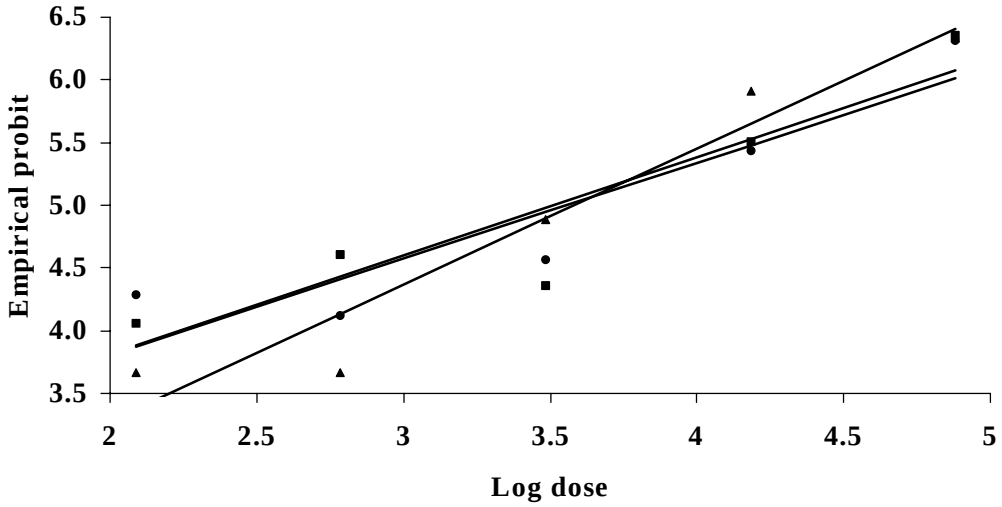

Fig. 3.5 Dose-response probit line for CrleGV-SA against neonate *C. leucotreta* larvae.

LC_{50} and LC_{90} estimated from the three assays were averaged to give 4.095×10^3 OBs/ml and 1.185×10^5 OBs/ml respectively. The 95 % fiducial limits of the LC_{50} and LC_{90} were estimated to range from $2.085 - 8.132 \times 10^3$ and $0.259 - 7.474 \times 10^5$ OBs/ml respectively. Other dose-response tests recorded with CrleGV against neonate *C. leucotreta* larvae estimated values for three different isolates to range from 3.93×10^3 to 5.35×10^3 OBs/ml for the LC_{50} and from 6.83×10^4 to 1.86×10^5 for the LC_{90} (Fritsch, 1989). One of these isolates was referred to as CrleGV-SA, however, detailed DNA characterisation is not reported and there is therefore insufficient evidence to show that it is the same isolate as the one referred to as CrleGV-SA in this dissertation. The values estimated in this study for CrleGV-SA were similar to those estimated by Fritsch (1989). However, one might have expected to obtain higher estimations of LC_{50} and LC_{90} in this study, because of the difference in experimental methodology.

Experimental methodologies used in the two studies were distinctly different. Fritsch (1989) incorporated the virus into the insect diet, whereas in this study, the virus was applied to the surface of the diet. As *C. leucotreta* larvae are internal feeders, once a larva has penetrated into surface-inoculated diet, there is little chance of it encountering and ingesting further OBs. Whereas, if the virus is included in the diet,

the larva could ingest OBs at any time during its tenure in the diet. Theoretically, whether virus is surface applied or included in the diet, a larva should in all probability encounter the same number of OBs, whether during initial penetration or during the several days that follow thereafter. However, in reality the biology and behaviour of the larva will influence this probability. When penetrating into a fruit, neonate *C. leucotreta* larvae are believed to chew their way through the rind without actually ingesting much (Hendrik Hofmeyr, personal communication). Feeding begins in earnest, once the larva has penetrated through to the albedo. This behaviour may have a significant bearing on dosage-mortality curves calculated from the different methods of diet inoculation. Jones (2000) suggests that in dose-response assays with internal feeders, the inoculum should be included in the diet. However, it was felt that surface inoculation of the diet would be more reflective of a field application situation, which was the ultimate end point of this study.

Two other possible reasons for any differences obtained in LC_{50} and LC_{90} estimations between this study and that of Fritsch (1989) are host susceptibility and virus pathogenicity. The two *C. leucotreta* cultures almost certainly originated from different field populations. The region of origin of the culture used by Fritsch (1989) is unclear; however, could have been the Cape Verde Islands. It is very possible that different populations of *C. leucotreta* could have different levels of susceptibility to a virus. Based on apparent differences in host preference in different regions it appears that different biotypes of *C. leucotreta* do occur (e.g. it is a significant pest on maize in east Africa (Reed, 1974) and on cotton in west Africa (Schulthess *et al.*, 1991), but not on either crop in southern Africa (Annecke & Moran, 1982)). Susceptibility to a virus could also be influenced by whether the virus is homologous for that population or not.

Finally, one or two of the isolates of CrleGV tested by Fritsch (1989) may simply have been more virulent than CrleGV-SA tested in this study.

3.3.1.3 Time-response bioassays

According to Finney (1971), subjects should ideally be tested only once each, in order to use these statistical techniques. This is in order to make the independent variables

(time) completely independent of one another. However, in practice this would not only be extremely wasteful of larvae but would be totally impractical. Instead of using 300 larvae (in three replicates: treated and untreated), more than 25000 larvae would be required.

During the first 32 hours of each bioassay replicate, a number of larvae were recorded dead in each control. Clearly, these deaths were not symptomatic of virus infection (section 2.3.1.1). This non-virus induced control mortality in the three replicates of the bioassay were 10 %, 10 % and 4 %. This was even lower than could be achieved in the pre-bioassay tests to check the suitability of the methodology. After 32 hours, very few larvae were recorded dead in the controls, and all of these appeared to be virus-induced deaths (a result of the low level of non-apparent virus in the *C. leucotreta* mother culture). Once the bioassays had been completed (i.e. no mortality had been recorded for 48 hours), diet was dissected to search for any survivors or cadavers. In each of the three bioassays one, four and two dead larvae were found; two, five and three live larvae were found; and a total of four, six and four larvae were missing (out of a total of 50 individuals exposed in each bioassay). These could have escaped or more likely could have died some while previously and disintegrated within the diet. As diet had been inoculated with the LC_{90} it was not surprising that an average (from the three replicates) of between 6.7 % (excluding missing larvae) and 16.0% (including missing larvae) of larvae had survived. This actually supported the estimation of the LC_{90}. These “non-responding” larvae were therefore not considered in the calculation of the time-response curve. This is known as biological truncation of the data (Bliss, 1935), which is applicable when the dosage is small enough to be survived by one or more of the individuals in the experiment. It could not be determined at what stage the cadavers, which were exhumed from the diet, had died, and as the purpose of these assays was to determine time to death, these larvae were also excluded from the calculation, as well non-virus induced control mortality. This is known as artificial truncation (Bliss, 1935), as truncation is not inherent in the process under investigation but is due to the method of experimentation and presumably could be eliminated by a change in technique. The total number of individuals considered exposed in each replicate, were therefore reduced from 50 to 38, 31 and 41.

Table 3.6 Mortality of neonate *C. leucotreta* larvae in time-response bioassays with the LC_{90} concentration of CrleGV-SA.

Time after treatment		**Cumulative larval mortality (corrected for non-virus induced control mortality) (%)**		
		Rep1 (n = 38)	**Rep2 (n = 31)**	**Rep3 (n = 39)**
Days	**Hours**			
0	16	0	0	0
1	0	0	0	0
	8	0	0	0
	16	0	6.45	2.56
2	0	2.63	6.45	7.89
	8	2.63	6.45	10.26
	16	5.26	6.45	12.82
3	0	5.26	6.45	17.95
	8	5.26	12.90	23.08
	16	7.89	25.81	41.03
4	0	10.53	29.03	53.85
	8	18.42	32.26	56.41
	16	21.05	45.16	63.16
5	0	28.95	58.06	69.23
	8	31.58	64.52	76.92
	16	39.47	64.52	87.18
6	0	44.74	74.19	92.31
	8	47.37	80.64	94.87
	16	60.53	87.10	100
7	0	65.79	87.10	
	8	71.05	96.77	
	16	89.47	100	
8	0	89.47		
	8	92.10		
	16	100		

The first virus induced mortality was observed 40 hours after larvae were placed onto the diet (Table 3.6). After eight days and 16 hours no further mortality was recorded. The time-mortality relationship (Fig. 3.6) was analysed using a logit version of a probit model (Appendix 3). From this, the LT_{50} and LT_{90} for each replicate and their standard errors were estimated. Lower and upper 95 % limits ranged from 3 days 19 h to 6 days 5 h for the LT_{50}, and from 5 days 19 h to 9 days 10 h for the LT_{90}. LT_{50} and LT_{90}, estimated from the three replicates, were averaged to equal 4 days 22 h and 7 days 8 h, respectively. These results would appear to indicate that CrleGV-SA is a fast GV or type 2 GV, which are highly infectious, have low LD_{50} values < 5 capsules per neonate and rapid speeds of kill. (Winstanley & O'Reilly, 1999). The slow GVs, type 1 GVs, have high LD_{50} values, with death being substantially prolonged, as long

as 10 - 20 days, far beyond the normal larval stage.

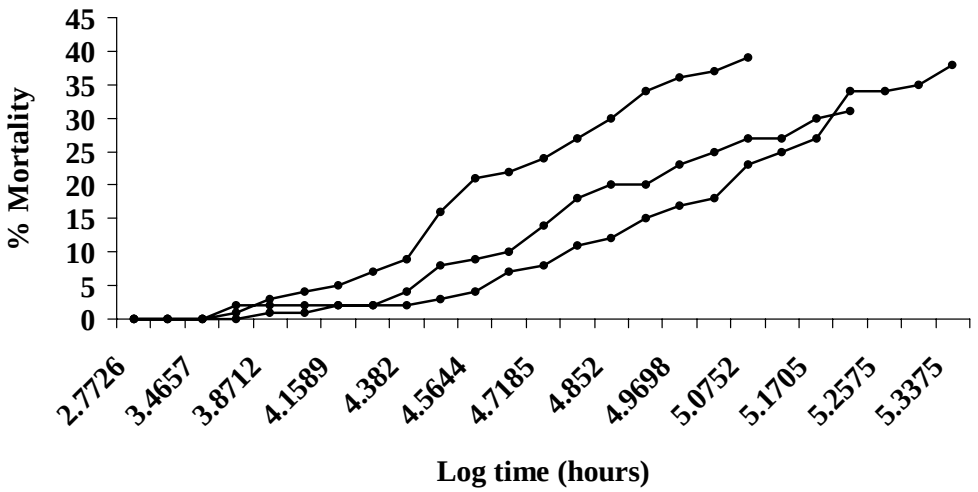

Fig. 3.6 Time-mortality relationship between *C. leucotreta* neonate larvae and CrleGV-SA applied at the LC_{90} concentration in three bioassay replicates.

Fritsch (1989) calculated time-response curves with concentrations of the isolate, which she referred to as CrleGV-SA, ranging from 1×10^5 to 1.6×10^6 OBs/ml (or 4×10^3 to 6.4×10^4 OBs per ml of diet, incorporated into the diet). With the concentrations that gave 78 % and 99 % mortality, LT_{50} was estimated at 4 days 8 h and 3 days 16 h, respectively. This was not dissimilar to the results obtained with CrleGV-SA in this study, in which LT_{50} was 4 days 22 h with a concentration which gave a mean mortality of 93.3 %. The slightly lower LT_{50} estimated from Fritsch's (1989) work could have been a result of the different methodology employed (discussed in detail in section 3.3.1.2). Frtisch incorporated the virus into the diet, whereas in this study, only the surface of the diet was inoculated. The latter method is a more accurate depiction of a real situation, as *C. leucotreta* is a cryptic feeder, and will therefore only be exposed to a virus inoculum during the brief period before it penetrates into its food source (i.e. usually a fruit in its natural environment).

Infectivity of CrleGV-SA to the homologous host is comparable with that of other GVs (Crook, 1991). Despite this, it must be questioned whether a LT_{50} to LT_{90} range of 4 days 22 h - 7 days 8 h is sufficiently rapid to cause a reduction in damage to fruit, significantly and acceptably.

Table 3.7 Penetration of first instar larvae into CrleGV-SA inoculated and control diets in three replicates of a time-response bioassay.

Time after treatment		**Larvae not penetrating the diet (%)**					
		Rep 1		**Rep 2**		**Rep 3**	
Days	**Hours**	**Control**	**Treatment**	**Control**	**Treatment**	**Control**	**Treatment**
0	16	32.6	47.9	28.7	42.0	31.1	41.2
1	0	17.0	25.5	22.1	30.4	27.3	39.9
	8	17.8	24.4	20.6	20.0	25.0	38.0
	16	8.9	17.8	12.5	11.6	25.0	36.0
2	0	6.7	15.9	9.4	18.6	19.2	34.3
	8	6.7	18.2	9.4	18.6	11.4	28.2
	16	8.9	18.6	6.8	16.3	8.2	26.6
3	0	8.9	16.3	7.6	16.3	0	22.0
	8	11.1	13.9	5.2	12.2	0	24.0
	16	9.1	14.3	4.8	13.5	0	26.0
4	0	4.5	17.1	2.0	16.7	0	26.0
	8	2.3	18.4	2.0	17.1	0	24.2
	16	11.4	32.4	3.9	22.6	0	24.2
5	0	4.5	23.5	2.6	17.9	0	26.0
	8	4.8	36.4	0	11.5	0	22.7
	16	7.1	30.0	0	19.2	0	25.4
6	0	4.8	10.7	0	34.8	0	26.8
	8	9.5	18.5	2.6	22.7	0	21.5
	16	7.3	4.0	2.0	20.3	0	21.5
7	0	7.3	8.0	0	19.2		
	8	9.8	15.8	0	18.6		
	16	10.0	15.8	0	16.5		
8	0	7.5	6.7				
	8	7.5	7.7				
	16	2.5	9.1				
Average		**9.14 ± 1.22**	**18.68 ± 2.00**	**6.46 ± 1.71**	**19.84 ± 1.57**	**7.75 ± 2.64**	**28.13 ± 1.47**

*A total of 50 larvae placed onto each of the inoculated and control diets.

In addressing this question, it must be noted that mortality was not the only benefit provided by infection. There was also a conspicuous difference in behaviour between larvae on inoculated diet and those on untreated diet, in all three of the assay replicates (Table 3.7). Even from as early as the first observation, made 16 hours after larvae were placed onto the diet there was a substantially higher number of larvae which had not penetrated into the treated diet (remaining on the surface of the diet and the walls and ceiling of the container) than on the control diet. In the second replicate, all larvae had penetrated into the diet from 5 days 8 h after initiation. In the third replicate, no larvae remained outside of the diet from as early as 3 days after trial

initiation. At these times, 11.5 % and 22.7% of larvae were recorded outside of the diet in these two replicates. Over the entire trial period, averages of 18.68 %, 19.84 % and 28.13 % of live larvae were recorded outside of the diet, in the three treated replicates, whereas, only 9.14 %, 6.46 % and 7.75 % of larvae had not penetrated into the untreated diets (Table 3.7). This was either a response to viral infection, preceding death, or a deterrent effect of the virus to penetration by the larvae into the diet, or a combination of the two.

A second factor, answering the question of whether a LT_{50} to LT_{90} range of around 4 - 7 days is sufficiently rapid, relates to the biology of the pest under natural conditions. A first instar larva takes approximately four days to penetrate through the rind and albedo of a citrus fruit (Hendrik Hofmeyr, personal communication). If the larva dies or if its behaviour changes (and it reverses out of the fruit) before it manages to penetrate through the albedo into the flesh of the fruit, the damage to the fruit may be insignificant. The penetration mark itself will be minute and will not lead to downgrading or export exclusion of the fruit. Secondary fungal infection, leading to rotting of the fruit, may also be avoided.

3.3.2 Detached fruit bioassays

Table 3.8 Rating of wetting and spreading ability of various adjuvant treatments on detached navel oranges.

Treatment	**Concentration per 100 ℓ water**	**Rating (1 is the best)**
Distilled water control	-	6
Orchex	250 ml	5
Kynobuff	100 ml	4
Bladbuff 5	200 ml	1
Citowett	25 ml	2
Agral 90	25 ml	3
Nu-Film 17	25 ml	4
Nu-Film P	30 ml	7

Based on visual appearance, the adjuvant Bladbuff 5 at 200 ml/100 ℓ water gave better coverage of fruit than did any of the other adjuvants tested (Table 3.8). Subsequently, it was found that at a reduced concentration of 75 ml/100 ℓ, coverage was still adequately enhanced. At lower concentrations the effect of Bladbuff 5 was noticeably reduced. Consequently, in all fruit dip bioassays, Bladbuff 5 was added to

all treatments at a concentration of 75 ml/100 ℓ water. (Dilutions are quoted in millilitres per 100 litres of water, as this is how concentrations are cited for field use (Grout *et al.*, 1998)).

A total of 26 out of the 30 control fruit (86.67 %) were infested with live *C. leucotreta* larvae 14 days after the trial was initiated (Table 3.9). In order to calculate a practically meaningful dose-response curve, the total number of fruit considered per treatment was reduced from 30 to 26 (Appendix 2). Control fruit were infested with an average of 2.67 larvae (considering all 30 fruit) (Table 3.9), which is a total of 80 larvae in all fruit. "Mortality" for treatments was calculated as the reduction in larvae from 80 (Appendix 2).

Table 3.9 *C. leucotreta* larval infestation of CrleGV-SA-treated detached fruit (navel oranges)*.

Treatment (CrleGV in OBs/ml)	**Fruit infested with one or more larvae (%)**	**Reduction in fruit infested relative to control (%)**	**Number of live larvae per fruit****	**Reduction in number of live larvae per fruit relative to control (%)**
Distilled water control	86.67		2.67a	
8.64×10^4	83.33	3.85	2.43a	8.99
4.32×10^5	66.67	23.08	1.67ab	37.45
2.16×10^6	66.67	23.08	1.77ab	33.71
1.08×10^7	63.33	26.92	1.17b	56.18
5.40×10^7	60.00	30.77	0.70b	73.78

*There was an average of 27.6 *C. leucotreta* eggs laid per fruit before fruit were treated (dipped).
**Values in the same column followed by the same letter are not significantly different ($P > 0.05$, Bonferroni LSD multiple range test).

The equation of the dose-response curve, calculated from reduction in fruit infested, could not be accurately determined. PROBAN calculated the equation to be $y = 4.0873 + 0.0000x$ (Appendix 2). LC_{50} and LC_{90} were estimated to be 1.087×10^8 and 2.613×10^8 OBs/ml respectively. These results can be rejected as being highly unreliable. G for fiducial limits was calculated to be ≥ 1. According to Van Ark (1995) values greater than approximately 0.25, mean that experimental procedures or the value of the probit line should come into question. The main problem may have been the high number of eggs per fruit. An average of 27.6 eggs was counted per fruit. This provided a very stringent test for the CrleGV-SA treatments, as even in an

orchard experiencing an exceptionally high level of *C. leucotreta* infestation, the average number of eggs recorded per fruit was 0.37, considering all fruit inspected, or 2.30, considering only fruit on which eggs were present (Moore & Fourie, 1999). Therefore, even if the calculations were sufficiently reliable, the relevance of the estimated dose-response relationship to concentrations that should be tested in the field, would have to be questioned.

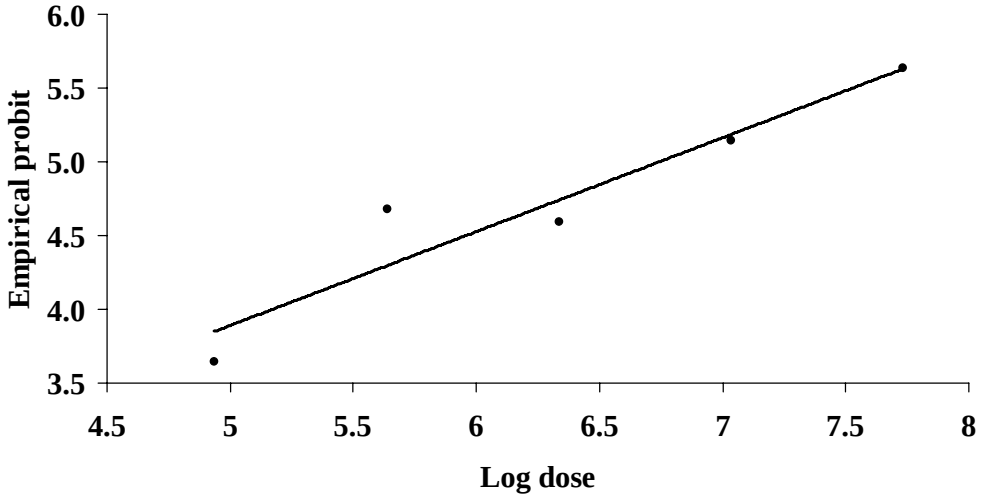

Fig. 3.7 Dose-response probit line for reduction in fruit infested with CrleGV-SA against neonate *C. leucotreta* larvae, when treatments were applied by dipping detached navel oranges with *C. leucotreta* eggs laid on them.

Although number of fruit without larvae should be a more important measurement in terms of practical control in the field, the dose-response curve calculated from the reduction in number of larvae infesting fruit in this bioassay must be considered to be more reliable. This is because of the extremely and unnaturally high number of eggs per fruit. The equation of this probit line (Fig. 3.7) was estimated as y = 0.9342 + 0.6043x (Appendix 2). The standard error of the slope was 0.0716. LC_{50} and LC_{90} were estimated to be 5.342×10^6 and 7.052×10^8 OBs/ml and G for fiducial limits was 0.0539. The 95 % fiducial limits of the LC_{50} and LC_{90} were estimated to be $3.228 – 9.563 \times 10^6$ and $0.225 – 4.180 \times 10^9$ OBs/ml respectively. The treatment of 1.08×10^7 OBs/ml was the lowest concentration for which there was a significant reduction in numbers of larvae (Table 3.9). The lethal concentrations estimated here were not dissimilar to those determined in other studies. Fritsch (1989) applied a

concentration of 1 x 10^8 OBs/ml of CrleGV for the control of *C. leucotreta* on citrus on Cape Verde. Concentrations of CpGV applied for the control of *C. pomonella* range from 6.6 x 10^6 OBs/ml (Cunningham, 1998) to 1 x 10^9 OBs/ml (Jacques *et al.*, 1994). Obviously, tank concentrations used should be viewed in conjunction with volumes applied per hectare, which provides the more important figure of OBs applied per hectare. Nevertheless, due to the obvious failing in the experimental procedure, discussed in the previous paragraph, this bioassay was not replicated.

In bioassays in which two neonate larvae were placed onto each fruit, a total of 17, 22 and 20 out of the 30 control fruit in each replicate were infested with live *C. leucotreta* larvae 14 days after the trial was initiated (Table 3.10). In order to calculate a practically meaningful dose-response curve, total number of fruit considered per treatment was reduced from 30 to 17, 22 and 20 (Appendix 2). Control fruit were infested with an average of 0.67, 1.0 and 1.0 larvae (considering all 30 fruit) in each of the three replicates (Table 3.10), which is a total of 20, 30 and 30 larvae in all fruit. "Mortality" for treatments was calculated as the reduction in larvae from these totals in each of the three replicates (Appendix 2).

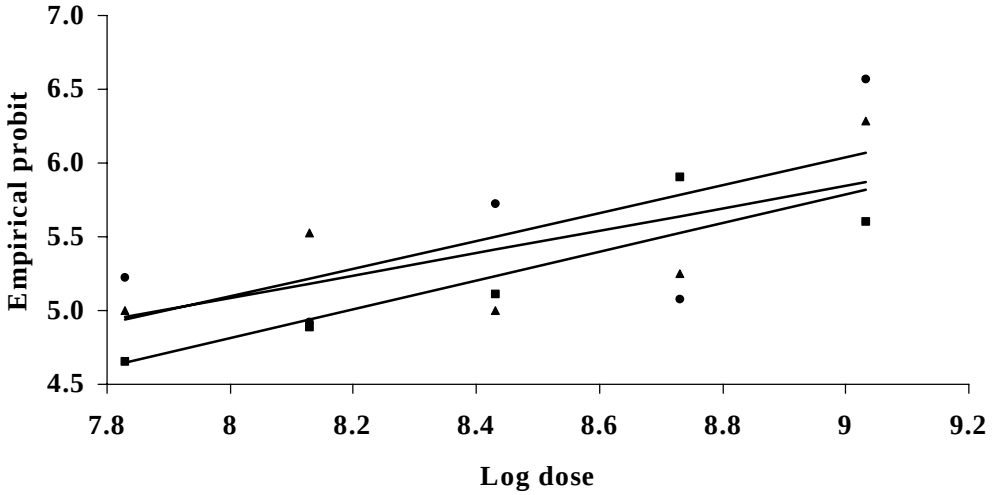

Fig. 3.8 Dose-response probit lines (3 replicates) for reduction in fruit infested, with CrleGV-SA against neonate *C. leucotreta* larvae, when treatments were applied by placing neonate larvae onto dipped detached navel oranges.

Table 3.10 *C. leucotreta* larval infestation and damage of CrleGV-SA-treated detached fruit (navel oranges)*.

Treatment (CrleGV in OBs/ml)	**Fruit infested with one or more larvae (%)**	**Reduction in fruit infested relative to control (%)**	**No. of live larvae/fruit****	**Reduction in no. of live larvae/fruit relative to control (%)**	**Fruit with *C. leucotreta* induced blemishes (%)**	**Reduction in no. of fruit with *C. leucotreta* induced blemishes (%)**
Replicate 1						
Control***	56.67	-	0.67	-	93.33	-
6.736×10^7	23.33	58.82	0.30	55.00	76.67	17.85
1.347×10^8	30.00	47.06	0.37	45.00	70.00	25.00
2.695×10^8	13.33	76.47	0.13	80.00	86.67	7.14
5.389×10^8	26.67	52.94	0.27	60.00	60.00	35.71
1.078×10^9	3.33	94.12	0.03	95.00	50.00	46.43
Replicate 2						
Control***	73.33	-	1.00	-	83.33	-
6.736×10^7	46.67	36.36	0.47	53.33	73.33	12.00
1.347×10^8	40.00	45.45	0.53	46.67	63.33	24.00
2.695×10^8	33.33	54.55	0.33	66.67	63.33	24.00
5.389×10^8	13.33	81.82	0.13	86.67	56.67	31.99
1.078×10^9	20.00	72.73	0.20	80.00	43.33	48.00
Replicate 3						
Control***	66.67	-	1.00	-	96.67	-
6.736×10^7	33.33	50.00	0.47	53.33	66.67	31.03
1.347×10^8	20.00	70.00	0.27	73.33	73.33	24.14
2.695×10^8	33.33	50.00	0.40	60.00	63.33	34.49
5.389×10^8	26.67	60.00	0.27	73.33	53.33	44.83
1.078×10^9	6.67	90.00	0.07	93.33	40.00	58.62

*Two neonate larvae were placed onto each fruit.

**Values in the same column followed by the same letter are not significantly different (P > 0.05, Bonferroni LSD multiple range test).

***Treated with distilled water.

In these bioassays, higher concentrations of CrleGV-SA had to be used in order to obtain mortality from which a dose-response curve could be calculated (Table 3.10). The equations of the probit lines (Fig. 3.8), calculated from the reduction in fruit infested, were y = 0.7252x - 0.6851 (SE of the slope = 0.5733), y = 0.9678x - 2.9357 (SE of the slope = 0.2985) and y = 0.3832x + 2.0552 (SE of the slope = 0.7062) (Appendix 2). The estimated value of G, in each of the three replicates, was too high, thus bringing experimental procedures into question. Fiducial limits could only be calculated for one of the three replicates. However, this was believed to be the result of an intrinsic difficulty with detached fruit assays, in which variation and unpredictability is inevitable. This being a result of variation in fruit both within and between assays e.g. age, colour, size, internal quality, rind integrity.

Deviations from two of the three lines were estimated to be heterogenous. Despite this, by using Bartlett's test for homogeneity of residual variances, the residual variances of the three lines were determined to be homogenous and their slopes were thus comparable (Appendix 2). The chi-squared test showed the lines to be parallel and their elevations to be comparable. The elevations of the three lines did not differ significantly from one another. LC_{50} and LC_{90} estimated from the three assays were averaged to give 9.195×10^{7} OBs/ml and 3.819×10^{10} OBs/ml.

From the same bioassays, the dose-response probit lines (Fig. 3.9) calculated from the reduction in number of larvae infesting fruit, were y = 0.9171x - 2.2602 (SE of slope = 0.5405), y = 0.8849x - 1.9996 (SE of slope = 0.2614) and y = 0.8287x - 1.4068 (SE of slope = 0.2668). Again, the estimated value of G for each of the three lines exceeded 0.25.

Standard error of the slope was 0.1549. LC_{50} and LC_{90} were estimated from the curve to be 9.310×10^{7} and 1.515×10^{9} OBs/ml. The 95 % fiducial limits of the LC_{50} and LC_{90} were estimated to be $0.574 - 1.282 \times 10^{8}$ and $0.933 - 3.447 \times 10^{9}$ OBs/ml.

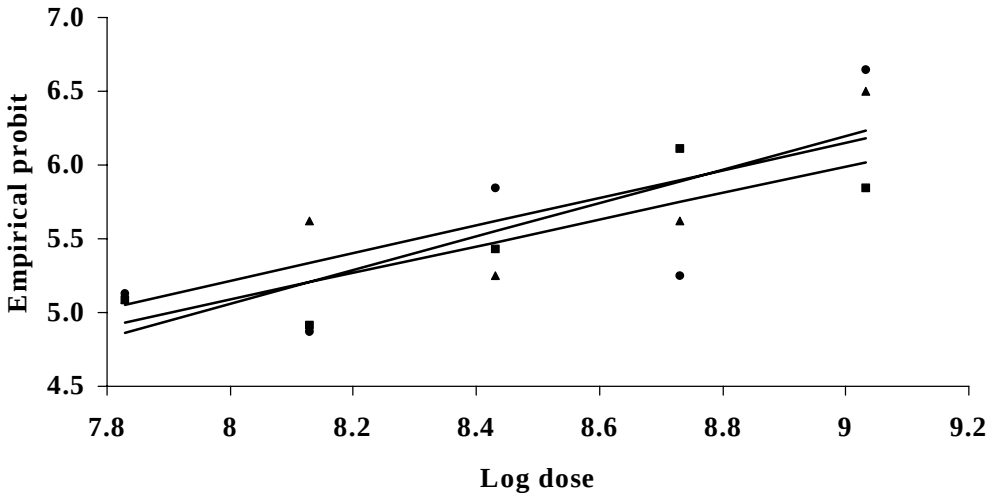

Fig. 3.9 Dose-response probit lines (3 replicates) for reduction in number of larvae infesting fruit, with CrleGV-SA against neonate *C. leucotreta* larvae, when treatments were applied by placing neonate larvae onto dipped detached navel oranges.

LC_{90} obtained in detached fruit assays should be used as a guide in deciding on the concentrations to be used in field trials. LC_{90} values estimated from the second bioassay (larvae placed onto fruit) were more than 10 times higher than the valid LC_{90} estimated from the first bioassay (calculated from the curve for reduction in number of larvae). One might have expected the inverse to have occurred, as only two larvae were placed onto each fruit compared to an average of 27.6 eggs per fruit in the first assay. This difference could be explained by a combination of the experimental technique and the biology of the target. To emerge from the egg, larvae need to chew their way through the chorion. If eggs have been treated with virus, neonate larvae can ingest their first OBs before they even begin to penetrate into the fruit. Neonate larvae that are placed onto fruit will have hatched up to a few hours previously. When placed onto the fruit, due to the already relatively long period between hatching and fruit penetration, these larvae may penetrate into the fruit more rapidly than they would under natural conditions. It is thus possible that they could ingest less virus than would a freshly hatched larva, which may wander around on the surface of the fruit for a bit longer, in the process possibly inadvertently ingesting OBs.

The results of the bioassays in which neonate larvae were placed onto the fruit may therefore have provided an exaggerated estimate of CrleGV-SA concentrations to be

used in the field. However, so too might have the bioassays in which eggs were laid on fruit, as the number of eggs were unrealistically high. Therefore, a lower concentration than indicated by these assays should also be tested in the field.

3.3.3 Surface dose bioassays with fifth instar larvae

3.3.3.1 General methodology

It was assumed that because of the larger size of fifth instar larvae, production of CrleGV-SA in this larval stage would be greater than in the younger (and smaller) larvae. A close and constant relationship between host weight at death and virus OB productivity for *Operophtera brumata* was found (Wigley, 1976). Consequently, it is the early fifth instar *C. pomonella* larvae that are inoculated for production of CpGV (e.g. Glen & Payne, 1984; Huber, 1981). Fifth instar *C. leucotreta* larvae have themselves been used for heterologous production of CpGV (Reiser *et al.*, 1993).

Table 3.11 A comparison of survival of *C. leucotreta* fifth instar larvae on artificial diet in three different containers.

Container		**Number of larvae per cell/pot/dish**	**Total number of larvae**	**% Larvae* surviving after 14 days**
25 cell bioassay tray		1	25	100
		2	50	94
Polypot		1	25	100
		2	50	100
		3	75	100
Petri dish	Small plug**	1	10	50
	Large plug**	1	10	90

*The majority of larvae had pupated.
**Dimensions and mean mass of large diet plugs were 22 mm x 32 mm x 4 mm and 2.73 g; dimensions and mean mass of the small diet plugs were 10 mm x 8 mm x 8 mm and 0.61 g.

Using diet plugs in Petri dishes appeared to be an unsuitable method for conducting bioassays with fifth instar larvae. Survival was particularly low on the small diet plugs (Table 3.11). This was because the diet desiccated too rapidly. Survival on the large diet plugs was far better, but not as good as the 100 % recorded in the bioassay trays (one larva per cell) and the 30 ml pots. It was decided that bioassay trays were the most suitable container in which to conduct dose-response bioassays. The containers were more compact and manageable than were the pots. The pots were

considered to be more suitable for time-response bioassays. More than one larva could be placed into each pot, and individual pots could be easily opened for frequent checking of mortality, without risking the escape of larvae from other units, as would be the case with the bioassay trays.

3.3.3.2 Dose-response bioassays

A small sample of larval cadavers from each treatment was inspected to confirm that death had occurred from viral infection. This was done by preparing Buffalo Black stained smears of individual larvae and inspecting them with light microscopy at 1000X magnification under oil immersion (see section 2.2.1.2 for full protocol).

The regression (probit) lines fitted to the data in Table 3.12 (Fig. 3.10) had the equations y = 1.0717 + 0.5368x (SE of slope = 0.0925), y = 1.1332 + 0.4984x (SE of slope = 0.0940) and y = 1.8891 + 0.4896x (SE of slope = 0.0925) (Appendix 3). Deviations from the lines calculated from the data from the first and second replicates were estimated to be homogenous, making the chi-squared test more applicable than would have been the case if deviations were heterogenous (Van Ark, 1995). Deviations from the line calculated from the data from the third replicate were estimated to be heterogenous, however, this was rectified by combining doses, resulting in homogenous deviations. The slopes of the lines (0.5368, 0.4984 and 0.4896) were rather gradual, 1 - 2 being the norm for virus assays (Jones, 2000). However, it is not unusual for the slope of the dosage-mortality curve for older instar larvae to be gradual, and it is certainly usually not as steep as for the younger instars. Dosage-mortality slope for fifth instar *C. pomonella* larvae with CpGV was estimated at 0.3478, compared to 1.3497 for first instar larvae (Sheppard & Stairs, 1977). Even from third to fourth instar *H. armigera* larvae, the slope of the dosage-mortality curve with HaNPV decreased from 2.200 to 0.923. The gradual gradient of a mean of 0.5083 in this case could probably be attributed mainly to the relatively small increase in mortality from the third (3.072×10^6 OBs/ml) to fourth (1.536×10^7 OBs/ml) treatment (the first treatment being the control), even with a 5-fold increase in concentration (Table 3.12). Strangely, the increase in mortality from the fourth treatment (1.536×10^7 OBs/ml) to the fifth treatment (7.681×10^7 OBs/ml) and even more so from the fifth to the sixth (3.841×10^8 OBs/ml) treatment, were far greater

Table 3.12 Mortality of fifth instar *C. leucotreta* larvae in dose-response bioassays with five concentrations of CrleGV-SA.

Treatment (CrleGV 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**
Distilled water control	4.00	-	-	4.00	-	-	4.00	-	-
6.145×10^5	24.00	20.83	4.188	16.00	12.50	3.850	48.00	45.83	4.895
3.072×10^6	40.00	37.50	4.681	40.00	37.50	4.681	52.00	50.00	5.000
1.536×10^7	44.00	41.67	4.790	36.00	33.33	4.569	64.00	62.50	5.319
7.681×10^7	60.00	58.33	5.210	48.00	45.83	4.895	68.00	66.67	5.431
3.841×10^8	80.00	79.17	5.812	72.00	70.83	5.548	96.00	95.83	6.731

(than from third to the fourth treatment). One would expect smaller increases in mortality as treatments become more concentrated, as the probability of a larva (particularly a large fifth instar larva which consumes relatively large volumes of food) ingesting a lethal dosage of OBs would increase by a diminishing factor. However, a similar trend was noted in bioassays with the African armyworm, *S. exempta* (Keith Jones, personal communication).

G for fiducial limits was calculated to be 0.1142, 0.1367 and 0.1372 (Appendix 4). According to Van Ark (1995) values greater than approximately 0.025, mean that the variation of mortalities is rather large. However, experimental procedures or the value of the probit line should only come into question if G exceeds 0.25. Mean LC_{50} and LC_{90} (for the three replicates) were estimated to be 2.678 x 10^7 OBs/ml and 9.118 x 10^9 OBs/ml respectively. The 95 % fiducial limits of the LC_{50} and LC_{90} were estimated to range from 5.683 x 10^5 - 1.751 x 10^8 and 2.361 x 10^8 - 1.387 x 10^{12} OBs/ml respectively.

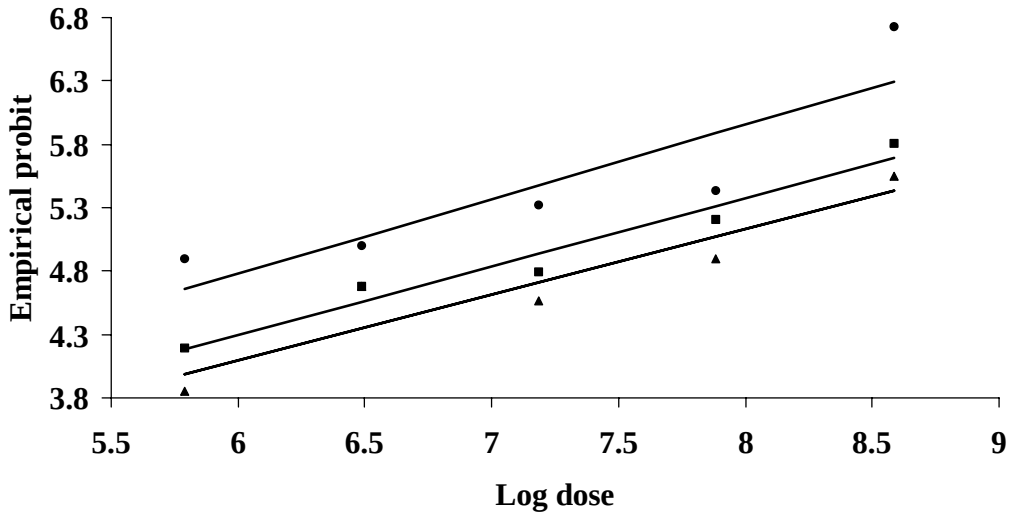

Fig. 3.10 Dose-mortality probit line for CrleGV-SA against fifth instar *C. leucotreta* larvae.

3.3.3.3 Time-response bioassays

As mentioned for the neonate assays, the data set generated by this methodology was

not ideal for the statistical technique (Finney, 1971). However, the alternative i.e. to test subjects only once each, would require very high numbers of larvae and would therefore be wasteful and impractical.

Table 3.13 Mortality of fifth instar *C. leucotreta* larvae in time-response bioassays (three replicates) with the LC_{90} concentration of CrleGV-SA.

Time after treatment		**Cumulative larval mortality (%)**		
		Rep1	**Rep2**	**Rep3**
Days	**Hours**	**(n = 38)**	**(n = 38)**	**(n = 36)**
6	0	0	0	11.1
	8	0	5.3	22.2
	16	2.6	10.5	27.8
7	0	10.5	26.3	50.0
	8	15.8	36.8	50.0
	16	21.0	42.1	50.0
8	0	47.4	63.2	72.2
	8	63.2	73.7	72.2
	16	78.9	84.2	80.5
9	0	89.5	94.7	83.3
	8	94.7	94.7	83.3
	16	94.7	94.7	83.3
10	0	97.4	94.7	83.3
	8	100	97.4	88.9
	16		100	94.4
11	0			94.4
	8			97.2
	16			100

The first virus induced mortality was observed 6 days (144 hours) post treatment (Table 3.13). In only one of the three replicates was there any control mortality: one larva died, which was symptomatic of viral infection. This was a result of the low level of non-apparent CrleGV-SA infection in the *C. leucotreta* culture, which sometimes became apparent when larvae were placed under some form of stress. It was occasionally observed that when fifth instar larvae were removed from rearing jars, whether to be used for bioassays or for virus production, CrleGV-SA infection would manifest in the larvae that were left in the jar. The disruption of diet to remove larvae was considered to cause sufficient stress to make the previously symptomless larvae susceptible to virus attack. As the purpose for conducting the bioassay was to estimate incubation time before harvesting, and as a level of virus (normally sub-lethal) was present in the *C. leucotreta* culture which was used for CrleGV-SA production, this negligible level of control mortality was considered to accurately

reflect the conditions which would normally prevail during virus production, and was therefore not considered in the calculation of the time-response relationship.

In the three replicates a total of 38 larvae (out of a total of 150) placed on treated diet, survived to pupate and eclose successfully. Total mortality of treated larvae was therefore 74.6 %. This was somewhat less than the 90 % mortality expected when diet is inoculated with the LC_{90}. It is possible that the larvae used in the time-response bioassays might have been slightly older (i.e. slightly closer to pupation) than the larvae used in the dose-response bioassays, even though they were all estimated to be fifth instar larvae. This is an important point to note when producing CrleGV-SA *in vivo* in fifth instar larvae. The youngest fifth instar larvae possible should be used, in order to minimise loss of productivity through pupation.

For the purpose of calculating the time-response relationship, larvae surviving to pupation (and eventual adulthood) were ignored. The total numbers of larvae, considered to be treated in each replicate, were therefore reduced to 38, 38 and 36. This is an acceptable omission, referred to as biological truncation (Bliss, 1935). As explained for the assays with neonate larvae, it is applicable when the dosage is small enough to be survived by one or more of the individuals in the experiment. Measurements of mean and standard deviation will have a consistent biological significance only if they represent the reaction times of those individuals that conform to the particular response under investigation (Bliss, 1935).

The first virus induced mortality was observed 6 days after larvae were placed onto the diet (Table 3.13). After 11 days and 16 hours no further mortality was recorded. The time-mortality relationship (Fig. 3.11) was analysed using a logit version of a probit model (Appendix 5). From this, the LT_{50} and LT_{90} for each replicate and their standard errors were estimated. Lower and upper 95 % limits ranged from 7 days 4 h to 8 days 5 h for the LT_{50}, and from 8 days 19 h to 10 days 11 h for the LT_{90}. LT_{50} and LT_{90}, estimated from the three replicates, were averaged to equal 7 days 17 h and 9 days 8 h, respectively. This appears to confirm the conclusion drawn from the time-response assays with neonate larvae, that ClGV-SA is a type 2, or fast GV. Federici (1997) states that type 2 GV infections typically last only 5 - 10 days in larvae infected during the fourth instar. These larvae were infected during the fifth instar.

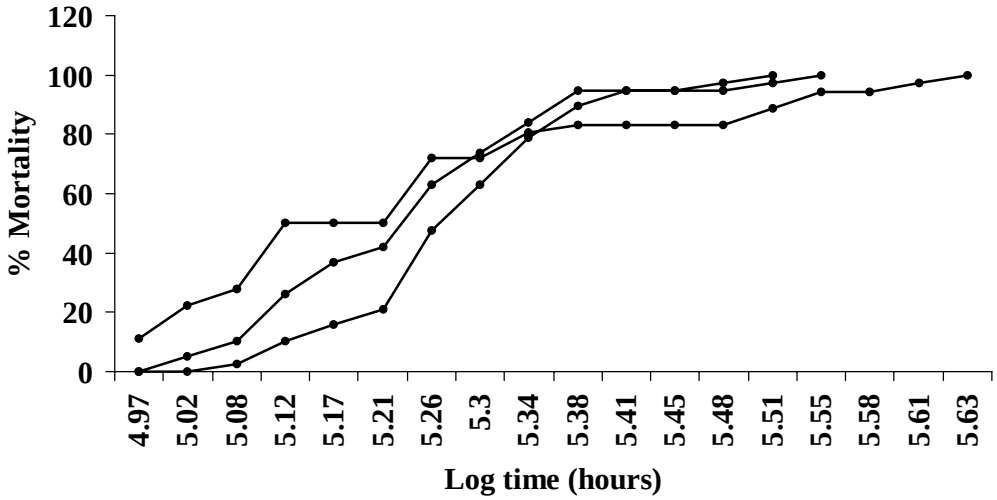

Fig. 3.11 Time-mortality relationship between *C. leucotreta* fifth instar larvae and CrleGV-SA applied at the LC_{90} concentration in three bioassay replicates.

The practical purpose of these time-response bioassays was to identify time scales pertinent to harvesting of larvae in which CrleGV-SA was being produced. More specifically, it could be determined how long after inoculation of larvae, harvesting should begin, when production would peak, and for how long after inoculation, harvesting would be likely to continue (assuming that larvae would be harvested individually as they became symptomatically infected). For these reasons, the lethal-time values calculated from the uncorrected data was as relevant as those calculated from the corrected data. Harvesting period was determined to be from five to 10 days post-inoculation, peaking from the seventh to the ninth days post-inoculation.

3.4 CONCLUSION

Effective surface dosing bioassay methods have been developed for use with *C. leucotreta* neonate larvae. Dose-mortality and time-mortality curves were calculated from such bioassays with CrleGV-SA against neonate *C. leucotreta* larvae. From probit lines, lethal concentrations were estimated (LC_{50} = 4.095 x 10^3 OBs/ml; LC_{90} = 1.185 x 10^5 OBs/ml) and from a logit version of probit analysis, lethal times were estimated (LT_{50} = 4 days 22 h; LT_{90} = 7 days 8 h). These values have provided a norm for pathogenicity, which can serve as a quality control standard against which all batches of CrleGV-SA can be tested in future. These values also confirmed that

CrleGV-SA has significant potential to be developed as a biological control agent for the management of *C. leucotreta* on agricultural crops. Further investigation was therefore warranted. This was done by conducting detached fruit assays. These assays provided an indication of the range of concentrations that should be tested in field trials. Accordingly, field trial concentrations should range approximately from the lowest concentration that significantly reduced infestation i.e. 1.08×10^7 OBs/ml up to the approximate LC_{90} values calculated i.e. a maximum value of 3.819×10^{10} OBs/ml. However, due to the stringent test imposed on the virus in the detached fruit bioassays (because of the high number of *C. leucotreta* eggs per fruit) lower concentrations should also be tested in the field.

Lethal concentrations (LC_{50} = 2.678×10^7 OBs/ml; LC_{90} = 9.118×10^9 OBs/ml) and lethal times (LT_{50} = 7 days 17 h; LT_{90} = 9 days 8 h) were also estimated for CrleGV-SA against fifth instar larvae. The LC_{90} will be used for surface inoculation of diet for virus production in fifth instar larvae. Larvae are likely to be ready for harvesting from five to 10 days post-inoculation.

4

HOST REARING

4.1 INTRODUCTION

Ripley *et al.* (1939) were the first to establish a method for the mass rearing of *C. leucotreta* using artificial diet. However, Theron (1948) was the first to provide a detailed account of the methods used to rear *C. leucotreta.* A maize meal diet was inoculated with a *Rhizopus* sp. fungus. Neither Ripley *et al.* (1939) nor Theron (1948) clarified the purpose of the fungus, but it probably produced metabolites, which converted carbohydrates in the maize meal into amino acids and vitamins, important to the development of *C. leucotreta* larvae.

The idea of mass rearing remained dormant for a couple of decades (bar a single report from Bot (1965)), until revived by Schwartz (1971; 1972), who suggested certain important improvements to the previously devised method. Most of these improvements related to better hygiene e.g. surface sterilisation of eggs. Schwartz (1971) originally reared *C. leucotreta* for general experimental purposes. This soon grew into a larger operation for the purpose of mass rearing and augmenting the *C. leucotreta* egg parasitoid, *Trichogrammatoidea cryptophlebiae* (previously known as *Trichogramma luteum*) (Schwartz, 1977; 1980; Schwartz *et al.*, 1982). Despite the recommendation of a more elaborate aseptic diet by Couilloud & Giret (1980), the methods of Ripley *et al.* (1939), Theron (1947) and Schwartz (1971) have been preferred in South Africa. This method has been used for mass rearing *C. leucotreta* for production of the egg parasitoid, at Zebediela Estate (Northern Province) and Goedehoop Citrus (now Cederberg Insectary) (Western Cape) for several years. However, two major problems exist with this method of rearing the host, making it unsuitable for mass production of CrleGV-SA.

Fungal contamination of diet occurs frequently, and the method is labour intensive, which would be a severe impediment when attempting to scale up for any commercial purpose e.g. baculovirus production, production of natural enemies, production for SIT (sterile insect technique). Three species of *Aspergillus* fungus were identified as the main contaminants in the diet. These were *A.* cf. *oryzae* (Ahlburg) Cohn (Plate 4.1), *A. tamarii* Kita (Plate 4.2) (in the *A. flavus* group) (Oloff O'Brien, personal communication) and *A. ascomycotina* (Elsabe Matthee, personal communication). Contamination was especially severe if humidity in the rearing room rose above 50 % RH or if the diet was too moist, causing humidity in rearing jars to become too high. *Aspergillus* spp. sporulate very aggressively, and unless such contamination is immediately curtailed, it can become devastating.

Plate 4.1 *Aspergillus* cf. *oryzae* contamination in a jar of *Rhizopus* inoculated maize meal diet for rearing *C. leucotreta* larvae.

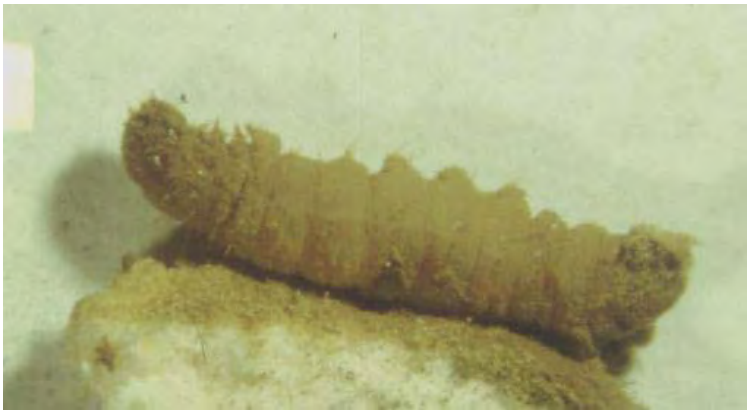

Plate 4.2 *C. leucotreta* larva attacked by *Aspergillus tamarii*, removed from a jar of contaminated *Rhizopus* sp. inoculated maize meal diet.

Apart from needing to address problems of contamination and labour intensiveness, which would be important regardless of the purpose for which *C. leucotreta* was reared, the ultimate objective of mass rearing *C. leucotreta* in this case must be remembered. Considerable numbers of large healthy larvae must be reared for optimal *in vivo* production of CrleGV-SA. Consequently, it was deemed necessary to improve all aspects of *C. leucotreta* rearing.

4.2 MATERIALS AND METHODS

4.2.1 Standard rearing protocol

The *C. leucotreta* larval culture was kept at approximately 27°C and 30 % relative humidity. Moths were held at the same temperature and approximately 60 % humidity. Moths were kept in groups of arbitrary numbers under inverted sieves (190 mm diameter) and were sustained with water-saturated cotton wool inserted into a 40 mm diameter hole made in the centre of the sieve. Sieves were placed on wax paper on which the moths oviposited (Plate 4.3). These egg sheets (Plate 4.4) were cut into small squares, each with a substantial but undetermined number of eggs. These were surface sterilised by briefly dipping in a 25 % formalin solution, and then placed on a septic maize meal diet, made up in honey jars (350 ml capacity) according to the following standard recipe (Ripley *et al*., 1939; Theron, 1948; Schwartz, 1971). Benlate (active ingredient, methyl-1-(butylcarbamoyl)-2-benzimidazole carbamate, commonly called benomyl) (0.165 g) was dissolved in distilled water (33 ml) and added to maize meal (40 g). This was autoclaved for 20 minutes at 121ºC and allowed to cool. *Rhizopus* sp. was grown on autoclaved maize meal. Once sporulating, a pinch (no specific measurement) of fungus was dispersed in a bottle (250 ml capacity) of sterile distilled water (with a drop of dishwashing detergent as an emulsifier) using sterilised forceps. A dash (no specific measurement) of the *Rhizopus* sp. suspension was discharged into each jar of diet, through a punctured (± 3 mm diameter hole) lid.

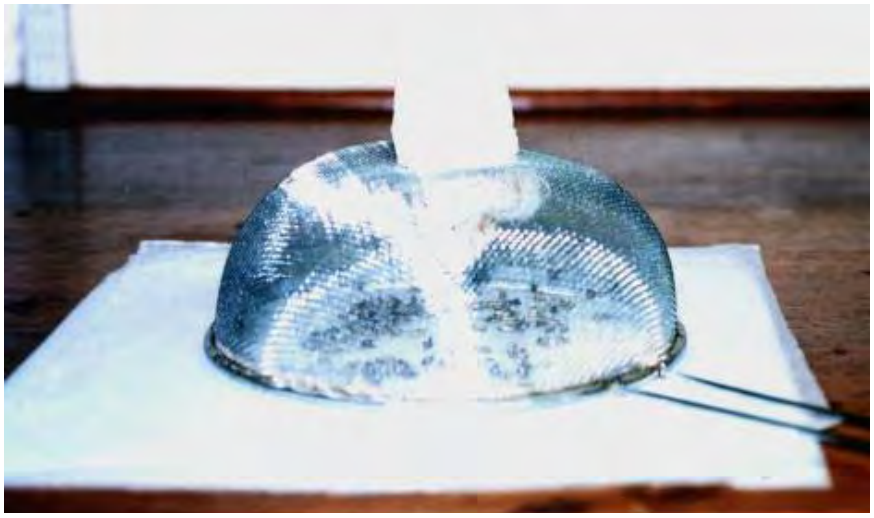

Figure 4.3 Inverted sieve on layers of wax paper, used for *C. leucotreta* oviposition.

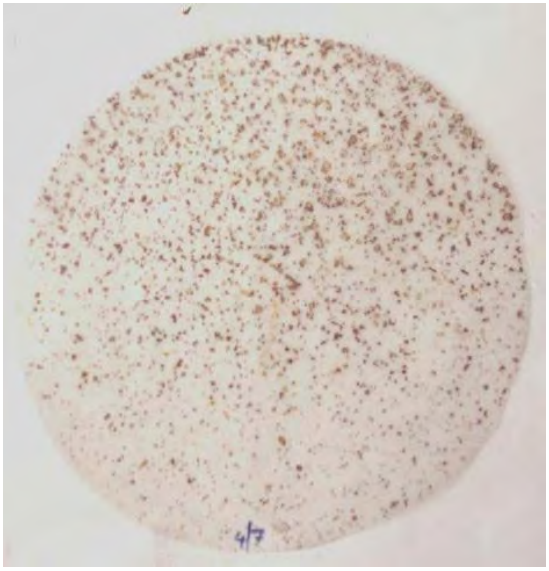

Plate 4.4 Egg (wax) sheet covered with *C. leucotreta* eggs.

Jars were stoppered with cotton wool plugs (Plate 4.5). By the time eggs had hatched and larvae began to penetrate into the diet (± 3 days after eggs were placed into diet jars), *Rhizopus* sp. had begun to grow vigorously on the diet. Most of the larvae pupated in the cotton wool. Once pupation occurred, stoppers (removed from bottles) were placed in wooden emergence boxes. If a large number of pupae had formed in the jars, then jars were also placed into emergence boxes. When moths emerged they were attracted

towards the light and moved through a hole into a 2 ℓ bottle, which was attached to the box (Plate 4.6), from which they were transferred into an oviposition sieve.

Plate 4.5 *Rhizopus* sp. fungus inoculated maize meal diet for rearing *C. leucotreta* larvae.

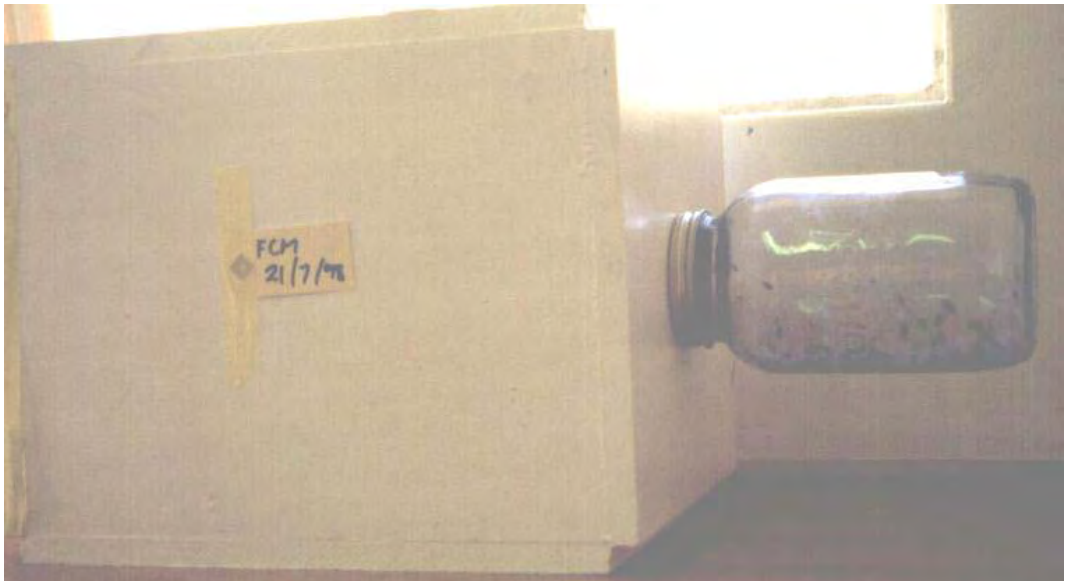

Plate 4.6 Emergence box and bottle used for holding *C. leucotreta* pupae and catching emerging moths.

4.2.2 Development of life stages

Ten egg batches of 50 eggs each, 10 batches of 100 eggs each, 10 batches of variable number but less than 100 eggs each (ranging from five to 95) and 10 batches of variable number but more than 100 eggs each (up to several hundred) were each placed on diet within honey jars. Jars were left for several weeks for moth development and eventually death to take place. The number of moths produced from each batch of eggs, was then recorded and means were compared.

Survival and development of each life stage was then determined. Firstly, egg viability was assessed. The eggs in 20 batches were defined as being clumped (in contact with one or more other eggs) and another 20 as being singly laid (not in contact with other eggs). Ten batches from each category (with a mean of 44.15 ± 6.27 (SE) eggs per batch) were briefly dipped in 25 % formalin, the standard surface sterilisation method. Egg batches were placed individually into Petri dishes. After eight days eggs were microscopically inspected to determine what proportion of eggs had hatched in each category and means were compared.

The effect of diet on egg viability was also tested by placing 20 egg batches directly onto the diet surface in honey jars, and comparing egg hatch with that of egg batches placed into Petri dishes. Mean number of eggs per batch was 5.55 ± 1.76 (SE).

Pupal viability was determined by placing 20 groups of 10 pupae into separate Petri dishes. Emerging moths were recorded. By determining survival of eggs and pupae, larval mortality could be deduced.

Sex ratio and mean weight of moths (separated into males and females) was examined when different numbers of eggs (50, 100, 150, 200, 300 and 400 eggs) were placed into jars of diet. Each treatment was replicated five times. This allowed assessment of the impact of *C. leucotreta* density on both population and individual fitness. Mean weights of moths were compared.

4.2.3 Oviposition

A new continuous production oviposition cage was designed (Design I) and built (Plate 4.7). The dimensions of the cage were: 1 m x 330 mm x 325 mm. The walls of the cage were made of a fine organdy gauze netting. The appropriateness of this material was first tested, as it was necessary to cover the cage with a substrate which was not suitable for oviposition. One pair of moths was placed in each of 20 Petri dishes. Half of the surface of the Petri dish was covered with wax paper and the other half with the netting. After five days, eggs on both substrates were counted.

Plate 4.7 Continuous production oviposition cage (Design I) for *C. leucotreta* moths.

Wax paper, on a roll, was fed into the cage through a thin slit at the bottom of the cage. The paper covered the floor of the cage and exited through a similar slit on the opposite side of the cage. Every 24 hours the wax paper, with eggs on it, was moved on by the length of the cage and cut. The eggs were used either for further production of *C. leucotreta* or production of egg parasitoids (*T. cryptophlebiae*). To test whether egg production using the continuous production cage was comparable with that of the existing method, initially 10 pairs of moths were placed in the cage. The wax paper was moved-on, daily and eggs counted until oviposition had ceased. A second test was conducted with 80 pairs of moths in the cage.

Simultaneously, one pair of moths was retained under each of 30 sieves for six days, by which time ovipostion had either ceased or declined dramatically. After each 24 hour period the wax sheet was replaced with a new one, and eggs counted. Consequently, fecundity per moth, with the sieve method, was recorded.

A second cage (Design II), which incorporated moth eclosion and oviposition into one unit, was designed, built and tested (Plate 4.8). A gauze net-covered cage was attached to the wooden emergence box into which the pupae were placed. Eclosed moths congregated in the cage. Wax paper was fed through the cage, conveyer belt style, in much the same manner as described for Design I.

Plate 4.8 Incorporated moth eclosion and oviposition unit (Design II) for *C. leucotreta* moths.

An identical number of pupae were placed into each of two emergence boxes, one with a 2 ℓ jar attached and one with an oviposition cage (Design II) attached. Moths in the jar were transferred into a sieve on a daily basis, where oviposition took place. The number of eggs laid was recorded for both systems.

Egg numbers with the Design II oviposition cage were unacceptably low and so modifications were made to improve the cage. This resulted in a third (Design III) and a fourth (Design IV) oviposition cage, both of which were tested in the same manner as was Design II.

4.2.4 Surface sterilisation of eggs

Firstly, the efficacy and safety of the standard egg surface sterilant, formalin (25 %), was tested. As results were not satisfactory, various durations of treatment (from five seconds to 15 minutes) and rinses (70% alcohol or distilled water) following treatment, were tested. Because adequate results were not achieved with any of these variations, the efficacy and safety of various alternative egg surface sterilants was tested. The products tested were alcohol (70 %), Virkon S (active ingredient: potassium peroxomonosulphate 50 % m/m; Antec (Healthcare) Africa, South Africa), glacial acetic acid, colloidal silver, formaldehyde, sodium hypochlorite and Sporekill (active ingredient: poly dimethyl ammonium chloride 120 g/ℓ; Hygrotech International, South Africa). Those treatments which showed any promise were tested again at various concentrations and for various durations. For each treatment, 20 small egg squares were prepared, with an average of 56.24 eggs per square. All treatments were conducted within a laminar flow cabinet. Dipped egg squares were held with alcohol and flame sterilised forceps. Dilutions of dip treatments were made with distilled water. Fumigant treatments were placed onto filter paper and sealed within 2 ℓ jars with the egg squares. After egg squares were subjected to treatments, 10 squares from each treatment were placed on Potato Dextrose Agar (PDA) in Petri dishes. This was done under the laminar flow cabinet to avoid aerial contamination of the agar. Petri dishes were then left at 27°C for four days before they were inspected for bacterial and fungal growth. PDA, being a non-selective fungal growth medium, was used – as the major problematic diet contaminants were fungi. PDA is, however, also commonly used as a bacterial growth medium.

The remaining 10 egg squares from each treatment were placed in Petri dishes, and left until hatching had occurred. Thereafter, eggs were microscopically inspected to determine mortality rate (i.e. whether they had successfully hatched or not). Mean proportions of eggs hatching were compared.

4.2.5 Diet sterilisation

Despite Benlate (a fungicide) being a standard ingredient in the diet, *Rhizopus* sp. growth was vigorous and *Aspergillus* spp. contamination still occurred. The value of Benlate in the diet was therefore examined. This was done by preparing five bottles of diet for each of six different concentrations of Benlate. Diet was prepared in the standard manner as previously described. *C. leucotreta* eggs were placed into each bottle. Fungal growth and larval development to adult stage were monitored. Mean numbers of moths produced per jar were compared.

Eggs, if not properly decontaminated, are a potential source of fungal contamination in diet (hence the importance of surface sterilisation of eggs). *Aspergillus* spp. spores (and other species of fungi) can also be inadvertently introduced when the diet is inoculated with *Rhizopus* sp.. A trial was conducted to test whether fungal contamination could also occur in sealed jars, entering through the cotton wool plug. A 20 % PDA solution in 50 ml distilled water was mixed in each of 20 jars. Ten of the jars were plugged with absorbent cotton wool and 10 were plugged with non-absorbent cotton wool. Jars were autoclaved for 15 minutes at 121ºC and then placed into the *C. leucotreta* larval culture room, without removing the cotton wool plugs at any stage. Jars were checked daily for fungal growth, for 21 days. Twelve days after preparation of PDA, the absorbent cotton wool plugs were thoroughly wet with water in an attempt to improve the possibility of fungal spores and hyphae reaching the PDA.

Eight trials were conducted to find an effective and safe anti-microbial agent as an alternative for Benlate. Diets were prepared in honey jars. Maize meal (40 g) and distilled water (33 ml) formed the basis of all diets, unless otherwise stated. Five jars of each treatment were prepared in all comparisons and an undetermined number of eggs placed into each jar, unless otherwise stated. In each of the trials fungal control and toxicity to *C. leucotreta* were monitored. Vigour of fungal growth was compared and fungal genera were identified in certain of the trials. Mean numbers of moths produced per jar were compared.

In the first trial various rates of Dithane (active ingredient, mancozeb) and formaldehyde (dissolved in the 33 ml distilled water) were tested, as well as Dithane and Benlate being sprayed (1 ml/jar of each particular solution) onto the surface of the diet. An undetermined number of eggs were placed into each jar.

In the second trial benzoic acid was tested, some of the jars being inoculated with *Rhizopus* sp.. This was compared with the standard *Rhizopus* sp. inoculated diet, with varying quantities of water added, which was autoclaved twice. It was postulated that a single autoclave for 20 minutes at 121ºC might not be adequate to kill all pathogens. Eggs were only placed on diets containing benzoic acid and on standard (single autoclaved) diets, but not on double autoclaved diets.

In the third trial sodium benzoate, sodium nitrate, colloidal silver, and alcohol were tested (without the addition of eggs). In the fourth trial nipagin (p-Hydroxybenzoic acid methyl ester) and glacial acetic acid were tested. In the fifth trial sodium benzoate, sodium nitrate and nipagin were retested at different dilutions. All diets in these last three trials were inoculated with *Rhizopus*, and in the last two trials, with eggs as well.

The last three trials were conducted to test the effect of the addition of sorbic acid to the diet. Various concentrations were used, in combination with nipagin and without it.

Level of fungal contamination in jars was subjectively assessed and categorised as none, very little, little, moderate or vigorous. Alternatively, the proportion of diet adjudged to be contaminated was given as a percentage. Identification of certain fungal genera – *Rhizopus*, *Aspergillus* and *Penecillium* – was also possible.

4.2.6 Diet improvement

If an anti-microbial agent is added to the diet to suppress contamination, including fungal growth, the use of a *Rhizopus* sp. in the diet as a source of nutrition becomes impossible.

It was therefore necessary to find a suitable replacement for the *Rhizopus* sp. diet. Four existing artificial diets were tested to determine if any one would be a suitable replacement for the existing septic diet. The first diet (Table 4.1) was developed by Bot (1965), specifically for *C. leucotreta*. The second (Table 4.2), was developed by Huber (1981) for rearing codling moth, but was used by Fritsch (1989) for rearing *C. leucotreta*. The third was developed by Guennelon *et al.* (1981) (Table 4.3) for codling moth, but is currently used successfully, by Horticulture Research International (HRI, Wellesbourne, UK) for rearing *C. leucotreta* (Doreen Winstanley, personal communication). The fourth diet was developed by Ware, according to an undisclosed recipe, primarily for rearing fruit flies (Tony Ware, personal communication). Ware's diet was combined with an equal quantity of maize meal, as a bulking agent. Six jars of each diet were made (dry plus wet ingredients weighing approximately 70 g per jar), all being inoculated with a similar number of eggs. Six additional jars of Guennelon diet and six of Ware's diet were inoculated with neonate larvae instead of eggs. Larval development in all jars was recorded and mean numbers of moths per jar produced per diet were compared.

Table 4.1 Bot's (1965) artificial diet ingredients tested for rearing *C. leucotreta*.

Ingredients	**Quantity for 20 jars**
Maize meal	800 g
Wheat germ	160 g
Brewer's yeast	240 g
Vitamin free casein	40 g
Ascorbic acid	6 g
Choline chloride	1.2 ml
Nipagin	12 g
Water	1000 ml

Table 4.2 Huber's (1981) artificial diet ingredients tested for rearing *C. leucotreta*.

Ingredients	**Quantity for 20 jars**
Maize meal	147 g
Yeast powder	147 g
Wheat germ	147 g
Ascorbic acid	5.5 g
Benzoic acid	5.5 g
Ethanol	20 ml
Nipagin	5.5 g
Agar	20 g
Water	2000 ml

Table 4.3 Guennelon *et al.*'s (1981) artificial diet ingredients tested for rearing *C. leucotreta*.

Ingredients	**Quantity for 20 jars**
Maize meal	380 g
Yeast powder	100 g
Wheat germ	92 g
Ascorbic acid	14 g
Benzoic acid	6 g
Formaldehyde (30 %)	3.2 ml
Nipagin	4.6 g
Agar	53.2 g
Water	2000 ml

As it was known that *C. leucotreta* was being successfully reared on the Guennelon diet, a dedicated attempt was made in this trial to adapt *C. leucotreta* to the diet. Four successive generations were reared on the Guennelon diet (10 jars per generation). Development of the first generation was compared with development on benzoic acid free Guennelon diet, as it was suspected that benzoic acid may be toxic to the early stages of *C. leucotreta.* A similar number of eggs were placed into each jar. So as to avoid contact with the diet (considering the possibility that the diet might negatively affect egg viability), each egg square was lightly wet with water and stuck to the inner side of the jar. Development was again recorded and mean numbers of moths compared.

Development of *C. leucotreta* was tested with each of five different concentrations of casein, ascorbic acid or wheat germ, as additives to the maize meal diet. Initial concentrations used were based on those reported by other authors for similar artificial diets for lepidopteran species (e.g. Ashby *et al.*, 1985; Guennelon *et al.*, 1981; Hunter-Fujita *et al.*, 1998; Reed & Tromley, 1985; Smith, 1998). The trial was repeated using only two concentrations of each additive, selected on the basis of the results of the first comparison. Development of *C. leucotreta* on these diets was compared with that on the standard maize meal diet, both with and without *Rhizopus* sp. inoculation. Mean numbers of moths produced per jar per treatment were compared.

Table 4.4 Recipes of new artificial diets.

Number of components (excluding water)	Ingredients	Quantity for 20 jars	Quantity per jar
4	Maize meal Wheat germ Casein (later replaced with milk powder*) Nipagin Distilled water	800 g 80 g 14.6 g 6 g 800 ml	40 g 4 g 0.73 g 0.3 g 40 ml
5	4-component diet plus Brewer's yeast	As above 40 g	As above 2 g
6	5-component diet plus Sorbic acid	As above 2.6 g	As above 0.13 g

*Nestlé® Nespray Instant Milk Powder.

Through testing these different additives, new four-, five- and six-component (dry ingredients) diets were developed (Table 4.4). Rate of development and number of moths developing on 20 jars of each diet were recorded and means compared. The effect of the addition of sucrose to the 5-component diet was also examined. Due to the expense of casein, this ingredient was eventually replaced with milk powder, after testing its suitability.

4.2.7 Statistical analyses

The computer programme, Statgraphics 2.0 (Statisical Graphics Corporation, 1996), was used for analysis of all data. Where there were multiple samples in a trial or where trials were replicated, means were either compared using a Students' T-test or were subjected to an ANOVA and then compared using the Bonferroni LSD multiple range test.

4.3 RESULTS AND DISCUSSION

4.3.1 Development of life stages

When honey jars of diet were inoculated with more than 100 eggs per jar, moth production appeared to decline (Table 4.5). However, moth production did not exceed a

mean of 64 % of eggs producing moths per honey jar, even when 50 or fewer eggs were introduced per jar (Table 4.5). It was then necessary to determine which life stage or stages were experiencing a high level of mortality.

Table 4.5 Rate of development of *C. leucotreta,* from egg to adult, on artificial diet.

Eggs/jar (10 jars of each)	**Percentage of eggs producing moths***
50	64.0a ± 4.7
100	61.5a ± 3.1
< 100 (variable numbers)	56.6a ± 8.3
> 100 (variable numbers)	37.6b ± 5.9

*Values in the same column followed by the same letter are not significantly different (P > 0.05; Bonferroni multiple range test).

To answer this question, percentage egg hatch was first examined. Formalin dipping, for surface sterilisation of eggs, was found to significantly reduce egg hatch (when data from singly laid and clumped eggs were pooled) (Table 4.6). The toxicity of formalin to eggs was also recorded in later trials (Tables 4.14 & 4.15). Egg hatch was also significantly reduced when eggs were laid in clumps (when data from formalin treated and untreated eggs were pooled) (Table 4.6). *C. leucotreta* larvae are known to be cannibalistic (Catling & Aschenborn, 1978). This behaviour may have been a factor responsible for reduced egg hatch. To reduce the occurrence of this phenomenon, it is necessary to ensure an optimum density of eggs on the wax sheets. This would be facilitated by exposing wax sheets to a predetermined number of moths for a specific length of time.

Table 4.6 Effect of formalin dipping and aggregational laying on *C. leucotreta* egg viability.

		% Eggs hatching		
		No formalin dip	**Formalin dipped**	**Mean**
% Eggs hatching	**Singly laid**	93.6a ± 1.8*	84.9a ± 3.8*	89.2a ± 2.3***
	Laid in clumps	87.8a ± 3.5*	71.2a ± 3.9*	79.5b ± 3.2***
	Mean	90.7a ± 2.0**	78.0b ± 3.1**	

*Values compared: values followed by the same letter are not significantly different (P > 0.05; Bonferroni multiple range test).
**Values compared: values followed by the same letter are not significantly different (P > 0.05; students T-test).
***Values compared: values followed by the same letter are not significantly different (P > 0.05; students T-test).

Egg viability was not reduced by placing the eggs directly onto the diet surface, relative to eggs being placed onto a neutral substrate (Table 4.7).

Table 4.7 The effect of diet on *C. leucotreta* egg viability.

Substrate on which eggs were laid	% Eggs hatching*
Eggs in Petri dish	91.1a ± 3.4
Eggs on diet	89.9a ± 3.1

*Values followed by the same letter are not significantly different (P > 0.05; students T-test).

A high level of pupal development and moth emergence was recorded (Table 4.8).

Table 4.8 Proportion of *C. leucotreta* pupae developing into moths.

Total no. of pupae	Total no. of moths emerging	% Pupae producing moths ± SE
200	191	95.5 ± 1.5

Mortality of 36.0 % was recorded from eggs to adulthood (with 50 eggs per jar) (Table 4.5). An egg mortality alone of 22.0 % (formalin treated) was recorded (Table 4.6). Therefore mortality of 14 percentage points of the total 36 % occurred in the larval and pupal stages. Pupal mortality was only 4.5 % (Table 4.8). Larval mortality therefore made up 9.5 percentage points. As egg production easily exceeds the numbers required for production of CrleGV-SA (but not for *T. cryptophlebiae* egg parasitoid production), a 22.0 % reduction in egg survival may be acceptable. However, a surface sterilisation treatment for eggs, which does not cause mortality, would be preferable. Even if it is not possible to reduce the larval mortality much below 20 %, it will be important to ensure that mortality does not increase beyond this level. This can be done by placing an optimal number of viable eggs into each jar of diet, thereby not increasing competition to an intensity, which would result in an excessive level of mortality. To obtain approximately 100 moths from each jar, at a 38.5 % reduction in numbers from egg to moth (Table 4.5), approximately 160 eggs should be placed into each jar.

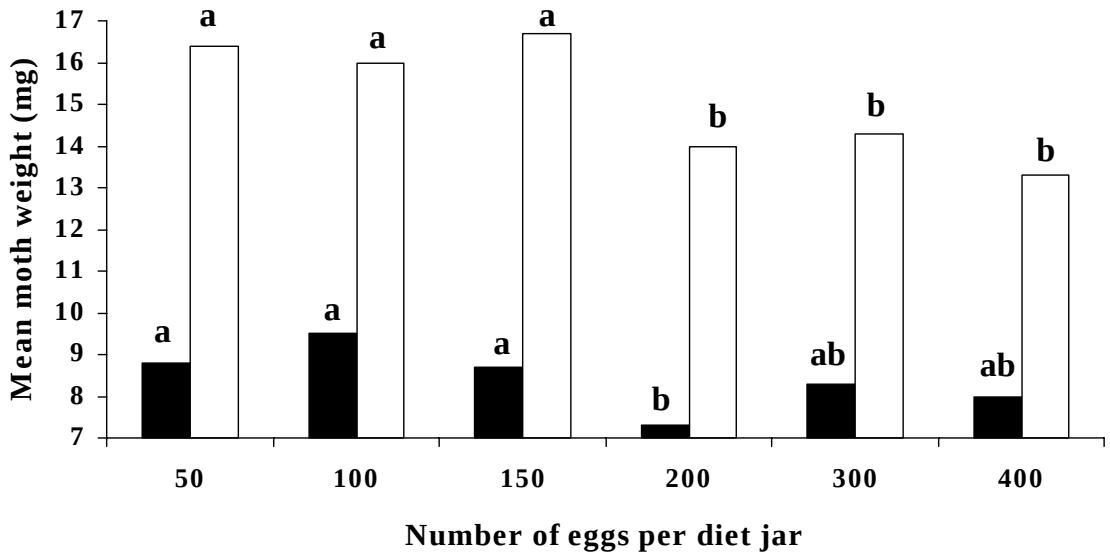

Fig. 4.1 Mean weight of male (black bars) and female (white bars) *C. leucotreta* moths that developed when different numbers of eggs were placed in jars of diet. Bars of the same colour with the same letter are not significantly different (P > 0.05; Bonferroni LSD multiple range test).

It is possible to produce more than 100 moths per jar (almost 300 larvae have been counted from one jar) but this appears to be at the cost of reduced fitness of female moths. There appeared to be no relationship between sex ratio and *C. leucotreta* density; sex ratio generally ranged from 1:1 to 4:3 (male:female) in jars of healthy individuals. Weight of male moths seemed to be affected by high population densities (Fig. 4.1), declining from a mean of 9.00 ± 0.25 mg per moth for the three lowest densities, to 7.87 ± 0.30 mg per moth for the three highest densities (a 12.2 % reduction in weight). Weight of female moths seemed to be even more affected (Fig. 4.1). Mean weight of moths declined from an average of 16.37 ± 0.20 mg each for the three lowest densities, to 13.87 ± 0.30 mg each for the three highest densities (a reduction of 15.2 %). In both cases (males and females) these means were statistically different (males: $t = 2.915$; $P < 0.05$; females: $t = 6.964$; $P < 0.05$). A decline in female moth weight would probably negatively affect their fecundity, as smaller moths would be unlikely to have the same egg load capacity as larger moths.

4.3.2 Oviposition

Fecundity of moths using the standard inverted sieve method was a mean of 256.2 eggs per moth (Table 4.9), ranging from 10 to 487. The mean compared favourably with that found by certain other researchers (Hepburn 1947; Stofberg 1939), but was well under that recorded by Daiber (1980). A conspicuous number of eggs were observed to be laid on the smooth rim of the sieve. This could account for a significant loss of eggs in the production cycle.

Table 4.9 Daily and total average number of eggs laid per female moth on wax paper underneath inverted sieves and in the continuous production oviposition cage (Design 1).

Day	**Mean eggs/moth**		
	Inverted sieves (n = 30)	**Continuous production cage (Design 1)**	
		10 pairs of moths (n = 1)	**80 pairs of moths (n = 1)**
1	0.2	5.70	14.42
2	59.4	7.40	24.69
3	73.4	2.80	20.12
4	60.8	3.60	14.55
5	48.4	0.50	3.00
6	10.8	0	2.27
7			2.99
8			0.17
9			0.31
Average total eggs/moth	256.20 ± 51.26	20.00	82.52

In the trial in which moths were given the choice of ovipositing on wax paper or on organdy netting, eggs were not laid on the netting. The netting was therefore regarded as a suitable material for enclosing the framework of the continuous production oviposition cage (Design I). Despite this, egg laying recorded for moths in the continuous production cage was unacceptably low (Table 4.9). Results could not be statistically compared with those achieved using the standard inverted sieve method as the trials in the continuous production cage were not replicated. However, due to the dramatic difference in egg

numbers with the two systems, replication was regarded to be unnecessary. The higher density of moths in the continuous production cage produced a higher mean number of eggs per female moth. It is possible that this trend may have been extended by placing higher moth densities in the cage. However, further work with the Design I cage was halted, as a cage which could be attached to the moth eclosion box was considered more suitable. This improved set-up would substantially save labour and time, would minimise the escape of moths experienced in the transferal process, and would decrease the presence of wing scales in the air (which could be allergenic).

Relative to the old system (inverted sieves), egg production was reduced by 45.1 % with the new Design II cage (which was attached to the moth eclosion box) (Table 4.10), and by 34.7 % with the new Design III cage (Table 4.11). The reduction occurred because eggs were being laid on the smooth wooden structure of the new oviposition cages, rather than on the wax paper. This problem was more severe with the Design II than the Design III cage, as there was a greater surface area of alternative oviposition substrate (wooden framework) in the former (Plate 4.8), than the latter.

Table 4.10 Comparison in egg production, between the old system and a new oviposition cage (Design II).

System	**Number of eggs (over 22 days)***	**Reduction in number of eggs (%)**
Standard sytem	31769	45.1
New Design II	17454	

*563 pupae placed into each identical moth eclosion box.

Table 4.11 Comparison in egg production, between the old system and a new oviposition cage (Design III).

System	**Number of eggs (over 16 days)***	**Reduction in number of eggs (%)**
Old system	29439	34.7
New design III	19223	

*511 pupae placed into each identical moth eclosion box.

The Design IV oviposition cage contained the wax paper conveyer-belt sheeting and was made with a thin wire frame, covered with organdy netting. There was therefore very little alternative surface area on which moths could oviposit. Sieve rims probably

provided a greater alternative surface area than did the framework of the Design IV cages. Consequently, the number of eggs produced in the Design IV cage was 9.3 % higher than in the sieve (Table 4.12). The Design IV cage was therefore an improvement on the original oviposition system.

Table 4.12 Comparison in egg production, between the old system and a new oviposition cage (Design IV).

System	**Number of eggs (over 19 days)***	**Increase in number of eggs (%)**
Old system	28872	9.3
New Design IV	31566	

*322 pupae placed into each identical moth eclosion box.

Plate 4.9 Incorporated moth eclosion and oviposition unit (Design IV) for *C. leucotreta*. The oviposition cage on the top right is not covered with netting so that the wax paper conveyer belt system can be demonstrated.

Ultimately, individual moth eclosion cages were abandoned. One unit consisting of 10 eclosion compartments was designed and constructed. Pupae from different dates were

placed into each compartment. New Design IV oviposition cages were attached to each compartment (Plate 4.9).

One problem that occurred with the new oviposition cage was the accumulation of dead moths in the cage, while more recently emerged moths were still fecund. These gravid moths could therefore be deprived of a large portion of their intended oviposition substrate (the wax paper). This problem was solved by turning oviposition cages by 90°, so that the wax paper was placed on the side of the cage rather than the bottom.

4.3.3 Surface sterilisation of eggs

In the first egg surface sterilisation trial the standard treatment, 25 % formalin dipping for five seconds, proved to be totally ineffective in controlling fungal growth (Table 4.13). Bacterial contamination was slightly, but not adequately, reduced. Mortality of these formalin treated eggs, 18 % higher than that of the untreated eggs (Table 4.13), was also unacceptably high. This was even higher than previously recorded (Table 4.6).

Table 4.13 Effectiveness of formalin as a surface sterilant of *C. leucotreta* eggs, and its effect on egg survival.

Treatment	**Egg hatch (%)* ± SE**	**Number of egg squares (out of 10) with contamination**			
		Bacteria	***Aspergillus***	***Penicillium***	**Any contamination**
Untreated	86.54a ± 1.26	10	10	1	10
25 % Formalin dip (5 sec)	71.20b ± 3.90	7	10	1	10

*Values in the same column followed by the same letter are not significantly different (P > 0.05; Bonferroni LSD multiple range test).

In a second trial, dipping times in formalin were extended, to try and further reduce contamination. The most effective treatment was a five minute formalin dip followed by an alcohol rinse (Table 4.14). The alcohol not only assisted in reducing contamination, being the only treatment which totally eliminated any evidence of bacteria, but also improved egg survival relative to eggs that were also treated with formalin for five minutes but not rinsed in alcohol (Table 4.14). Despite the alcohol rinse, survival of these eggs was still substantially lower than survival of untreated eggs. Dipping in

formalin for 15 minutes, not only killed almost all of the eggs but did not reduce bacterial contamination (Table 4.14). A subsequent rinse in distilled water dramatically reduced the deleterious effect of the formalin.

Table 4.14 Effectiveness of various formalin treatments as *C. leucotreta* egg surface sterilants and their effect on egg survival.

Treatment	**Egg hatch (%)* ± SE**	**Number of egg squares (out of 10) with contamination**			
		Bacteria	***Aspergillus***	***Penicillium***	**Any contamination**
Untreated	90.32a ± 1.83	10	10	8	10
25 % Formalin dip (5 min)	57.02b ± 2.83	10	0	0	10
25% Formalin dip (5 min) and 70 % alcohol rinse	63.45c ± 12.62	0	0	1	1
25 % Formalin dip (15 min)	2.99d ± 0.94	10	0	2	10
25 % Formalin dip (15 min) and distilled H_2O rinse	79.00b ± 2.78	10	0	2	10

*Values in the same column followed by the same letter are not significantly different (P > 0.05; Bonferroni LSD multiple range test).

Table 4.15 Effectiveness of various treatments as *C. leucotreta* egg surface sterilants and their effect on egg survival.

Treatment	**Egg hatch (%)* ± SE**	**Number of egg squares (out of 10) with contamination**			
		Bacteria	***Aspergillus***	***Penicillium***	**Any contamination**
Untreated	89.32a ± 2.23	10	10	9	10
70 % Alcohol dip (15 min)	80.22b ± 1.65	0	10	9	10
1 % Virkon S dip (15 min)	57.28c ± 8.02	10	10	9	10
0.2 % Sodium hypochlorite dip (15 min)	0d	0	0	0	0
0.4 % Sporekill dip (1 h)	78.04b ± 1.77	0	1	0	1
Colloidal silver dip (1 h)	84.51ab ± 3.70	10	10	8	10
0.8 % Glacial acetic acid dip (1 h)	82.39ab ± 2.67	10	10	10	10
Formaldehyde fumigation (55 µℓ/2 ℓ bottle for 6 h)	83.33ab ± 2.01	5	9	8	10

*Values in the same column followed by the same letter are not significantly different (P > 0.05; Bonferroni LSD multiple range test).

It was therefore evident that formalin either did not adequately sterilise the egg surfaces or substantially reduced survival of eggs, or both. Consequently, a range of other treatments was tested (Table 4.15). Formaldehyde (as a fumigant), sodium hypochlorite and Sporekill demonstrated sufficient potential to surface sterilise eggs to warrant further testing. Sodium hypochlorite was highly toxic to eggs but concentrations and immersion times could be reduced. Alcohol eliminated bacterial contamination but was ineffective

against fungi and was therefore not tested further. Virkon, colloidal silver and glacial acetic appeared to be completely ineffective.

Table 4.16 Effectiveness of formaldehyde fumigation as a *C. leucotreta* egg surface sterilant, and its effect on egg survival.

Treatment	**Egg hatch (%)* ± SE**	**Number of egg squares (out of 10) with contamination**			
		Bacteria	***Aspergillus***	***Penicillium***	**Any contamination**
Untreated	89.32a ± 1.79	10	10	10	10
Formaldehyde fumigation (55 µℓ/2 ℓ bottle for 6 h)	76.52b ± 4.15	8	8	9	10
Formaldehyde fumigation (200 µℓ/2 ℓ bottle for 6 h)	0e	4	4	6	10
Formaldehyde fumigation (1 ml/2 ℓ bottle for 6 h)	0e	0	4	4	6
Formaldehyde fumigation (55 µℓ/2 ℓ bottle for 8 h)	51.84c ± 2.83	3	8	4	10
Formaldehyde fumigation (55 µℓ/2 ℓ bottle for 15 h)	0e	1	4	4	8
Formaldehyde fumigation (55 µℓ/2 ℓ bottle for 6 h) + 25 % formalin dip (2 min) + 70 % alcohol dip (2 min)	40.90d ± 3.43	1	3	1	4

*Values in the same column followed by the same letter are not significantly different (P > 0.05; Bonferroni LSD multiple range test).

Fumigation with formaldehyde at concentrations higher than 55 µℓ/2 ℓ bottle and for longer than six hours, negatively affected egg survival (Table 4.16). Even at the highest concentration (1 ml/2 ℓ bottle) and longest duration (15 hours) of fumigation, sterilisation was not adequate. A series treatment of formaldehyde fumigation, followed by formalin dipping and rinsing in alcohol was the most effective treatment in this particular trial, reducing contamination by 60 % (Table 4.16). However, mortality of eggs was unacceptably high. Formaldehyde was therefore not considered to be a sufficiently effective or safe egg surface sterilant.

Because the sodium hypochlorite treatment initially used killed all eggs (Table 4.15), lower concentrations and shorter immersion times were subsequently tested (Table 4.16). Unfortunately, none of these treatments were adequately effective in reducing either bacterial or fungal contamination. Even after rinsing in distilled water, sodium hypochlorite treatments of five and 15 minutes dramatically reduced egg survival (Table

4.17). Sodium hypochlorite was therefore not an appropriate surface sterilant for *C. leucotreta* eggs.

Table 4.17 Effectiveness of sodium hypochlorite for surface sterilisation of *C. leucotreta* eggs and its effect on egg survival.

Treatment	**Egg hatch (%)* ± SE**	**Number of egg squares (out of 10) with contamination**			
		Bacteria	***Aspergillus***	***Penicil-ium***	**Any contamination**
Untreated	88.57a ± 9.94	10	10	7	10
0.2 % Sodium hypochlorite dip (5 sec) & sterilised distilled H_2O rinse	82.95a ± 2.57	8	10	7	10
0.2 % Sodium hypochlorite dip (5 min) & sterilised distilled H_2O rinse	10.00b ± 10.00	8	10	2	10
0.2 % Sodium hypochlorite dip (15 min) & sterilised distilled H_2O rinse	11.35b ± 4.62	4	10	2	10

*Values in the same column followed by the same letter are not significantly different (P > 0.05; Bonferroni LSD multiple range test).

Table 4.18 Effectiveness of Sporekill for surface sterilisation of *C. leucotreta* eggs and its effect on egg survival.

Treatment	**Egg hatch (%)* ± SE**	**Number of egg squares (out of 10) with contamination**			
		Bacteria	***Aspergillus***	***Penicil -lium***	**Any contamination**
Untreated	88.57a ± 2.67	0	10	10	10
0.4 % Sporekill dip (5 s)	76.82a ± 5.53	0	10	0	10
0.2% Sporekill dip (15 min)	85.83a ± 4.89	0	0	0	0
0.2 % Sporekill dip (4 min)	75.46a ± 3.28	0	9	9	10
0.1 % Sporekill dip (15 min)	81.19a ± 6.36	0	3	0	3
0.1 % Sporekill dip (4 min)	86.82a ± 4.42	0	4	2	6

*Values in the same column followed by the same letter are not significantly different (P > 0.05; Bonferroni LSD multiple range test).

It was therefore evident that formalin either did not adequately sterilise the egg surfaces or substantially reduced survival of eggs, or both. Consequently, a range of other treatments was tested (Table 4.15). Formaldehyde (as a fumigant), sodium hypochlorite and Sporekill demonstrated sufficient potential to surface sterilise eggs to warrant further testing. Sodium hypochlorite was highly toxic to eggs but concentrations and immersion times could be reduced. Alcohol eliminated bacterial contamination but was ineffective against fungi and was therefore not tested further. Virkon, colloidal silver and glacial acetic appeared to be completely ineffective.

4.3.4 Diet sterilisation

Benlate was found to have no effect in suppressing fungal growth, even at a rate 20 times the standard rate (Table 4.19). As a result of the severe contamination of *Aspergillus* spp., development of *C. leucotreta* was poor. It was therefore not possible to ascertain to what extent the Benlate, particularly the higher rates, may have retarded *C. leucotreta* development. As Benlate was shown to be ineffective as a fungicide, this question was irrelevant. Through years of exposure of *Rhizopus* sp. and *Aspergillus* spp. to Benlate, these fungi may have developed resistance to the active ingredient.

Table 4.19 Effect of different concentrations of Benlate on fungal growth when added to the *Rhizopus* sp. inoculated maize meal diet.

Benlate rate (g/ℓ distilled H_2O)	**Moths produced/jar* ± SE (2 jars/treatment)**	**Fungal growth**
0.5 (standard rate)	2.0a ± 1.0	All jars with strong growth of *Rhizopus* sp. and *Aspergillus* spp. – no apparent suppression of fungal growth.
1.0	10.5a ± 1.5	
2.0	4.0a ± 2.0	
3.0	4.5a ± 3.5	
5.0	13.0a ± 7.0	
10.0	2.0a ± 1.0	

*Values in the same column followed by the same letter are not significantly different (P > 0.05; Bonferroni LSD multiple range test).

None of the PDA jars, both those plugged with absorbent cotton wool and those plugged with non-absorbent cotton wool, showed any signs of fungal infection. Contamination of diet can therefore not occur once the diet jar is plugged. Contamination most likely enters with egg or *Rhizopus* sp. inoculation. It is therefore important to surface sterilise eggs effectively, and to replace Benlate with an effective fungicide. This in turn would necessitate replacing the *Rhizopus* sp. fungus with alternative dietary additives.

Neither Dithane nor formaldehyde was a suitable fungicide. At the rates at which they controlled fungi, they also inhibited or even prevented *C. leucotreta* development (Table 4.20). Development of *C. leucotreta* on the standard *Rhizopus* sp. inoculated diet was poor. This was due to *Aspergillus* spp. contamination. Interestingly, development in the

diet which was surface-sprayed with Benlate, was very good. The explanation is that not only is Benlate not toxic to the larvae, as are Dithane and formaldehyde, but also that *Aspergillus* spp. Contamination, which is highly toxic to larvae, was substantially less than that observed on the standard *Rhizopus* sp. diet.

Table 4.20 Effect of different concentrations of Dithane, formaldehyde and Benlate on fungal growth and *C. leucotreta* development, when added to a maize meal diet.

Treatment	**Moths produced/jar* ± SE (2 jars/treatment)**	**Fungal growth**
Standard *Rhizopus* sp. diet	19.5b ± 10.5	Vigorous
Dithane 0.02 g/ℓ	26.5ab ± 1.5	Vigorous
Dithane 0.05 g/ℓ	8.0bc ± 1.0	Little
Dithane 0.10 g/ℓ	9.5bc ± 9.5	Little
Dithane 0.50 g/ℓ	0c	Little
Dithane 1.00 g/ℓ	0c	None
Formaldehyde 0.01 ml/ℓ	16.0b ± 2.0	Vigorous
Formaldehyde 0.05 ml/ℓ	24.0b ± 22.0	Little
Formaldehyde 0.10 ml/ℓ	13.5bc ± 2.5	*Aspergillus*; no *Rhizopus*
Formaldehyde 0.50 ml/ℓ	9.5bc ± 6.5	None
Formaldehyde 1.00 ml/ℓ	0c	None
Benlate 0.50 g/ℓ surface sprayed	62.5a ± 9.5	Vigorous
Dithane 0.20 g/ℓ surface sprayed	30.5ab ± 8.5	Vigorous

*Values in the same column followed by the same letter are not significantly different (P > 0.05; Bonferroni LSD multiple range test).

Benzoic acid appeared to be even more toxic to *C. leucotreta* than were Dithane and formaldehyde. *C. leucotreta* development was already prevented at rates that were too low to fully control fungal growth (Table 4.21). Double autoclaving of diet was shown to be unnecessary, as no fungal growth was observed on single autoclaved diet (Table 4.21).

A trial conducted with various other products, showed that alcohol was an effective but unsuitable additive. Alcohol at 70 % dilution, completely suppressed fungal growth, but also caused desiccation of the diet (Table 4.22). Neither sodium benzoate nor sodium nitrate was effective in controlling fungal growth at the rates used. Colloidal silver, if the diet was not autoclaved, showed some potential to suppress fungal growth.

Table 4.21 Effect of different concentrations of benzoic acid (as a diet additive) on fungal growth and *C. leucotreta* development, and effect of double autoclaving on fungal growth.

Treatment	**Autoclaved**	***Rhizopus* inoculated**	**Moths produced/ jar* ± SE**	**Fungal growth**
Standard *Rhizopus* sp. diet	1x	Yes	48.0a ± 3.0	Vigorous
Benzoic acid 0.2 g/jar	1x	No	5.0b ± 1.0	Very little
Benzoic acid 0.4 g/jar	1x	No	0c	None
Benzoic acid 0.8 g/jar	1x	No	0c	None
Benzoic acid 1.2 g/jar	1x	No	0c	None
Benzoic acid 0.2 g/jar	1x	Yes	0c	Little
Benzoic acid 0.4 g/jar	1x	Yes	0c	Very little
Benzoic acid 0.8 g/jar	1x	Yes	0c	No *Rhizopus* sp.; very little *Aspergillus* spp.
Benzoic acid 1.2 g/jar	1x	Yes	0c	None
Standard diet	1x	No	No eggs	None
No benzoic acid (33 ml H_2O)	2x	No	No eggs	None
No benzoic acid (40 ml H_2O)	2x	No	No eggs	None
No benzoic acid (45 ml H_2O)	2x	No	No eggs	None
No benzoic acid (50 ml H_2O)	2x	No	No eggs	None

*Values in the same column followed by the same letter are not significantly different ($P > 0.05$; Bonferroni LSD multiple range test).

Table 4.22 Effect of different concentrations of various anti-microbial agents on fungal growth, when added to the standard diet. (*C. leucotreta* eggs were not included).

Treatment	**Fungal growth**		
	Rhizopus	***Aspergillus***	***Penicillium***
Standard *Rhizopus* sp. diet	Vigorous	Vigorous	Vigorous
Sodium benzoate 0.02 g/jar	Moderate	Moderate	None
Sodium benzoate 0.04 g/jar	Moderate	Moderate	Moderate
Sodium benzoate 0.06 g/jar	Moderate	Moderate	None
Sodium benzoate 0.08 g/jar	Little	Little	Little
Sodium nitrate 0.02 g/jar	Vigorous	Vigorous	Vigorous
Sodium nitrate 0.04 g/jar	Moderate	Moderate	Moderate
Sodium nitrate 0.06 g/jar	Moderate	Moderate	Moderate
Sodium nitrate 0.08 g/jar	Moderate	Moderate	Moderate
Alcohol 70 %	None	None	None
Alcohol 35 %	Very little	Very little	None
Alcohol 17.5 %	Little	Little	Little
Colloidal silver	Moderate	Moderate	Moderate
Colloidal silver (not autoclaved)	None	None	Moderate

Table 4.23 Effect of different concentrations of nipagin and glacial acetic acid on fungal growth and *C. leucotreta* development, when added to the standard diet.

Treatment	Moths produced/jar* ± SE	Fungal growth
Standard *Rhizopus* sp. diet	16.5abc ± 1.5	Vigorous
Nipagin 0.2 g/jar	30.0a ± 11.0	Little
Nipagin 0.4 g/jar	7.5bcd ± 1.5	None
Nipagin 0.6 g/jar	6.0cd ± 3.0	None
Nipagin 0.8 g/jar	2.0d ± 1.0	None
Nipagin 1.2 g/jar	1.0d ± 0	None
Glacial acetic acid 165 µℓ/jar	28.0ab ± 1.0	Vigorous
Glacial acetic acid 330 µℓ/jar	3.0cd ± 1.0	None
Glacial acetic acid 660 µℓ/jar	0.5d ± 0.5	None
Glacial acetic acid 990 µℓ/jar	0d	None
Glacial acetic acid 1650 µℓ/jar	0d	None

*Values in the same column followed by the same letter are not significantly different (P > 0.05; Bonferroni LSD multiple range test).

Both nipagin and glacial acetic acid appeared to be more promising anti-microbial additives than did any of the products which were tested in the previous trials. At concentrations which completely controlled fungal growth, development of *C. leucotreta* still occurred. The ideal concentration of nipagin, to control fungal growth and not impede *C. leucotreta* development, appeared to be somewhere between the two lowest rates tested (Table 4.23). (Development on the standard *Rhizopus* sp. inoculated diet was poor, due to *Aspergillus* sp. contamination).

Table 4.24 Effect of different concentrations of nipagin, sodium benzoate and sodium nitrate on fungal growth and *C. leucotreta* development, when added to the standard diet.

Treatment	Moths produced/jar* ± SE	Fungal growth
Standard *Rhizopus* sp. diet	22.0a ± 3.0	Vigorous
Nipagin 0.3 g/jar	23.5a ± 1.0	None
Sodium benzoate 0.10 g/jar	3.5b ± 1.0	Moderate
Sodium benzoate 0.15 g/jar	4.5b ± 2.0	Moderate
Sodium benzoate 0.20 g/jar	5.0b ± 1.5	Moderate
Sodium nitrate 0.10 g/jar	1.0b ± 0	Moderate
Sodium nitrate 0.15 g/jar	4.0b ± 2.0	Moderate
Sodium nitrate 0.20 g/jar	2.0b ± 1.0	Moderate

*Values in the same column followed by the same letter are not significantly different (P > 0.05; Bonferroni LSD multiple range test).

Consequently, sodium benzoate and sodium nitrate were tested at higher concentrations, but were ineffective at eliminating contamination. Nipagin was tested at a concentration of 0.3 g/jar. It was both effective in controlling fungal contamination and was not toxic to *C. leucotreta* (Table 4.24).

In trials, nipagin appeared to eliminate fungal contamination sufficiently (Table 4.24). However, once adopted as an ingredient in the diet, this was not always the case. It became evident that either the dosage of nipagin would have to be increased or another anti-microbial agent would have to be included in the diet along with nipagin. This led to the inclusion of sorbic acid, which proved effective in eliminating fungal contamination, but initially appeared to be detrimental to larval survival (when nipagin was also included) (Table 4.25).

Table 4.25 Effect of different concentrations of sorbic acid on fungal growth and *C. leucotreta* development, when added to diet.

Treatment	**Moths produced/jar* ± SE**	**Fungal growth**
5 component diet**:	28.3abc ± 6.1	Very little
Plus 0.10 g sorbic acid/jar	14.3c ± 6.9	None
Plus 0.13 g sorbic acid/jar	15.0c ± 3.8	None
Plus 0.20 g sorbic acid/jar	19.7bc ± 8.7	None
Plus 0.25 g sorbic acid/jar	14.7c ± 9.7	None
5 component diet** without nipagin:		
Plus 0.10 g sorbic acid/jar	47.0a ± 4.7	Vigorous
Plus 0.13 g sorbic acid/jar	42.7ab ± 6.4	Little
Plus 0.20 g sorbic acid/jar	29.0abc ± 11.8	None
Plus 0.25 g sorbic acid/jar	15.3c ± 4.0	None

*Values in the same column followed by the same letter are not significantly different (P > 0.05; Bonferroni LSD multiple range test).
**See Table 4.4.

In a subsequent trial, three concentrations of sorbic acid (two of them with nipagin) were used in diet, purposely inoculated with a suspension of *A. tamarii* fungal spores. This test was too severe, and all treatments displayed a high level of fungal growth (Table 4.26). There was also some reduction in larval survival.

Table 4.26 Effect of different concentrations of sorbic acid on fungal growth and *C. leucotreta* development, when added to diet (diets were also inoculated with *A. tamarii*).

Treatment	**Moths produced/jar* ± SE (8 jars/treatment)**	**Proportion (%) of diet estimated to be contaminated with *Aspergillus* sp.**
5 component diet**	79.6a ± 5.7	77.9
Plus 0.10 g sorbic acid/jar	54.9b ± 8.4	71.5
Plus 0.13 g sorbic acid/jar	36.2b ± 3.6	51.6
Plus 0.17 g sorbic acid/jar (no nipagin)	39.9b ± 4.3	68.6

*Values in the same column followed by the same letter are not significantly different (P > 0.05; Bonferroni LSD multiple range test).
**See Table 4.4.

Table 4.27 Effect of different concentrations of sorbic acid on fungal growth and *C. leucotreta* development, when added to diet (repeat of Table 3.29 trial, but without *Aspergillus tamarii* inoculation of diet).

Treatment	**Moths produced/jar* ± SE (8 jars/treatment)**	**Fungal contamination 9 DAT*****	**Proportion (%) of diet with fungal contamination 26 DAT*****
5 component diet**	63.9a ± 5.1	Superficial.	78.1b ± 3.9
Plus 0.10 g sorbic acid/jar	69.1a ± 8.0	Clean.	63.7b ± 10.5
Plus 0.13 g sorbic acid/jar	53.6a ± 8.5	Clean.	4.4a ± 2.6
Plus 0.17 g sorbic acid/jar (no nipagin)	72.6a ± 7.0	Superficial.	75.6b ± 3.9

*Values in the same column followed by the same letter are not significantly different (P > 0.05; Bonferroni LSD multiple range test).
**See Table 4.4.
***DAT = days after treatment.

Consequently, the trial was repeated without inoculating the diet with fungal spores. The addition of 0.1 g sorbic acid per jar was sufficient to eliminate fungal contamination without negatively affecting larval development (Table 4.27). Experience later showed that it was necessary to add 0.13 g sorbic acid per jar to consistently produce contamination free diet. This concentration also did not significantly affect *C. leucotreta* survival (Table 4.27).

It was decided that both nipagin and sorbic acid would be added to the diet, to suppress contamination. These were added as 0.3 g nipagin and 0.13 g sorbic acid to each jar of

diet (46.73 g of other dry ingredients – see section 4.3.5 for details). However, it did remain important to prepare the diet as hygienically as possible. This included the autoclaving of all necessary equipment, working under a laminar flow hood, working with clean hands and surface sterilising the eggs as effectively as possible. If this was not done then contamination could still occur. To more completely suppress contamination, higher dosages of nipagin and sorbic acid could be added to diet, but any increase in these rates would mean an increase in cost and a possible increase in mortality.

4.3.5 Diet improvement

Table 4.28 Development of *C. leucotreta* on three artificial diets and on the standard *Rhizopus* sp. inoculated maize meal diet.

Diet	**Eggs or neonate larvae placed onto diet**	**Moths produced/jar***	**Observations**
Bot	Eggs	0a	Eggs hatched, no larval penetration; no contamination.
Guennelon	Eggs	1.33a	Contact with diet surface appeared to impede egg development; no contamination.
Guennelon	Larvae	1.33a	Majority of larvae died on diet surface.
Ware	Eggs	0a	Contact with diet surface appeared to impede egg development; no contamination.
Ware	Larvae	3.12a	The only jar in which any development took place was one in which there was fungal contamination.
Rhizopus sp. inoculated	Eggs	56.8b	Normal development. No contamination in diet.

*Values in the same column followed by the same letter are not significantly different ($P > 0.05$; Bonferroni LSD multiple range test).

C. leucotreta production on all of the three artificial diets tested, was inferior to the *Rhizopus* sp. inoculated diet (Table 4.28). No further testing was conducted with either the Bot or Ware diets.

As it was previously determined that benzoic acid was toxic to *C. leucotreta* (Table 4.21), it was speculated that the poor development of *C. leucotreta* on Guennelon diet (Table 4.28) might be due to the benzoic acid constituent. By rearing *C. leucotreta* on

Guennelon diet without benzoic acid and on the normal Guennelon diet containing benzoic acid, it was established that there was no difference in developmental success (Table 4.29). It was then hoped that with continued rearing of several consecutive generations on the Guennelon diet, *C. leucotreta* would adapt to the diet. After four generations this had not happened, and development remained at an unacceptably low level (Table 4.29).

Table 4.29 Development of four successive generations of *C. leucotreta* on Guennelon diet.

Diet	**Moths produced/jar ± SE**
Guennelon without Benzoic acid	0.5 ± 0.5
Guennelon (1st generation)	1.0 ± 0.3
Guennelon (2nd generation)	1.5 ± 0.3
Guennelon (3rd generation)	5.3 ± 0.3
Guennelon (4th generation)	3.3 ± 0.3

Table 4.30 Development of *C. leucotreta* on maize meal diet with different concentrations of casein, ascorbic acid or wheat germ added.

Additive to diet	**Rate/jar (g)**	**Moths produced/jar (1 jar/treatment)**	**Contamination**
Casein	0.73	36	No
	1.46	32	No
	2.92	22	No
	5.84	1	Yes
	11.68	0	Yes
Ascorbic acid	0.4	58	Yes
	0.8	58	No
	1.2	24	Yes
	1.6	39	Yes
	2.0	48	No
Wheat germ	4	45	No
	6	35	No
	8	32	No
	10	45	No
	12	35	Yes

As none of the existing artificial diets tested, proved to be suitable, different concentrations of various ingredients were tested separately to determine their nutritional value to *C. leucotreta*. Development of *C. leucotreta* on a maize meal diet with casein at

0.73 g/jar, ascorbic acid at 0.4 g/jar, or wheat germ at 4 g/jar, appeared to be good (Table 4.30). Unfortunately there was some contamination, as no anti-microbial agents were added. It was therefore necessary to repeat the trial, however, this was done with only two concentrations of each additive (Table 4.31).

Table 4.31 Development of *C. leucotreta* on maize meal diet with different concentrations of casein, ascorbic acid or wheat germ added.

Additive*	**Moths produced/jar** ± SE (5 jars/treatment)**	**Duration of development from egg to start of pupation (days)**	**Contamination**
None	33.8bc ± 4.8	28	None in any of the diets.
Rhizopus sp.	74.2d ± 5.7	12	
Ascorbic acid 0.2 g	19.0ab ± 2.8	29	
Ascorbic acid 0.4 g	3.8a ± 2.1	30	
Casein 0.36 g	42.8bcd ± 10.7	22	
Casein 0.73 g	50.2bcd ± 6.1	21	
Wheat germ 2 g	69.6cd ± 8.6	20	
Wheat germ 4 g	70.6cd ± 5.9	18	

*Nipagin (0.3 g) was added to each jar (to prevent fungal contamination) except to the standard *Rhizopus* sp. inoculated diet.
**Values in the same column followed by the same letter are not significantly different (P > 0.05; Bonferroni LSD multiple range test).

Ascorbic acid, at the concentrations used, either had no nutritional value for *C. leucotreta*, or was somewhat toxic to *C. leucotreta*, with moth production down by 74.4 % and 94.9 % (for the lower and higher concentrations respectively) relative to the *Rhizopus* sp. inoculated diet (Table 4.31). (The apparently promising results with ascorbic acid in the previous trial (Table 4.30) may have been due to the beneficial *Rhizopus* sp. contamination). Pupation was also delayed by 17-18 days (Table 4.31). When using casein or wheat germ as an additive, moth production was better than on the diet in which there were no additives but not as good as on the *Rhizopus* sp. inoculated diet, although not significantly worse (Table 4.31). Both casein and wheat germ therefore offered some nutritional advantage to *C. leucotreta*, although pupation was delayed by 6 - 10 days (Table 4.31).

Based on the results achieved with casein and wheat germ (Table 4.31) a 4-component diet (Table 4.4) was devised. This included nipagin, based on results achieved in the tests with anti-microbial agents (Table 4.24). *C leucotreta* development (measured by number of pupae) on the 4-component diet was not significantly lower than on the *Rhizopus* sp. diet. Addition of yeast (the fifth ingredient in the 5-component diet) to the 4-component diet resulted in improved production and rate of development to that achieved on the 4-component diet, which was even marginally better than on the *Rhizopus* sp. diet (Table 4.32).

Table 4.32 Development of *C. leucotreta* on two new artificial diets (see Table 4.4 for recipes) relative to the standard *Rhizopus* sp. inoculated diet.

Diet	**Development (pupae/jar)* ± SE (5 jars/treatment)**	**Duration of development from egg to pupa (days)**	**Duration of development from egg to moth (days)**
Rhizopus sp.	55.8ab ± 2.7	13	21
4-component	45.0b ± 4.5	17	27
5-component	57.8a ± 4.8	16	26

*Values in the same column followed by the same letter are not significantly different (P > 0.05; Bonferroni LSD multiple range test).

Sucrose offered no added nutritional advantage to the development of *C. leucotreta* larvae (Table 4.33).

Table 4.33 Development of *C. leucotreta* on two new artificial diets (see Table 4.4 for recipes) relative to the standard *Rhizopus* sp. inoculated maize meal diet.

Diet	**Development (moths/jar)* ± SE (5 jars/treatment)**	**Duration of development from egg to pupa (days)**
Rhizopus sp.	34.00a ± 5.1	12
5-component	45.20a ± 7.2	16
5-component + sucrose	36.00a ± 6.1	16

*Values in the same column followed by the same letter are not significantly different (P<0.05; Bonferroni LSD multiple range test).

Production of pupae on the 5-component diet (Table 4.4) was comparable to that on the old standard *Rhizopus* sp. inoculated diet (Table 4.34). However, pupation on the new diet was delayed by four days (Table 4.36). This would not be a problem in a continuous production system. Egg laying of the F1 generation was 19.83 % higher when reared on

the new diet compared to the old diet (per equivalent number of moths) (Table 4.34). A further observation in favour of the new diet, was that there appeared to be far greater synchrony of development. Nutritional value of the new diet is obviously similar throughout the medium, whereas proximity to the *Rhizopus* sp. growth affects nutritional value in the old diet. This results in a conspicuous spread of life stages in the *Rhizopus* sp. diet. Greater synchrony of life stages is certainly desirable for efficient and organised mass rearing.

Table 4.34 Development of *C. leucotreta* on a new artificial diet (see Table 3.4 for recipes) relative to the standard diet.

Diet	**Development (pupae/jar)* (20 jars/treatment)****	**Duration of development from egg to pupa (days)**	**Eggs/240 moths**	**Contamination (evaluated 16 days after introducing eggs onto diet)**
Rhizopus sp.	34.35a ± 1.6	16	18882	*Aspergillus* growth appeared in 100 % of jars.
5-component	33.10a ± 1.7	21	22626	*Aspergillus* growth appeared in 15 % of jars.

*Values in the same column followed by the same letter are not significantly different (P > 0.05; Students' T-test).
**Average of 67 eggs placed into each jar for each treatment.

All jars of the standard diet became contaminated with *Aspergillus* sp., but this only occurred once larvae had begun pupating – this was too late to have any detrimental effect on production. *Aspergillus* spp. contamination usually sets in earlier, and can then significantly reduce production. Only three bottles of the new diet became contaminated, but this too, occurred at a late stage. Through repeated use and observation of the new 5-component diet, it was noted that the occurrence of *Aspergillus* spp. contamination was not entirely precluded. It eventually became necessary to incorporate an additional anti-microbial agent in the diet, namely sorbic acid (Tables 4.25, 4.26 & 4.27), which proved adequately effective and safe. This now constituted the 6-component diet (Table 4.4).

Due to the expense of casein, this ingredient was replaced with milk powder, with no detrimental effect (Table 4.35).

Table 4.35 Investigation of milk powder as an alternative to casein in a 6 component artificial diet for rearing *C. leucotreta* larvae (see Table 4.4 for recipes).

New artificial diet with either casein or milk powder	**Development (pupae/jar)* (20 jars/treatment)****	**Duration of development from egg to pupa (days)**	**Eggs/80 moths**	**Contamination (evaluated 17 days after introducing eggs onto diet)**
Casein	84.3a ± 11.6	17	9464	None
Milk powder	79.1a ± 9.5	17	9509	None

*Values in the same column followed by the same letter are not significantly different (P > 0.05; Students' T-test).
**Average of 127 eggs placed into each jar for each treatment.

Consequently, the diet developed for rearing *C. leucotreta* larvae (the 6-component diet) consisted of maize meal (2000 g), wheat germ (200 g), milk powder (36.5 g), Brewers' yeast (100 g), nipagin (15 g), sorbic acid (6.5 g) and distilled water (2000 ml). The dry ingredients were thoroughly mixed together in a bucket. A mass of 47 g of the mixture was placed into each 350 ml honey jar and 40 ml distilled water was added to each. Measurements were easily and conveniently made with containers pre-sized for the exact quantities. Jars were plugged in the normal fashion and autoclaved for 20 minutes. The quantities of ingredients mixed into the bucket were therefore sufficient to produce 56 jars of diet or 5600 moths (at 100 moths per jar).

4.4 CONCLUSION

A significant improvement has been made in reducing the level, and frequency of occurrence, of contamination in the larval diet, both by treatment of the diet and of the host eggs placed onto the diet. Dipping eggs into a 0.15 % solution of Sporekill for 15 minutes was a highly effective and safe surface sterilisation treatment. Both nipagin and sorbic acid were added to the diet to further control contamination.

Simultaneously, an artificial diet which consistently produces good numbers of moths has been developed. This consists of maize meal (2000 g), wheat germ (200 g), milk powder (36.5 g), Brewers' yeast (100 g), nipagin (15 g), sorbic acid (6.5 g) and distilled water

(2000 ml). To date around 18 generations of *C. leucotreta* have been reared on the new diet. Production has been consistently good, with approximately 100 moths produced per jar. Almost 20 % more eggs were produced from moths reared on the new diet compared to moths reared on the old diet. A further 9 % improvement in egg production and a reduction in the labour required to produce eggs, has been made with the development of a new oviposition cage attached to the moth eclosion box.

Consequently, a more reliable and less labour intensive source of material is available for the *in vivo* production of CrleGV-SA.

5

VIRUS PRODUCTION

5.1 INTRODUCTION

Baculoviruses can be produced *in vivo*, usually in the homologous host, or *in vitro*, in cell culture. However, despite the recent development of cell lines with improved susceptibility to virus, and greater productivity (Granados *et al.*, 1994; Davis & Granados, 1995; Granados & McKenna, 1995; Shuler *et al.*, 1995), it is not yet possible to produce virus on a commercial scale. GVs are also far more difficult to produce in cell culture than are NPVs. To date, only two GVs, those of *Cydia pomonella* and *Trichoplusia ni*, have been successfully propagated *in vitro* (McIntosh, 1994). *In vivo* production of CrleGV-SA in the homologous host, *C. leucotreta*, was therefore investigated in this study.

The utilisation of viruses for pest control is dependent on economic production of sufficient quantities to meet user standards (Yearian & Young, 1982). The ultimate, practical goal of *in vivo* virus production, is to reduce the cost of a microbial control agent whilst maintaining efficacy and purity (Shapiro, 1982). It is necessary to determine and optimise those factors that influence virus production. This includes host factors (density, age, stage, sex), virus inoculum factors (concentration, activity, purity), environmental factors (container type, temperature, humidity, photoperiod, diet) and the production scheme. Regardless of the insect virus produced and the variation in methodology used by different workers, the basic production scheme involves virus

inoculation, virus incubation, harvest of larvae, processing of virus, and storage of virus (Shapiro, 1982).

Optimal production results in the maximum utilisation of larval tissue (Ignoffo, 1966). In other words, optimal production is one that produces the greatest yield of biologically active virus that conforms to quality control standards. The production method must be such that the virus can be formulated into an efficacious product that will meet the safety requirements established by regulatory authorities (Yearian & Young, 1982). Preparations of virus propagated *in vivo* are invariably contaminated with a variety of bacteria and fungi (Podgewaite *et al.*, 1983) and sometimes other entomopathogenic viruses too (Hunter-Fujita *et al.*, 1992). Production should aim to not only exclude human or veterinary pathogens from the final formulation but also to limit total number of contaminating organisms, regardless of toxicity. The product must be characterised on the basis of biological activity, microbial contaminants (quantitative and qualitative), and safety (Martignoni, 1978).

This study is by no means a comprehensive investigation of all of the factors mentioned for optimisation of production. Based on the work of others, several assumptions are made in this study in establishing production protocols, which if not optimal can most certainly, not be far wrong. This study focuses on harvesting the maximum amount of virus possible, using the LC_{90} for fifth instar larvae (see section 3.3.3.2), with as little labour as possible. The labour element is an important factor, if the ultimate aim is to produce the virus on a commercial scale at a competitive cost.

5.2 MATERIALS AND METHODS

5.2.1 Virus replication

The containers in which the bioassays were conducted (see section 3.2.3) were too small for mass production of CrleGV-SA. Therefore, CrleGV-SA was mass produced in the

220 ml glass pie dishes. The artificial diet (94 g dry ingredients with 100 g distilled water) was prepared in the same manner as previously described (section 3.2.1.1). The only difference between the diet used for virus production and the diet used for host rearing was that 33 % higher concentrations of the anti-microbial agents (nipagin and sorbic acid) were used in the virus production diet (see section 4.3.5 for diet recipe). Once cooled, the surface of the diet was inoculated with an adjusted LC_{90} concentration of CrleGV-SA (for fifth instar larvae). LC_{90} for fifth instar larvae, estimated to be 9.118 x 10^9 OBs/ml, was calculated from inoculating diet with 200 µl of virus per 400 mm^2 (surface area of diet in one cell of a bioassay tray) (sections 3.2.1.1 and 3.2.3.2). This translated to a volume of 19 ml of a solution of LC_{90} to inoculate the surface of a pie dish of diet (38013 mm^2) for virus production. However, such a volume was clearly far more than was needed to thoroughly wet the diet surface. It was determined that 14 ml was sufficient to adequately and evenly wet the diet surface in a pie dish.

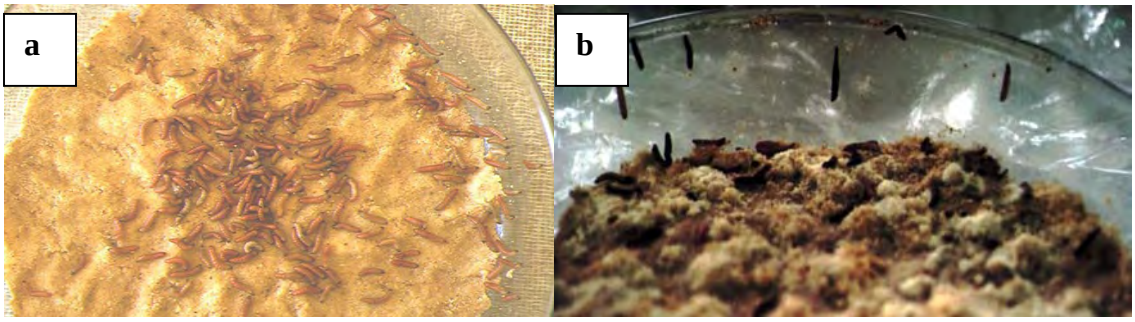

Plate 5.1 Artificial diet in a pie plate, inoculated with CrleGV-SA and fifth instar *C. leucotreta* larvae for *in vivo* production of CrleGV-SA (**a**), and CrleGV-SA infected larvae ready for harvesting five to 10 days later (**b**).

Obviously, the important factor for virus production is not the concentration of the inoculum, but the concentration of OBs on the diet surface (provided by the inoculum) – a total of 1.732 x 10^{11} OBs in this case. To ensure that the surface of the diet was inoculated with this number of OBs, it was sprayed with 14 ml of 1.237 x 10^{10} OBs/ml. (Correct volumes and concentrations of purified virus were aliquotted and stored at -40ºC). This was applied as a fine spray using a 1 ℓ plastic spray bottle. The inoculated diet was placed in a laminar flow cabinet for ± 30 minutes, to dry. A total of 200, 250, 300 or 400 fifth instar larvae were placed on the diet surface (Plate 5.1a). The pyrex dish

was covered with a double layer of “Gladwrap” (plastic stretch film) to prevent larvae from escaping. Further production of virus was continued in this manner, using the number of larvae per pyrex dish, which proved to be the most productive (in the preceding trial).

5.2.2 Virus harvesting

Inspections were conducted once or twice a day, and any larvae showing symptoms of viral infection (Fig. 5.1b) were collected and stored at -40°C. An infected larva was identified by the characteristic features, described in section 2.3.1.1. Number of larvae harvested from each pyrex dish were recorded and data analysed by ANOVA using Statgraphics 2.0 (Statistical Graphics Corporation, 1996). Means (for each total of larvae placed onto inoculated diet) were compared using the Bonferroni LSD multiple range test.

Once numbers of larvae harvested were recorded, they were grouped in the freezer, regardless of from which pyrex dish they were harvested.

An alternative method of harvesting was also investigated. Three pie dishes of diet were prepared and inoculated. Three hundred fifth instar larvae were placed into each dish. After eight, nine and 10 days the entire contents of one pie dish was harvested and frozen at -40°C.

5.2.3 Semi-purification of virus from diet

The methodology used was based on that suggested by Jones (2000) for NPVs, with slight modification, necessary and appropriate for semi-purification of this GV.

Large numbers of larvae (or larvae and diet) were defrosted and virus liberated either by crushing with a mortar and pestle or homogenising in a Dupont omni-mixer. The product was then filtered through a double layer of muslin cloth, 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 minutes, 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 minutes. Thereafter, the slide was washed under running tap water for 10 seconds. The slide was left to air dry, and examined through a light microscope at 1000X magnification under oil immersion. Once it was established that no virus was present in the pellet, it was discarded.

The supernatant was then spun at 9000 rpm (using a JA-20 Beckman rotor) for 30 minutes. 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 minutes. 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.

5.2.4 Quantification of virus

Initially, enumeration of the filtered unpurified virus had been attempted using a 0.02 mm depth Thoma bacterial counting chamber at 400X magnification under dark field light microscopy. There was too much contamination to accurately estimate the concentration of OBs. It was therefore necessary to semi-purify the homogenate as described in section 5.2.3.

It was then possible to enumerate the semi-purified virus using the Thoma counting chamber at 400X magnification under dark field. Before this was done, the virus (in a microtube) was placed in an ultra-sonic water bath for 60 seconds to separate any clumped OBs (Hunter-Fujita *et al.*, 1998). A suitable dilution of virus, determined through a process of trial and error, was then prepared. A 1000X dilution was usually examined initially, although it was in one case, necessary to dilute a CrleGV-SA suspension by up to 40000X before it could be accurately counted. 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 moved, by sliding, to fully cover the chamber. Warm breath was exhaled onto the coverslip and pressure applied to it on either side of the chamber until Newton's rings were visible on both sides. 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 (Plate 5.2). Five separate and independent samples of the same dilution were prepared from a virus stock and each one enumerated in the counting chamber. Therefore, a total of 25 large squares (containing 400 small squares) were counted for each calculation.

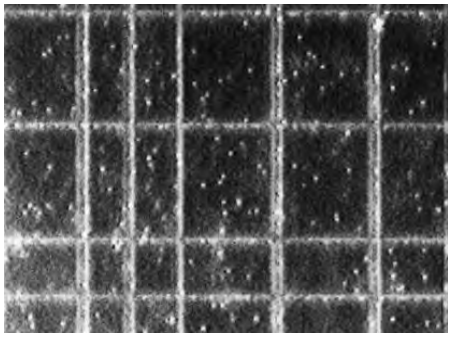

Plate 5.2 OBs of CrleGV-SA in the small squares of a 0.02 mm depth Thoma bacterial counting chamber, viewed at 400X under dark field light microscopy.

The concentration of virus, in number of OBs per ml, was calculated by the formula:

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

where 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. Virus quantification was conducted with six batches of virus, ranging from 233 - 7906 cadavers, harvested over a period 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.

5.2.5 Microbial contamination

A comparison of the level of microbial contamination in five different preparations was conducted. These were virus purified by glycerol gradient centrifugation (as described in section 2.2.2.3), and four batches of semi-purified virus: from larvae individually harvested when symptomatically infected and from larvae harvested with their diet after eight, nine and 10 days. Three dilutions of each treatment were made in a 10-fold series: 10X, 100X, 1000X, 10000X, 100000X and 1000000X dilutions in sterile distilled water. Eight droplets (110 μl) of each dilution were placed in circular formation onto each of a PDA (potato dextrose agar) plate (plastic Petri dish) and a WA (water agar) plate (Baxter *et al.*, 1999). Droplets did not merge together and did not touch the edge of the container. This was done within a laminar flow cabinet. Once the droplets had dried, the lids were placed onto the plastic Petri dishes and they were incubated at 27°C for three days. After two days the number of bacterial colony forming units (CFUs) was counted and the ratio of CFUs to OBs for each preparation was determined. Even though three agar plates were used per dilution, the data was not statistically analysed. Due to the relatively small sample size, variation would have been too great to obtain any reliable significance. No attempt was made to identify the dominant bacterial species present. After three days number of droplet points with fungal growth were counted.

5.3 RESULTS AND DISCUSSION

5.3.1 Virus replication

A 33 % higher concentration of the anti-microbial agents (nipagin and sorbic acid) were used in the virus production diet compared to the diet used for host rearing (section 4.3.5). This was found to be necessary, as fungal contamination tended to be worse in the virus production diet. As non sterile fifth instar larvae were introduced onto the diet and as it was necessary to regularly open the diet container to remove virus infected larvae, the opportunity for fungal infection was far greater on this diet than on the host diet.

For production of a virus, a suspension of the virus can either be incorporated into the artificial diet or simply be placed on the diet surface (Shapiro *et al.*, 1981). Both methods have been used in production; however, application to the diet surface is now the generally preferred method (Hunter-Fujita *et al.*, 1998). If virus is incorporated into the diet, this must be done before the diet gels (i.e. 50°C for agar) and after the diet has cooled sufficiently, to avoid virus inactivation (i.e. 58°C (Shapiro, 1982)). There is therefore a very small "window" during which time the virus can be effectively and safely added to the diet. If carageenans, with a higher gelling temperature, are used instead of agar, it is impossible to incorporate virus into the diet. As the diet used for *C. leucotreta* did not contain any gelling agent and was autoclaved, it was not possible to even consider incorporation of virus. The diet would have to be changed in order to fulfil this purpose. However, this was not considered a viable investigation. Shapiro *et al.* (1981) found that LdNPV yield from *in vivo* production in the homologous host (*Lymantria dispar*), was more than three times higher per larva when inoculum was applied to the diet surface, as opposed to it being incorporated in the diet. Also, less inoculum was required to attain infection from surface application. However, unlike *C. leucotreta, L. dispar* is a surface feeder.

5.3.2 Virus harvesting

A higher recovery of infected larvae relative to introduced larvae was obtained with 300 larvae per container than with any other number tested (Table 5.1). Increasing the density per container by 33 % (to 400) seemed to detrimentally affect the harvest of virus infected larvae. With increasing density, larvae also appeared to die more rapidly. The greater density probably caused this in two ways. Firstly, it increased the opportunity for horizontal transmission of the virus and therefore larvae were ingesting greater quantities of virus. Secondly, a greater level of stress could have been experienced by the larvae due to "overcrowding" which in turn caused a manifestation of the usually non-apparent level of infection (Cherry *et al.*, 1997) (which was known to be present in the *C. leucotreta* culture) on top of the induced epizootic.

Table 5.1 Harvest of virus infected larvae from diet inoculated with the LC_{90} (9.118 x 10^9 OBs/ml).

Larvae introduced	Replicates	Larvae harvested (ave.)	Harvest (%)	Period of harvesting (DAT*)	Peak harvest (DAT*)
200	5	62.2	31.1a ± 4.2	7 - 15	9.2
250	4	121.7	48.7b ± 8.3	6 - 12	8.5
300	4	167.0	56.0b ± 3.2	6 - 11	8.5
400	3	154.0	38.5ab ± 7.9	3 - 9	7.0
Average			43.1 ± 5.5		8.3 ± 0.5

*DAT = days after treatment.
*Values in the same column followed by the same letter are not significantly different (P > 0.05; Bonferroni LSD multiple range test).

Only 43.1 % of all larvae introduced onto the diet were eventually harvested as virus infected larvae (Table 5.1). This was unacceptably low, although the harvest of 56.0 % when 300 larvae were placed onto inoculated diet, was somewhat better. Cherry *et al.* (1997) harvested between 36 % and 44 % of beet army worm, *Spodoptera exigua*, larvae, infected with SeNPV, when larvae were held communally, but identified no relationship between level of density and percentage harvested. However, they did record that when larvae were held individually, percentage harvested jumped to an impressive 83 %. This they attributed to the avoidance of cannibalism. As *C. leucotreta* is only cannibalistic as early neonate larvae (and not greatly so), this would probably not be a consideration in

the *in vivo* production of virus in fifth instar larvae. Some larvae have been recorded to become more cannibalistic, when infected with virus, but such behaviour was not observed with *C. leucotreta*.

It is speculated that the most important factor contributing towards the low percentage of larvae harvested could be larval behaviour. As *C. leucotreta* larvae are internal feeders, one is dependent on the larvae exiting the diet when symptomatically infected with virus, in order to be able to harvest larvae individually. It is usual for baculovirus-infected larvae to move away from their food, disperse, or climb an elevated location to hang by their caudal prolegs (Tanada & Kaya, 1993). This was usually observed to be the case with *C. leucotreta*; however, several virus infected larvae seemed to die undetected within the diet. In one of the virus production trials, where 300 larvae were placed onto virus inoculated diet, 43 % of larvae were harvested over a period of six days. Thereafter, the remaining diet was broken up by hand and examined for larvae. A further 24 virus infected larvae (several of which were already dead) were found within the diet (bringing the percentage harvest up to 51 %). Numerous other infected larvae could have remained within the diet, undetected, during the previous six days, and by the time the diet was broken up and inspected these larvae could have ruptured and disintegrated beyond recognition. It is not clear why a minority (albeit a significant minority) of infected larvae do not demonstrate the typical behaviour of emerging from the diet. It could be due to a conflict in behavioural cues: an instinct to seek an environment suitable for pupation on the one hand and therefore remain within the diet, and an instinct to respond to the viral infection on the other hand. However, although the majority of larvae do emerge from the diet to pupate, it is usual for a conspicuous number of larvae to pupate within the diet. This relates to their behaviour in nature, where pupation takes place within the soil.

A number of larvae would attempt to pupate. However, the majority of them became sick before pupation was complete. Most of these sick larvae would exit the silk case before pupation was completed, where they were easily identified as being infected and were harvested. Some sick larvae remained within the silk case but did not complete

pupation. In one production trial, 13 out of 300 larvae were lost to seemingly successful pupation. This represents a small 4.3 % loss. However, these pupae were harvested with the virus infected larvae, in the hope that they too carried a substantial load of virus.

Harvesting of the entire production dish – larvae and diet, was investigated, as the harvesting of larvae individually was labour intensive, and recovery rate of larvae (i.e. number of infected larvae harvested per number of healthy larvae placed on inoculated diet) was considered poor. Diet dishes were checked daily. Symptomatically infected larvae were first noted on the diet seven days after inoculation (in small numbers). However, this was considered marginally too soon to begin harvesting. By 10 days post-inoculation the diet surface was covered with dead larvae.

5.3.3 Semi-purification of virus from diet

If virus was required for DNA extraction or for conducting bioassays then it was necessary to purify the virus as described in sections 2.2.2.1 - 2.2.2.3. For use in field trials it was not necessary to do any more than semi-purify the virus, at most. This was actually preferable to purified virus, as the spray suspension would then contain more small particles which could offer protection to the virus against harmful ultra-violet (UV) rays. Simple filtration of the initial homogenate (of infected larvae or larvae and diet) may even be sufficient for use in field trials. However, semi-purification was necessary and sufficient to be able to enumerate the virus using a counting chamber (Jones, 2000).

5.3.4 Quantification of virus

There was great variation in the virus production per larva and per gram of larva (Table 5.2). It was not possible to associate this variation with any differences in the virus production techniques, as larvae were harvested over a period of time and pooled. Virus was extracted from each batch of pooled larvae. Larvae within a batch are likely to have been subjected to different regimes (e.g. density of larvae, stage at which harvested). The purpose of this production was not to determine the ideal conditions and parameters for

virus production, but simply to produce large quantities of virus for laboratory and field use.

Different densities of larvae is a factor which could affect the OB yields per larva. Shapiro *et al.* (1981) found decreasing yields of LdNPV per larva with increasing densities of gypsy moth, *L. dispar*, in rearing containers. Cherry *et al.* (1997) identified a generally decreasing yield of SeNPV OBs per *S. exigua* larva with increasing larval density. However, one also needs to be cognisant of OB yield per container and the greater labour and expense required in rearing fewer larvae per container. For example, as *S. exigua* larval density increased from 25 to 200 larvae per container, OB yield per larva decreased, but OB yield per container still increased (Cherry *et al.*, 1997). To rear only 25 larvae per container, Cherry *et al.* (1997) would have had to use 5.4 times as many containers as they would if they had reared 200 larvae per container. This could signify a substantial increase in cost and labour. The OB yield per larva at different densities was not measured for the production of CrleGV-SA in *C. leucotreta*.

The productivity ratio (PR), defined as the difference between the size of the infecting inoculum and the per-larval OB yield, is calculated as:

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

If the mean number of OBs per larva is 1.158×10^{11} (Table 5.2) and the number of infecting OBs per mm^2 is 9.135×10^6 (OBs introduced onto diet surface ÷ surface area), then PR = 1.268×10^4. This compares well with other recorded PRs, which range between 8.4×10^2 (Kelly & Entwistle, 1988) and 1.2×10^6 (Smits & Vlak, 1988). Hunter-Fujita *et al.* (1998) tabulated several baculovirus PRs, all of which were for NPVs (including the above mentioned two). The PR calculated here for CrleGV-SA fell well within the range of values listed. However, one would expect PRs for GVs to be higher than those for NPVs, as generally larval equivalents for GVs are higher than those for NPVs (Crook, 1991) (probably due to the smaller size of GVs).

Table 5.2 Yield of CrleGV-SA by *in vivo* production in fifth instar *C. leucotreta* larvae and harvesting of larvae individually.

Number of CrleGV-SA infected larval cadavers	**Total Mass**	**Mass per larva**	**Total OBs**	**Mean OBs per larva (larval equivalent) ± SE**	**Mean OBs per gram ± SE**
376	19.93 g	0.053 g	3.731×10^{13}	9.918×10^{10}	1.872×10^{12}
1291	66.24 g	0.051 g	9.815×10^{13}	7.603×10^{10}	1.482×10^{12}
397	18.27 g	0.046 g	2.436×10^{13}	6.136×10^{10}	1.333×10^{12}
233	10.81 g	0.046 g	8.301×10^{12}	3.563×10^{10}	7.679×10^{11}
1121	53.58 g	0.048 g	3.412×10^{12}	3.043×10^{9}	6.367×10^{10}
7906	382.60 g	0.048 g	3.318×10^{15}	4.196×10^{11}	8.672×10^{12}
Mean				$1.158 \times 10^{11} \pm 6.225 \times 10^{10}$	$2.361 \times 10^{12} \pm 1.287 \times 10^{12}$

OB production per final mg of body weight for GVs in Lepidoptera seems to be about 2×10^{7} (Entwistle & Evans, 1985). The mean production of 2.361×10^{9} OBs per mg of final body weight (Table 5.2) achieved with CrleGV-SA in *C. leucotreta* was therefore very impressive.

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

$$PE = \text{OBs per larvae} \div \text{weight of larva (mg)}$$

PE of fifth instar *C. leucotreta* larvae for CrleGV-SA production is therefore 2.363×10^{9} (if mean final weight of larvae is 49 mg).

Glen & Payne (1984) produced a yield of 9×10^{9} OBs of CpGV per *C. pomonella* larva. By adding the juvenile hormone analogue, methoprene, to the diet, they managed to raise the yield to 1.6×10^{10} OBs per larva. Reiser *et al.* (1993) achieved a maximum yield of 1.7×10^{10} OBs of CpGV in *C. pomonella* larvae and a maximum yield of 1.8×10^{10} OBs of CpGV in *C. leucotreta* larvae. Fritsch (1989) produced 1.067×10^{10} OBs of CrleGV-

CV (a Cape Verde isolate of CrleGV) per *C. leucotreta* larva. In comparison, the production of 1.158 x 10^{11} OBs per larva, achieved as an average in this trial, is quite outstanding. Despite this good yield, further attempts were made to improve productivity and reduce the labour involved. This was done by harvesting the entire contents (larvae and diet) of containers after different lengths of time, post-inoculation.

Table 5.3 CrleGV-SA yield from harvesting larvae with diet (300 larvae per treatment).

Time of harvesting (days after inoculation)	**Number of OBs harvested**	**Number of OBs per larva (300 larvae)**	**Number (and %) of pupating larvae**
8	5.995 x 10^{12}	1.998 x 10^{10}	16 (5.3 %)
9	5.086 x 10^{12}	1.695 x 10^{10}	8 (2.7 %)
10	5.280 x 10^{12}	1.760 x 10^{10}	4 (1.3 %)

OB yield per dish and per larva was highest when contents were harvested eight days after inoculation, compared to nine and 10 days after inoculation (Table5.3). However, differences were not great and may not have been significant. Yield per larva, in all three cases, was lower than average yield per larva when larvae were harvested individually (Table 5.2). However, it was more important to compare yield per container (pie dish). When 300 larvae were placed onto inoculated diet, the average harvest was 56.0 % of larvae (Table 5.1). At the average yield per larva of 1.158 x 10^{11} (Table 5.2), yield per container would then be 1.945 x 10^{13} OBs. Therefore the optimum yield achieved by harvesting larvae individually was 3.2 times the maximum yield measured for harvesting larvae and diet together. However, it must be noted that at 56 % harvest (assuming 300 larvae per container), yield per larva must exceed 3.568 x 10^{10} OBs, in order to achieve a better yield per container than that achieved by harvesting the entire contents of the container at eight days post inoculation. At the overall average harvest of 43.1 % (Table 5.1) (again assuming 300 larvae per container), yield per larva must exceed 4.636 x 10^{10} OBs, in order to achieve a better yield per container than achieved with the whole dish harvest. In four out of six batch studies (Table 5.2), these per larval yields were exceeded. It must be realised though, that to justify individual harvesting of larvae over

harvesting of the entire contents, percentage harvest and yield per larva must be excellent.

The labour saving involved (and consequently the cost saving) in harvesting the entire contents of the production dish ought to be substantial when producing virus on a commercial scale. In addition to the labour saving, a further benefit of harvesting the diet with the larvae, is that the fine diet particles, if present in the final viral preparation for field use, could offer some protection against UV inactivation. Such a preparation will have to be well formulated so as not to block spray nozzles.

A decreasing number of pupae were recorded with increasing time of incubation (Table 5.3). Larvae, appearing to be healthy, would obviously begin pupating. During the pupation process, viral infection would manifest symptomatically and larvae would either exit the pupal covering, which was in the process of being constructed, or would die within the partially constructed pupal covering. Stairs (1964) noted that metamorphosis (such as pupation) significantly extended the incubation period of a virus. GmNPV-induced death in the larval stage of *Galleria mellonella* usually occurred 11 - 12 days after inoculation; virus induced death in the pupal stage usually occurred 14 days after inoculation; virus induced death in the adult stage took even longer. Stairs (1964) concluded that embryonic regenerative cells are resistant to virus infection, and that the new tissues formed during periods of rapid cell proliferation, such as those which occur at pupation and eclosion, must be infected before the virus will kill the host.

5.3.5 Microbial contamination

Obviously bacterial and fungal growth was more prominent on the PDA plates than the WA plates. As it was possible to determine distinct CFUs of at least one dilution of each preparation on PDA, but not so on WA, only CFU counts conducted on PDA were considered for comparative purposes.

Contamination per volume of preparation ranged from 1.164 x 10^5 CFUs/ml to 3.182 x 10^7 CFUs/ml, a 273-fold difference (Table 5.4). This cannot be regarded as a large range of bacterial contamination, when one considers that contamination in different batches of the same virus (SlNPV), prepared in the same way in one facility, differed by up to 1000 fold (Grzywacz *et al.*, 1997).

Table 5.4 Microbial contamination of different preparations of CrleGV-SA.

Harvested	Purification	CFUs/ml	CFUs/larva	CFU : OB	Fungal contamination/ml*
Larvae	Glycerol gradient centrifugation	2.364 x 10^6	6.349 x 10^5	1:4 793	4.545 x 10^3
Larvae	Semi-purification	3.182 x 10^7	2.014 x 10^6	1:208 342	9.090 x 10^5
Larvae and diet: 8 days after inoculation	Semi-purification	6.445 x 10^5	4.608 x 10^4	1:433 594	9.090 x 10^1
Larvae and diet: 9 days after inoculation	Semi-purification	1.164 x 10^5	8.323 x 10^4	1:203 652	3.636 x 10^4
Larvae and diet: 10 days after inoculation	Semi-purification	1.248 x 10^6	9.988 x 10^4	1:176 211	6.363 x 10^4

*Fungal contamination was defined as the number of droplets, out of the total of eight, which had fungal contamination (at the highest dilution at which fewer than eight droplets were contaminated) per millilitre of the original preparation.

Surprisingly, microbial contamination (bacteria:virus ratio i.e. CFU:OB) was greatest in the preparation of gradient purified virus (Table 5.4). This ratio was strongly influenced by the low yield of OBs per larva (3.043 x 10^9) from this particular batch, being well below the average yield (Table 5.2). At an application rate of 10^{14} OBs/ha (a common rate of application of CpGV against *C. pomonella* on apples (Cross *et al.*, 1999)) the number of contaminant microbes would be equivalent to 2.086 x 10^{10} microbes/ha. However, even this is not excessive, considering the top 15 cm of fertile soil may contain up to 4000 kg of bacteria and fungi per hectare. Grzywacz *et al.* (1997) also concluded that the centrifugation procedures used for purification of SlNPV were not effective in reducing contamination by vegetative cells or spores. This together with the costs of centrifugation casts serious doubts on the value of this particular purification procedure. Centrifugation, in this study on CrleGV-SA, did appear to reduce the level of fungal

contaminants (relative to the semi-purified virus extracted from individually harvested larvae).

CFU:OB ratio in all of the semi-purified preparations was favourably low and substantially lower than in the purified preparation. The lowest level of contamination, both bacterial and fungal, occurred in the semi-purified preparation of larvae and diet which was harvested eight days after inoculation (Table 5.4). Bacterial contamination was less than half of that of any other preparation; the next least contaminated preparations being semi-purified larvae with diet, harvested nine days after inoculation; and semi-purified individually harvested larvae. Fungal contamination in the semi-purified contents harvested after eight days was 50X less than in the purified preparation and at least 400X less than in any other preparation. At eight days post-inoculation, there were obviously many larvae which were heavily infected but not yet dead (LT_{90} estimated from control corrected data being 9 days 18 h). Bacterial contamination was lower in larvae harvested before death (Jones *et al.*, 1993a). Fungal contamination probably increased on the diet with time, rather than in the larvae. Greater fungal contamination could be observed on the surface of diets harvested after nine and 10 days, than on the diet harvested after eight days.

This survey of microbial contamination was fairly superficial and should be replicated in order to obtain satisfactorily significant results. A more detailed study on microbial contamination of CrleGV-SA-SA was conducted by Awosulu (2001).

5.4 CONCLUSION

An efficient and effective means of producing large quantities of CrleGV-SA at a high yield per larva and with low levels of contamination, was developed. However, this does not mean that the method cannot be further improved, for commercial production purposes. The harvesting of an average of only 56 % of larvae placed onto inoculated diet should, if possible, be increased. Production per larva (a mean of 1.158×10^{11} OBs)

might also be improved by the addition of a juvenile hormone analogue to the diet. Harvesting of larvae individually on a large scale will become fairly labour intensive. Harvesting of the entire contents of the production container requires far less labour; however, yield per container was only a third of the optimum yield when larvae were harvested individually. Although, as the former preparation was less contaminated than the latter, with both bacterial particles and fungal spores, it may be worthwhile investigating means of improving the yields from whole container harvests. It should also be tested whether there is any benefit in UV protection and hence field persistence, with virus harvested in this manner.

6

FIELD TRIALS: PERSISTENCE AND EFFICACY

6.1 INTRODUCTION

Field trials are the experimental step to logically follow laboratory based bioassays, and must represent the culmination of all investigations which have preceded them. Several successes have been achieved throughout the world with the use of viruses, and particularly baculoviruses, for the control of insect pests on agricultural crops (Cunningham, 1988; Harper, 1987; Hunter-Fujita *et al.*, 1998; Lacey & Goettel, 1995). Currently 16 commercially available baculovirus formulations are listed in the Directory of Microbial Control Products and Services (Shah & Goettel, 1999). Four of these are CpGV formulations. It is helpful to review the successes achieved with CpGV, because of the close relation between *C. pomonella* and *C. leucotreta*, in both biology and behaviour, and the close relation of the two pathogens (CpGV and CrleGV) (Jehle *et al.*, 1992a). In the past 30 years numerous field trials have demonstrated good activity of CpGV against *C. pomonella* in various regions across Europe, Australia, New Zealand, South America, Canada and the United States (Burgerjon, 1986; Jaques *et al.*, 1987; Lacey & Knight, 1998). The normal dosage of CpGV used against codling moth in the field, ranges from 10^{13} to 10^{14} OBs per hectare. This had some influence on the CrleGV-SA concentrations chosen in this study to test against *C. leucotreta* on citrus.

Unlike CpGV, CrleGV has not been exploited for biological control (of *C. leucotreta*) on agricultural crops. Only one record exists of a small-scale field trial with CrleGV, on citrus and Spanish pepper on the Cape Verde Islands (Fritsch, 1988). In the virus sprays, concentrations of 10^8 and 10^9 OBs/ml were used, and only skimmed milk powder and a wetting agent were added to the virus suspensions. *C. leucotreta* damage was reduced by 77 % in citrus and 65 % in Spanish pepper (Fritsch, 1988).

A principal disadvantage of the use of baculoviruses in the field, is their short residual activity due to their inactivation by UV irradiation (Huber, 1990; Shapiro, 1995). It was thought that damage mainly occurred as a result of UV-B radiation, but work by Shapiro & Robertson (1992) suggests that there is also a strong effect from UV-A. This lack of persistence is one of the primary factors necessitating the protective formulation of baculoviruses (Jones *et al*., 1997). This study therefore aims to determine the effective persistence of CrleGV-SA in the field. Consequently, it will be possible to determine whether and to what extent formulation is required to improve the persistence of CrleGV-SA. Field persistence trials will also indicate how soon after initial application, a second treatment might be necessary.

6.2 MATERIALS AND METHODS

6.2.1 Persistence

A high density orchard (1664 trees planted per ha) of 10 year old Delta Valencias on the Outspan Foundation Block outside Uitenhage (33º 45' S, 25º 24' E), Eastern Cape Province, was selected for this trial. On 21 August 2001 seven trees were each sprayed with 17 ℓ of a crude CrleGV-SA concentration of 7.67×10^7 OBs/ml using a 1000 ℓ capacity high pressure spray machine (made by Janisch, South Africa) with hand held spray guns (Plate 6.1). Pressure was set at 20 bars and 2 mm diameter nozzles were used in the guns. This rate of application would translate into a rate of 2.17×10^{15} OBs/ha. The application was more thorough than would normally be necessary; however, it was

important to ensure absolute coverage of the fruit for the purpose of this trial. Daily weather data (rainfall, maximum and minimum temperatures, wind speed and direction, humidity and sunrise and sunset times) was provided by the South African Weather Service for the Uitenhage area (Appendix 6 – for the most relevant measurements). All weather parameters obtained have been summarised in Table 6.1.

Plate 6.1 High pressure application of CrleGV-SA to mature navel orange trees. Despite the mammalian safety of baculoviruses, precautions were taken by wearing protective clothing (overall, boots, gloves, hat and respirator).

At 0 days (immediately after the spray had dried), 1 day, 3 days, 6 days, 7 days, 14 days and 21 days after treatment, 30 fruit were picked from the outer northern (sunny) side of sprayed trees, 30 fruit from the inner southern (shady) side of sprayed trees, and 60 fruit from unsprayed trees. Thirty of the unsprayed fruit were dipped in a CrleGV-SA concentration of 7.67×10^7 OBs/ml in the laboratory and allowed to dry on a wire mesh drying rack. The other 30 fruit were left untreated. Two neonate larvae were placed onto each fruit, using a size 000 paint brush. A separate brush was used for each treatment and all brushes were sterilised in 2 % sodium hypochlorite and rinsed in sterile distilled water before use. Fruit were kept at 27°C and were inspected and dissected 14 days after

the larvae were placed onto them. Recordings were made of decaying fruit, fruit with penetration marks and fruit infested with live larvae. Original activity remaining (OAR) of the virus, on both the northern and southern aspects of trees, were estimated at various time intervals. Approximate virus half-life was also estimated by comparison of efficacy at different time intervals with dose-response data in detached fruit assays (section 3.3.2).

6.2.2 Efficacy

In all trials, sprays were applied using the same spray machine (and hand guns) at the same settings as described for the persistence trial (Plate 6.1). All trials, except the one conducted at Citrusdal, were laid out as single tree treatments replicated 10 times in a randomised block design (Fig. 6.1). Order of treatments within blocks was determined using random number tables. Application of treatments was commenced shortly after pheromone traps indicated an increase in moth activity. Bladbuff 5, at a concentration of 75 ml/100 ℓ, was added to the tank for all CrleGV-SA treatments, in order to improve wetting and spreading. Spraying was always conducted from late afternoon to evening (between 15h00 and 19h00) so as to avoid immediate UV inactivation of the virus. Alsystin (benzoylated urea: triflumuron), an insect growth regulator (chitin synthesis inhibitor), was often included in the trial as a chemical standard.

A week before evaluation of trials began (which was between 1 – 4 weeks after application), fruit lying on the ground underneath the trees were removed. Each week thereafter, fruit dropped from each tree were collected in paper bags, which were labelled with the treatment (name or number). This was continued either until fruit were harvested or there was no longer any difference between treatments. Fruit were inspected and dissected in the laboratory, and the cause of drop recorded. Before inspection, the labelled side of the bag was faced away from the inspector. Only once the inspection results for the fruit from a particular bag had been recorded, was the label noted. This was done to avoid any bias in the inspection process.

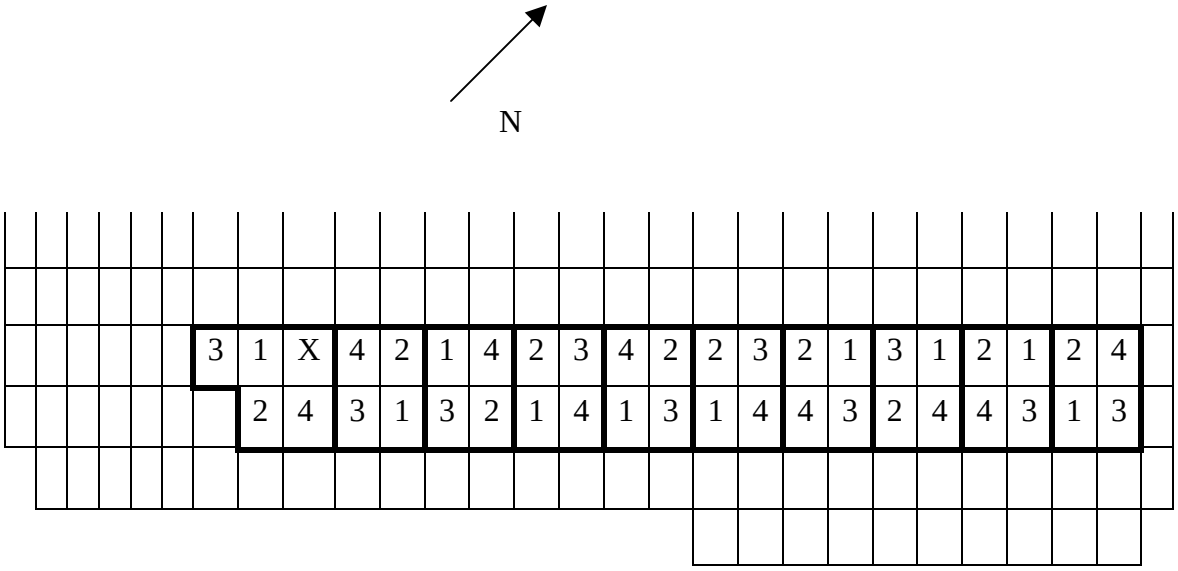

Fig. 6.1 CrleGV-SA field trial layout at Sun Orange Farm, Eastern Cape Province. Each block represents one tree. (1 = untreated; 2 = Alsystin treated; 3 = CrleGV-SA at 8.31 x 10^6 OBs/ml; 4 = CrleGV-SA at 7.46 x 10^7 OBs/ml; X = inferior tree not suitable for inclusion in the trial).

Table 6.1 Summarised weather data, obtained from the South African Weather Service, for areas in which field trials were conducted.

Weather station	**Trial duration**	**Average value for duration of trial (except rainfall: total for trial period)**					
		Temp. (ºC)	**Sunshine time (h : min)**	**Rainfall (mm)**	**Wind speed (km/h)**	**Wind direction**	**Relative humidity**
Uitenhage	21 Aug – 11 Sep 2001	14.54	11:23	47.0	25.00	SSW	***
Patensie	14 Apr – 2 Jun 2000	16.88	10:51	13.8	17.75	SW	79.76
Porterville*	14 Apr – 26 May 2000	17.80	10:12	16.5**	12.29	SE	***
Addo	5 Apr – 12 Jun 2001	25.12	10:48	85.6	2.35	N	85.9

*The nearest weather station to Citrusdal.
**Rainfall was measured at Citrusdal.
***No humidity sensors at these stations.

Daily weather data (rainfall, maximum and minimum temperatures, wind speed and direction, humidity and sunrise and sunset times) were provided by the South African Weather Service for each area in which a field trial was conducted (Appendix 4 – for the most relevant measurements), except Mpumalanga, from which no meaningful trial

results were obtained. Unfortunately, only rainfall data was available from Citrusdal (32º 38' S, 19º 02' E). Other weather data was obtained from the nearest station, at Porterville (33º 01' S, 19º 00' E). Weather data for each area has been summarised in Table 6.1.

During 2000, two small scale field trials were conducted to test the efficacy of CrleGV-SA against *C. leucotreta* on citrus. The first trial was applied on 16 year old Robyn navel orange trees on the farm Nikalandershoek near Patensie (33º 45' S, 24º 52' E) in the Gamtoos River Valley, Eastern Cape Province, on 14 April 2000. Treatments were CrleGV-SA at 1.08 x 10^6 OBs/ml (translates to 2.05 x 10^{13} OBs/ha), Alsystin (10 ml/100 ℓ) and an untreated control. An average of 43 ℓ of spray mix was applied per tree. Assessment of fruit drop began one week later, and continued for seven weeks until fruit were harvested. Mean fruit dropped per tree, both weekly and for a four week period, during which time the virus appeared to be effective, was calculated. Data was analysed by ANOVA and means compared using the Bonferroni LSD multiple range test, with the computer package, Statgraphics (Statistical Graphics Corporation, 1996).

The second trial was applied on mature Palmer navel orange trees on the farm of Martin Esterhuizen at Citrusdal (32º 38' S, 19º 02' E), Western Cape Province, on 18 April 2000. Ten trees adjacent to one another in a single row were sprayed. CrleGV-SA was applied at a concentration of 5.9 x 10^7 OBs/ml at a rate of 7.37 x 10^{14} OBs/ha. Spraying was initiated at 18h45 and approximately 30 ℓ was applied per tree. Assessment of fruit drop was initiated four weeks post treatment and conducted for only two weeks until fruit were harvested. Fruit drop and *C. leucotreta* infestation was compared with that from 10 untreated trees in an adjacent row. Statistical analysis of the data from this trial was not possible, as evaluations were conducted by Hendrik Hofmeyr (Citrus Research International, Citrusdal), who pooled the evaluated fruit per treatment.

During 2001, a further three field trials were conducted to test the efficacy of CrleGV-SA against *C. leucotreta* on citrus, one of which was conducted in conjunction with egg parasitoid augmentation and is reported on in Chapter 7.

The first 2001 trial was conducted on extremely large (± 6.5 m tall) mature navelate orange trees on Crocodile Valley Estate near Nelspruit (25° 30' S, 30º 0' E) in Mpumalanga Province. Three treatments were applied to 10 trees each, arranged in a random block design with another 10 untreated trees. An average of 77.8 ℓ was applied per tree. The treatments used were two concentrations of CrleGV-SA (5.8×10^6 OBs/ml at a rate of 1.09×10^{14} OBs/ha and 4.98×10^7 OBs/ml at a rate of 8.47×10^{14} OBs/ha) and Alsystin (10 ml/100 ℓ). CrleGV-SA sprays were applied between 17h00 and 19h00 (29 March 2001). From three weeks after treatment, until six weeks after treatment dropped fruit was collected and dissected to determine cause of drop.

The second trial was conducted on mature Palmer navel orange trees on Sun Orange Farm near Addo (33º 34' S, 25º 40' E) in the Sundays River Valley, Eastern Cape Province. Again two CrleGV-SA treatments (8.31×10^6 OBs/ml at a rate of 1.22×10^{14} OBs/ha and 7.46×10^7 OBs/ml at a rate of 1.01×10^{15} OBs/ha) and Alsystin (10 ml/100 ℓ) were applied. An average of 38.3 ℓ of spray mix was applied per tree. Sprays were applied between 15h00 and 17h30 on 5 April 2001. Fruit drop was evaluated from three weeks (21 days) to nine weeks and five days (68 days) after application. Statistical analysis of the data was conducted in the same manner as described for the data from the trial at Nikalandershoek Farm.

6.3 RESULTS AND DISCUSSION

6.3.1 Persistence

Including all fruit sampled, means of 26.36 % of untreated fruit, 22.5 % of treated fruit from the northern aspect of trees, 14.64 % of treated fruit from the southern aspect of trees, and 17.5 % of laboratory treated fruit showed signs of decay, when the trial was evaluated 14 days after neonate larvae had been placed onto the fruit (Fig. 6.2). Obviously, *C. leucotreta* penetration and infestation had some influence on the health of fruit; however, it cannot be presumed that it was the sole cause of decay. Once fruit have

been harvested they are prone to decay, if not treated with an appropriate post-harvest fungicide. Even the smallest fissure in the rind could lead to decay.

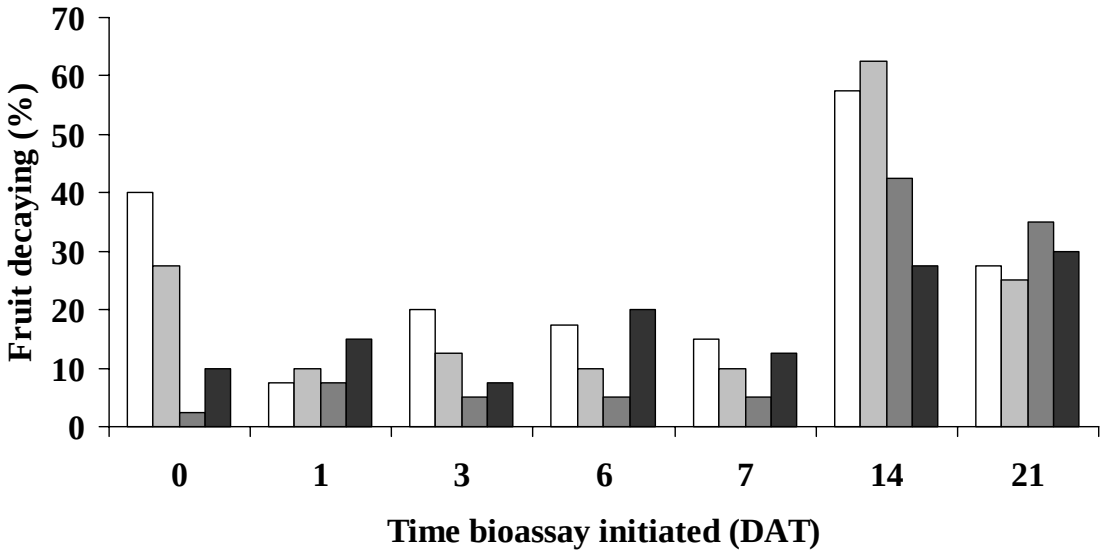

Fig. 6.2 Percentages of fruit decaying when assayed at various times after treatments with CrleGV-SA. Bioassays were initiated on the indicated days after treatment (DAT) by placing neonate larvae on fruit treated with CrleGV-SA either on the northern (pale grey bars), or southern aspect of trees (dark grey bars), in the laboratory (black bars) or untreated (white bars) and recording decay 14 days later.

Including all fruit sampled, 21.71 % of untreated fruit, 16.86 % of treated fruit from the northern aspect of trees, 15.29 % of treated fruit from the southern aspect of trees, and 13.86 % of laboratory treated fruit, had penetration marks (whether superficial or deep) (Fig. 6.3).

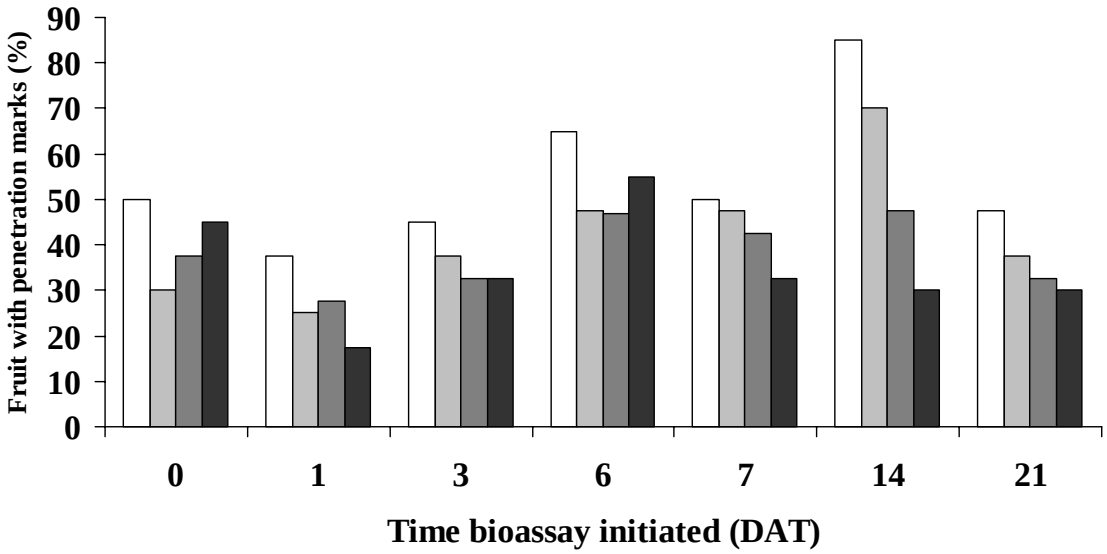

Fig. 6.3 Percentages of fruit with *C. leucotreta* penetration marks when assayed at various times after treatments with CrleGV-SA. Bioassays were initiated on the indicated days after treatment (DAT) by placing neonate larvae on fruit treated with CrleGV-SA either on the northern (pale grey bars) or southern aspect of trees (dark grey bars), in the laboratory (black bars) or untreated (white bars) and recording penetration marks 14 days later.

By far, the biggest differences between treated and untreated fruit were recorded for fruit actually infested with *C. leucotreta* larvae. A total of only 35.36 % of control fruit (from all dates) were infested when fruit were dissected 14 days after initiation of bioassays. The probable reason for this low level of infestation, was the cultivar of orange used. *C. leucotreta* are known to prefer navel oranges to Valencia oranges (Georgala, 1968). Levels of infestation observed in the field, are consistently far higher on navel oranges. This can be as much due to the suitability of the host, as to the preference of the pest. *C. leucotreta* infestation in the other treatments was 16.79 % of treated fruit from the northern aspect, 9.14 % of treated fruit from the southern aspect, and 8.57 % of laboratory treated fruit (Fig. 6.4).

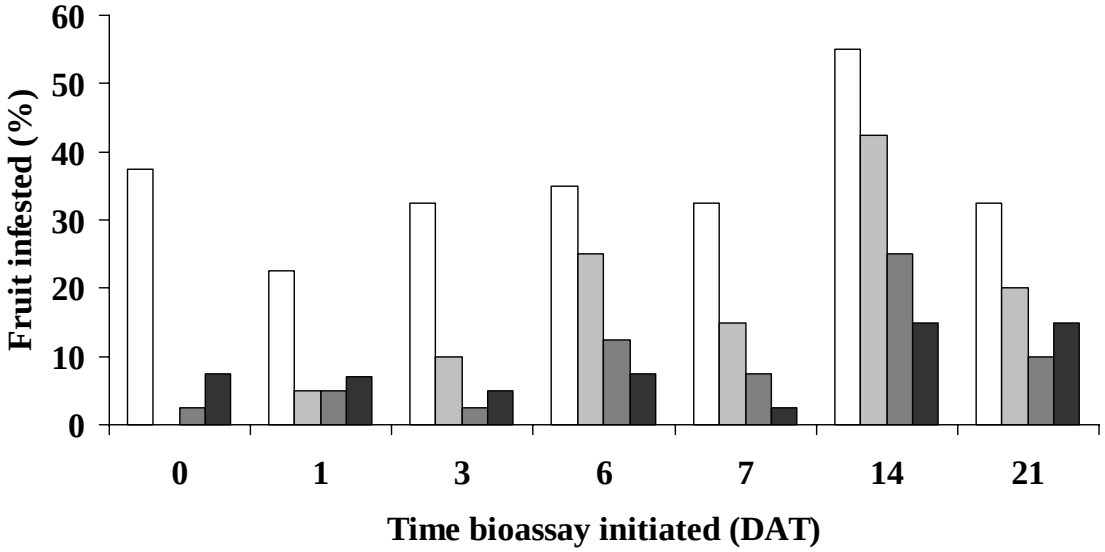

Fig. 6.4 Percentages of fruit infested with *C. leucotreta* larvae when assayed at various times after treatments with CrleGV-SA. Bioassays were initiated on the indicated days after treatment (DAT) by placing neonate larvae on fruit treated with CrleGV-SA either on the northern (pale grey bars) or southern aspect of trees (dark grey bars), in the laboratory (black bars) or untreated (white bars) and recording penetration marks 14 days later.

Various parameters for viral inactivation have been employed, for instance the proportion of OBs surviving (e.g. Witt and Hink, 1979), extrapolation from previously constructed log dose-probit mortality curves of the actual AOB dose corresponding to a particular observed mortality level (Richards & Payne, 1982) or, most commonly, in terms of mortality relative to the original activity (OAR) of the unexposed virus isolate. Reduction in infestation of the three treatments was determined relative to the infestation in the control fruit (Fig. 6.5). OAR of the virus on the northern aspect of trees appeared to be have declined by 50 % between four and five days after treatment. However, reduction in infestation again rose above 50 %, and when the trial was terminated 21 days post application, infestation was still 38 % lower than in untreated fruit. Efficacy dropped below that of the laboratory applied virus, from between one and three days post treatment.

OAR of the virus on the southern aspect of trees did not decline to the same extent as on the northern aspect during the 21 day period of the trial. At 21 days after treatment,

infestation was 69 % lower than in untreated fruit. This was an even greater reduction in infestation than that given by the laboratory treatment. However, it appeared that efficacy of the virus on the southern aspect was generally lower than the efficacy of the laboratory applied virus, from between three and six days after treatment.

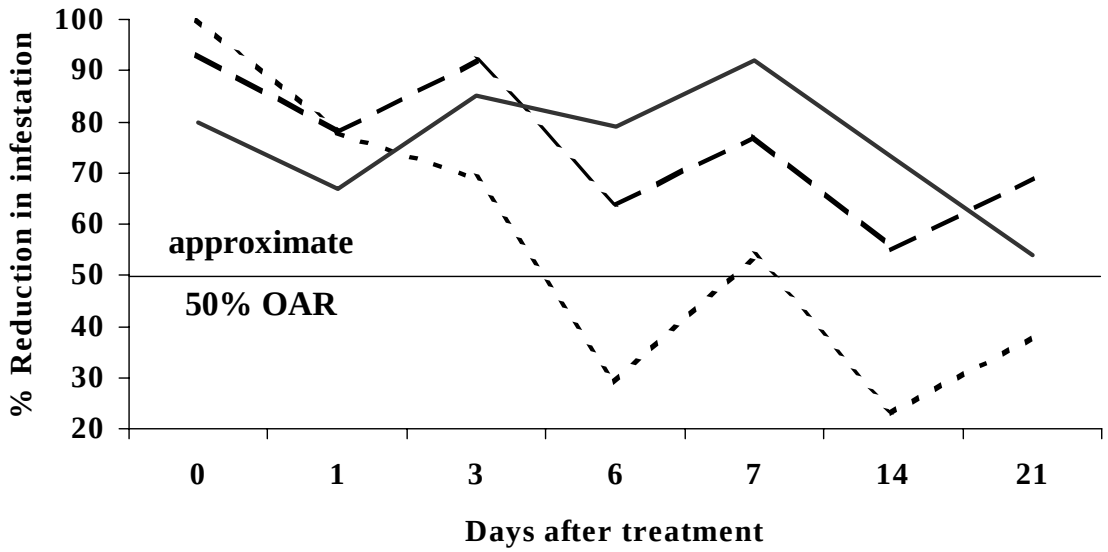

Fig. 6.5 Reduction in *C. leucotreta* infestation of fruit (Delta Valencia oranges), relative to untreated fruit (0 % on y-axis), when treated with CrleGV-SA on the northern aspect of trees(- - - -), on the southern aspect of trees (– – –) and in the laboratory (——). (OAR = original activity remaining).

It would appear that the half-life of the virus on the northern aspect of trees was 4 - 7 days or even slightly longer, and exceeded 21 days on the southern aspect of trees. However, because the dose-mortality relationship is sigmoid, rather than linear, the calculation of OAR provides a misleading guide to the actual extent of virus inactivation (Hunter-Fujita *et al.*, 1998). The best indication of virus half-life in this case can probably be obtained from inspection of the detached fruit assay dose-response data reported in Chapter 3. In the assay in which neonate larvae were placed onto treated fruit (Section 3.2.2), in much the same way as was done in this persistence trial, a 50 % reduction in virus concentration (from 1.347×10^8 to 6.736×10^7 OBs/ml) resulted in a mean (from three replicates) reduction in efficacy (measured by fruit infested) of 11.1 % (Table 3.10 & Fig. 3.8). The half-life of the virus in the field would therefore probably

relate to about an 11 % reduction in efficacy (mortality). A reduction of 11 % in the original activity of the virus on the northern aspect of trees would be recorded as an 89 % reduction in infestation. This was reached within the first 24 hours of the trial (Fig. 6.5). A reduction of 11 % in the original activity of the virus on the southern aspect of trees would be recorded as an 81 % reduction in infestation. Original activity dropped just below this level within the first 24 hours of the trial, however, a further two days later was up above this level again. It therefore appears that half-life of the virus on the southern aspect of trees was between 3 - 6 days. Replication of the trial would have assisted in confirming these observations. However, due to persistent rainfall after the first trial, this was not possible. Fruit was harvested shortly thereafter.

A field half-life for CrleGV-SA of less than 1 day on the sunny side of trees and of more than 3 days on the shady side of trees is satisfactory. In the only other recorded measurements of this value, CrleGV half-life in the field was estimated to be around two days in the tropical conditions of the Cape Verde Islands, and from 2.0 - 6.5 days (depending on average sunshine hours per day) in temperate European conditions (Fritsch & Huber, 1989). The majority of trials conducted to test the UV stability of baculovirus formulations in the field, have been conducted in temperate climates (Jaques, 1977; Richards & Payne, 1982). However, even under these conditions, a half-life of only a few days or even less is reported. Vail *et al.* (1991) reported that seven day old field weathered CpGV on walnut trees, still resulted in more than 80 % mortality of *C. pomonella* larvae. Nonetheless, the persistence of CpGV in the field, usually on apples, is considered to be so brief (Cross *et al.*, 1999), that weekly applications are deemed a viable option (Dickler & Huber, 1986). In Iran, a field half-life of only 1 - 4 h was reported for CpGV (Rezapanah *et al.*, 1996).

Three factors which may have enabled the virus to persist, seemingly fairly well (particularly on the southern aspect of trees), have been identified. Season, crop architecture and timing of application, can all influence the rate of UV inactivation of the virus.

The trial was applied in August, which falls during the South African winter. Average sunshine hours per day are obviously less than during summer, when most of the sprays against *C. leucotreta* will be applied. It can be expected that half-life in the summer months will probably be shorter. The biological half-life of PbGV on cabbage leaves in England differed by a factor of more than twofold between June and October and correlated well with integrated monthly UV flux data (Richards & Payne, 1982).

Generally, half-life of baculoviruses on non-tree crops is shorter than that on tree crops. Furthermore, in a single crop, it was shown that CpGV survived better on larger than on smaller apple trees (Richards, 1984). Mature citrus trees are probably denser than any other crop on which baculoviruses are used or tested, and can therefore offer a greater degree of natural shading against UV radiation. This manner of protection probably enabled 100 % control of *H. armigera* on citrus, with a single application of HaNPV (Moore, 1998).

In order to avoid immediate and substantial inactivation of the virus, applications should be made in the evening. Not only does this allow neonate larvae to ingest unaffected virus for several hours until sunrise the following morning, but wet virus has been found to inactivate more rapidly than dry virus (David, 1969). In this trial CrleGV-SA was applied at 16h00, shortly before the sun set.

Due to the satisfactory persistence demonstrated (particularly through the estimation of OAR), it was decided not to formulate CrleGV-SA with UV protectants, for the initial field trials designed to measure efficacy. The CrleGV-SA applied, was in any case, a crude suspension and body parts and natural debris therein, would provide substantial UV protection (David, 1969). There is also considerable indication that formulation of a microbial insecticide is not essential to its success (Jones *et al.*, 1997). Initial field efficacy trials would provide a benchmark for efficacy and would indicate the need for formulation.

It must be realised, however, that UV inactivation is not the only means by which virus can be lost. UV inactivation can be regarded as true environmental loss (Hunter-Fujita *et al.*, 1998). Other possible causes of loss of virus from the plant, can be rain, wind and leaf fall. However, here the virus is retained in the environment (in the soil) and could serve as a significant source of inoculum, which could be returned to the plant by rain splash (Hunter-Fujita *et al.*, 1998). It is highly improbable that rain and wind had any role to play in the lesser persistence of CrleGV-SA on the northern side of trees, as the average wind direction in the Uitenhage area, over the time period of the trial, was SSW (Appendix 4). Rain would also have come from this direction. A total of 47 mm of rain fell during the trial period (Table 6.1) – 99 % of which, fell from 28 - 31 August (Appendix 4). The average wind direction during this time was also calculated to be SSW (Table 6.1). Therefore, if anything, the wind and rain would have reduced the persistence of the virus on the southern side of trees. Certain studies have actually identified pronounced rainfastness of baculoviruses on plant surfaces e.g. CpGV on apple leaves (Burgerjon & Sureau, 1985). Also, caging plants at night to prevent dew formation, did not reduce virus (HzNPV) inactivation (Young & Yearian, 1974). It must therefore be assumed that UV radiation was responsible for the difference. Unfortunately the UV intensity could not be measured as no radiometer, or similar instrument, was available. However, mean daily sunshine hours (time between sunrise and sunset) for the period of the trial was measured as 11 h 23 min (Table 6.1).

Because of the difference in rate of breakdown between virus on the northern and southern aspects of trees, control strategies with CrleGV-SA could be influenced. It may be necessary to spray the northern side of trees more frequently than the southern side, or to include a UV protectant in tank mixes applied to the northern side, but not to the southern side. This might not be an impractical option if application protocols are properly planned.

6.3.2 Efficacy

Severe rains were experienced in the area of Nikalandershoek Farm (Patensie, Eastern Cape Province) during the first four months of 2000 (Appendix 4). From January to April, 411.6 mm, more than the average annual rainfall, were recorded. Consequently, conditions were ideal for fungal proliferation. Fruit decay was severe and occurred rapidly once fruit dropped from the tree. Therefore, in most cases it was not possible to determine what the injury was that caused the fruit to drop. Accordingly, only four *C. leucotreta* infested fruit were identified out of a total of 295 dropped fruit, in this trial. An underestimation of the level of *C. leucotreta* infestation may have resulted. Despite only a few infested fruit being found, there was less fruit drop from both CrleGV-SA and Alsystin treated trees, from three to six weeks after treatment (with the exception of the Alsystin treated trees during the fifth week after treatment), than from untreated trees (Fig. 6.6). A *C. leucotreta* infested fruit generally takes three to five weeks, after initial infestation, to drop off the tree (Hendrik Hofmeyr, personal communication). Fruit drop was not significantly less in the CrleGV-SA treatment than the untreated control during any particular week (Fig. 6.6). Data over weeks three to six were pooled for each treatment, and reduction in fruit infestation was calculated as:

$$100 \times \left(1 - \frac{\text{number of infested fruit in treatment}}{\text{number of infested fruit in control.}}\right)$$

Fruit drop was 33.87 % lower in the CrleGV-SA treatment and 37.10 % lower in the Alsystin treatment than in the untreated control (Fig. 6.7). However, these differences were not significant ($P<0.05$; Bonferroni multiple range test). Data from week seven showed negligible differences in fruit drop between the three treatments. This was probably due to there being little *C. leucotreta* infestation, even in untreated fruit, as drop from untreated trees was notably lower during this week than any previous week. Data from week seven was therefore not included in the analysis.

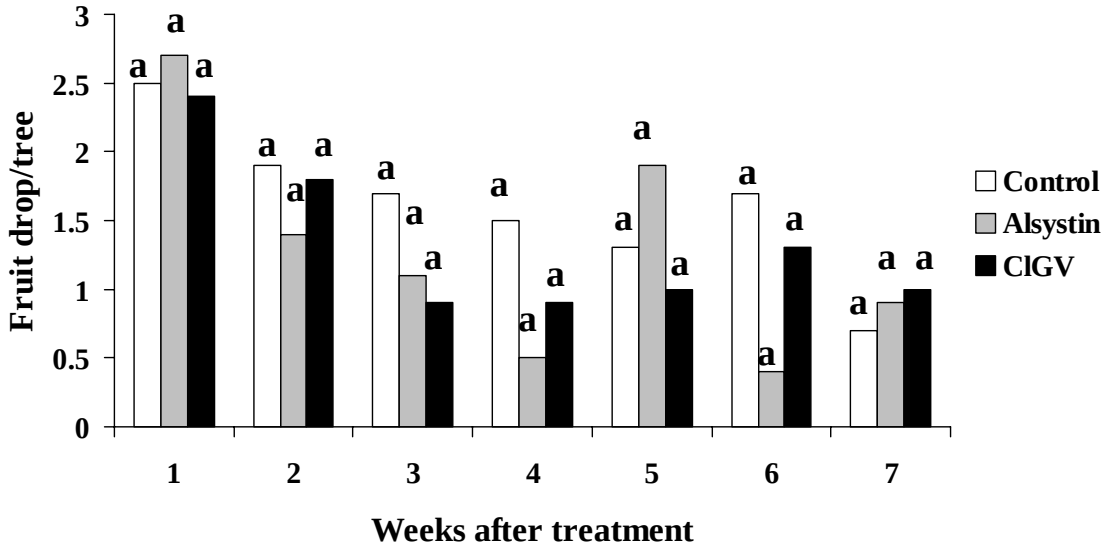

Fig. 6.6 Weekly fruit (Robyn navel oranges) drop for treatments applied for control of *C. leucotreta* at Nikalandershoek Farm, Eastern Cape Province, on 14 April 2000, when untreated (white bars), treated with Alsystin (grey bars) and with CrleGV-SA (black bars). Bars per week with the same letter are not significantly different (P > 0.05; Bonferroni LSD multiple range test).

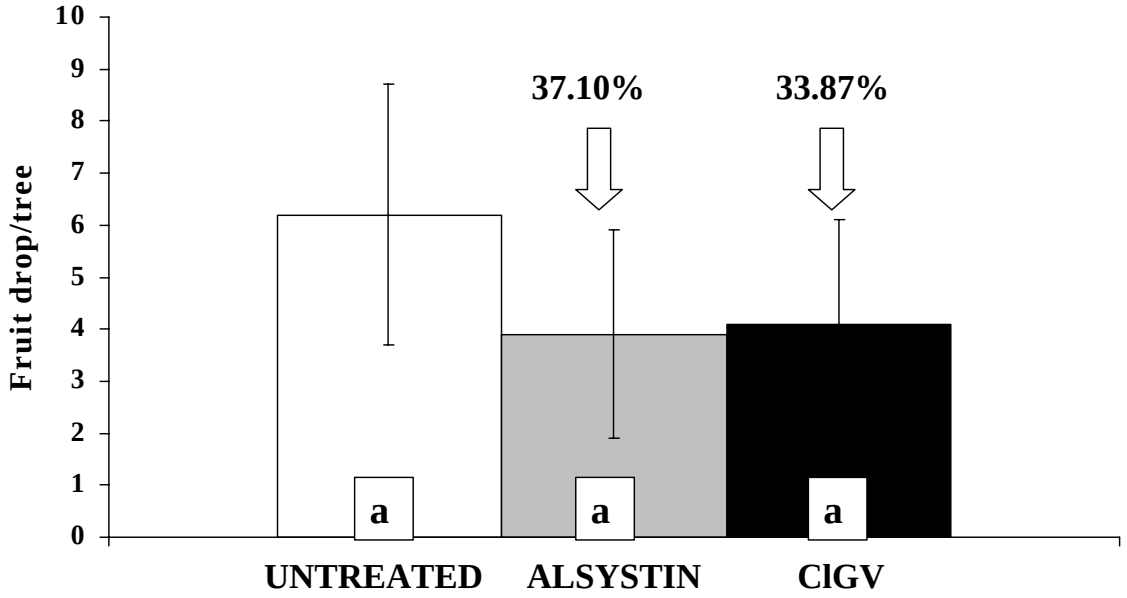

Fig. 6.7 Mean (and SE) fruit (Robyn navel oranges) drop per tree for a period of 3 - 6 weeks after treatments applied for control of *C. leucotreta* at Nikalandershoek Farm, Eastern Cape Province, on 14 April 2000. Bars with the same letter are not significantly different (P > 0.05; Bonferroni LSD multiple range test).

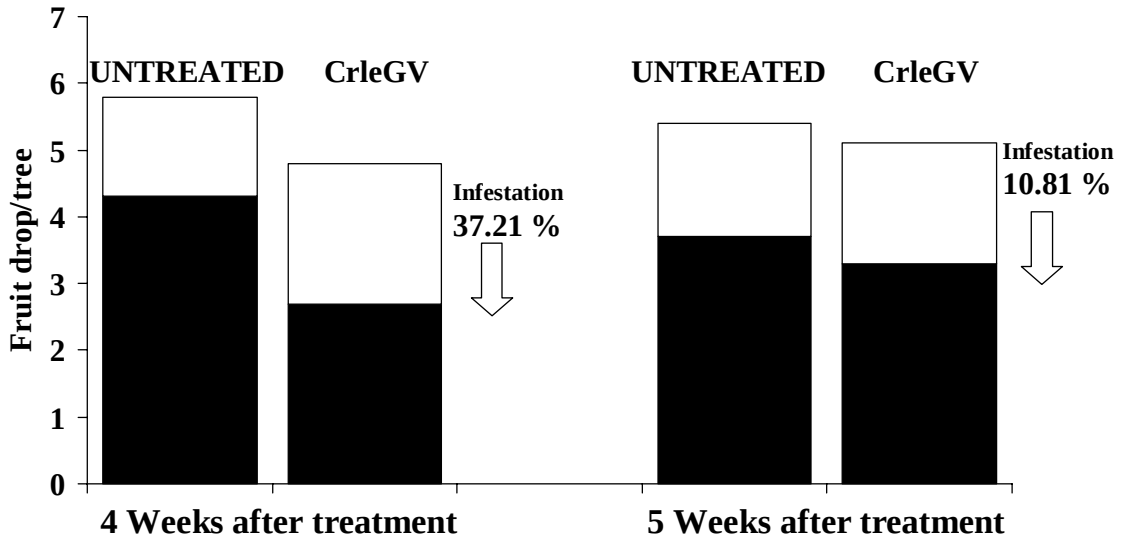

Fig. 6.8 Weekly total fruit (Palmer navel oranges) drop (entire bar) and *C. leucotreta* infested fruit drop (dark portion of bar) for treatments applied for control of *C. leucotreta* at Martin Esterhuizen's Farm, Western Cape Province, on 18 April 2000. Arrows indicate reduction in infestation relative to untreated trees.

In the Citrusdal trial, *C. leucotreta* infestation in dropped fruit was 37.21 % lower from the CrleGV-SA treated trees than from the untreated trees, four weeks after treatment (Fig. 6.8). As fruit from all trees within each treatment were pooled, it was not possible to determine whether this difference was statistically significant. It is unlikely that the 10.8 % difference in fruit drop during the following week would have been significant (Fig. 6.8). Fruit were harvested during the week thereafter.

Mean reduction in infestation in the CrleGV-SA treatment, over the two weeks of monitoring of the Citrusdal trial, was 24.01 %. The most likely reason for the virus not reducing infestation by a greater margin, was the level of *C. leucotreta* infestation in this orchard – infestation was exceptionally high. Due to their slow speed of kill and lack of persistence, baculoviruses should only be applied against low to moderate levels of infestation, preferably in conjunction with other means of biological control, such as parasitoid augmentation, or biorational control, such as mating disruption (Cross *et al.*, 1999).

From the Mpumalanga trial only three infested fruit were found from all treatments, over a four week period. There were therefore no significant or even noteworthy differences between treatments. Infestation at this site was far too low to obtain any meaningful results.

The most meaningful results from all trials were those recorded in the trial conducted at Sun Orange Farm. From three weeks after treatment until fruit were harvested at nine weeks and five days after treatment, *C. leucotreta* infestation was lower for all three treatments than the untreated control (Fig. 6.9). During no particular week were these differences significant, probably a reflection on the relatively small number of replicates (i.e. 10 per treatment). During weeks three and four after application, reduction in infestation for the CrleGV-SA treatments was greater than for the Alsystin treatment (Fig. 6.9). It is understandable that the efficacy of CrleGV-SA would become apparent before that of Alsystin, as Alsystin is an ovicide, only effective against eggs that are laid on an existing residue, and CrleGV-SA is a larvicide. There would therefore be an apparent time delay in the working of Alsystin, particularly if there were numerous *C. leucotreta* eggs laid before spraying. CrleGV-SA would, however, be immediately effective against any neonate larvae. Despite the difference in mode of activity, it is surprising that a notable reduction in infestation of fallen fruit from Alsystin treated trees, was recorded only from six weeks after application. Thereafter, *C. leucotreta* infestation in Alsystin treated fruit was lower than in CrleGV-SA treated fruit.

The sudden rise in *C. leucotreta* infestation in CrleGV-SA treated fruit, from five weeks after treatment and the higher level of infestation than in Alsystin treated fruit from the following week, may have been caused by the rainfall during the first three weeks after application (80.3 mm) (Appendix 4). Rainfall was particularly high during the second week after application (50 mm). It is possible that CrleGV-SA is not as rainfast as has been reported for other baculoviruses on other crops (David & Gardiner, 1966; Burgerjon & Grison, 1965; Burgerjon & Sureau, 1985).

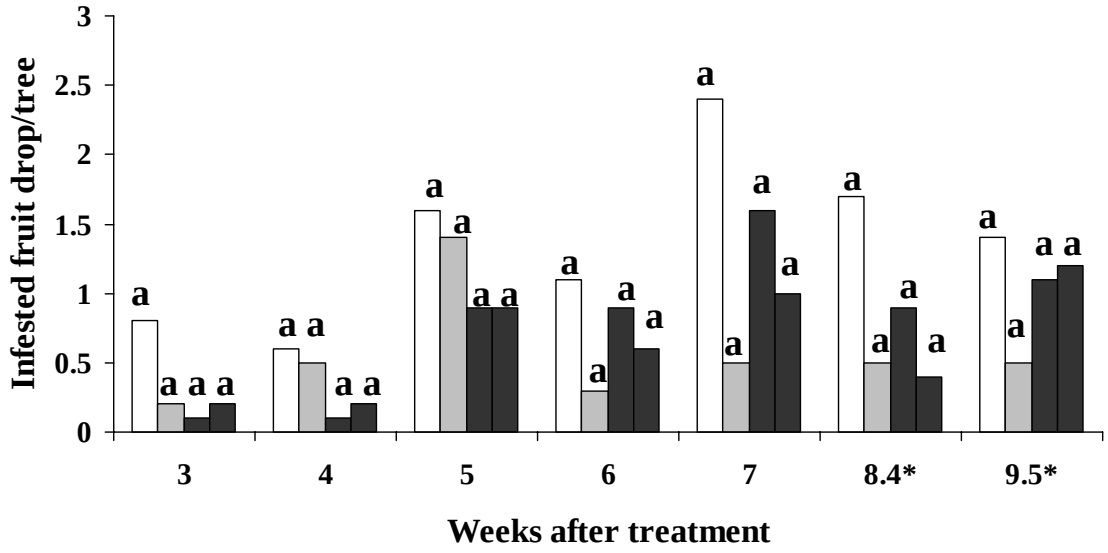

Fig. 6.9 Weekly *C. leucotreta* infested fruit (Palmer navel oranges) drop from untreated control trees (white bars), Alsystin treated trees (grey bars), trees treated with 8.31 x 10^6 OBs/ml CrleGV-SA (first of two black bars) and trees treated with 7.46 x 10^7 OBs/ml (second of two black bars), at Sun Orange Farm, Eastern Cape Province, on 5 April 2001. Bars per week with the same letter are not significantly different (P > 0.05; Bonferroni multiple range test). (*8.4 = 8 weeks and 4 days; *9.5 = 9 weeks and 5 days).

Until six weeks (42 days) after treatment, there was little difference in infestation of fruit treated with the two different concentrations of CrleGV-SA. From 42 - 60 days after treatment, *C. leucotreta* infestation was lower in fruit from trees treated with the higher concentration of CrleGV-SA (Fig. 6.9). In the final evaluation at 68 days (9 weeks and 5 days) after treatment, there was little difference in fruit infestation between the CrleGV-SA treatments and the untreated control. If this was indicative of a breakdown in the efficacy of the CrleGV-SA treatments, and if infested fruit took three weeks (21 days) to fall, then CrleGV-SA had protected the fruit for less than 47 days. If a breakdown in efficacy could have been recorded immediately after the penultimate evaluation (8 weeks and 4 days after treatment) then the fruit would have been protected for 39 days. Alsystin residues are known to remain effective for eight weeks. A breakdown in Alsystin efficacy would therefore be detected in infestation of dropped fruit from 11 weeks after application.

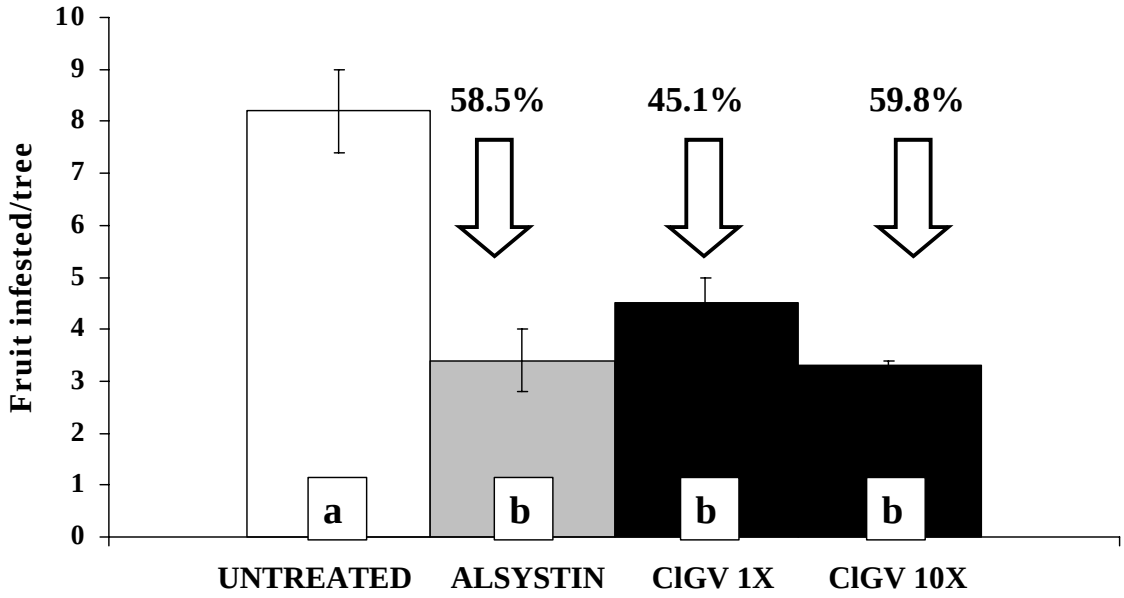

Fig. 6.10 Mean (and SE) *C. leucotreta* infested fruit (Palmer navel oranges) drop over the period, 21 - 60 days after application, for treatments applied at Sun Orange Farm, Eastern Cape Province, on 5 April 2001. Arrows indicate reduction in infestation relative to untreated trees. Bars with the same letter are not significantly different ($P > 0.05$; Bonferroni multiple range test). (CrleGV-SA 1X = 8.31×10^6 OBs/ml; CrleGV-SA 10X = 7.46×10^7 OBs/ml).

If fruit infestation per tree per week was averaged over a period of 21 - 60 days, all treatments significantly reduced infestation (Fig. 6.10). Infestation was 58.5 % lower in Alsystin treated fruit and 45.1 % and 59.8 % lower for the two CrleGV-SA treatments.

The efficacy of CpGV against *C. pomonella* in numerous field experiments has ranged from 70 to over 90 % control, somewhat lower than for the best insecticides to which *C. pomonella* populations have not developed resistance (Cross *et al.*, 1999). This is substantially better than recorded here with CrleGV-SA against *C. leucotreta* (Fig. 6.10). However, *C. leucotreta* is considered to be an even more difficult pest to control than *C. pomonella*, particularly with a baculovirus. Firstly, *C. leucotreta* does not overwinter (Angelini & Labonne, 1970; Reed, 1974) as does *C. pomonella*. The moth is therefore potentially present throughout the year, and there is always an overlap of generations. Secondly, *C. leucotreta* is an active flyer. Even though control of the pest within an

orchard may be good, high numbers of moths can rapidly reinfest an orchard. Thirdly, time between hatching of *C. leucotreta* larvae and their penetration into fruit is briefer than that for *C. pomonella*. This is due to the difference in oviposition sites. A large proportion of *C. pomonella* eggs are deposited on leaf surfaces (Alford, 1984), whereas *C. leucotreta* eggs are only deposited on fruit surfaces. *C. pomonella* larvae may even briefly feed on foliage before moving onto the fruit, whereas *C. leucotreta* only feed on the fruit (while boring). There may therefore, be less opportunity for interception of *C. leucotreta* by the virus. Lastly, the foliage of a citrus tree is denser than that of an apple tree. It is therefore more difficult to achieve adequate coverage of a citrus tree with an insecticide, than it is to achieve adequate coverage of an apple tree.

Only one other record exists of a small-scale field trial with CrleGV against *C. leucotreta.* On Cape Verde Islands concentrations of 10^8 and 10^9 OBs/ml, with the addition of skimmed milk powder and a wetting agent, reduced *C. leucotreta* damage by 77 % in citrus and 65 % in Spanish pepper (Fritsch, 1988). It could not be ascertained exactly how the trial was evaluated i.e. interpretation of damage, period of time, frequency of inspection.

If reduction in fruit infestation in this study was calculated in the same way as was done in the Cape Verde trial i.e.

$$100 \times \left(1 - \frac{\%\ \text{infested* fruit in treatment}}{\%\ \text{infested* fruit in control}}\right)$$

(* Fritsch (1988) referred to damaged fruit rather than infested fruit.)

then Alsystin would have reduced damage by 42.72 %, the lower concentration of CrleGV-SA by 44.53 % and the higher concentration of CrleGV-SA by 52.83 %. However, this method was believed to be less accurate or meaningful, as it is real numbers of lost fruit, rather than percentages of fruit lost, that translate into monetary loss.

Table 6.2 Estimated average figures for production and sale of navel oranges (Shaun Brown, Capespan, personal communication).

Export volume/ha (tons)	**Export cartons/ha (at 15 kg/ carton)**	**Gross income/ carton**	**Cost/carton (production, packing and shipping)**	**Net income per carton (profit)**	**Mean number of fruit/carton**	**Net value/ fruit**
35 000	2 333	R30	R15	R15	72	R0.21

During the period that the trial at Sun Orange was monitored, the untreated control lost around one fruit per tree per week to *C. leucotreta.* If CrleGV-SA reduced fruit infestation by 59.8 % (Fig. 6.10) for 39 days, and there are 555 trees per hectare (the most common modern planting spacing), then each application of CrleGV-SA could prevent a loss of 1 849 fruit. If the average profit value of a navel orange is R0.21 (Table 6.2) then this would be a saving of R388.31 per hectare. For an application of CrleGV-SA to be justified, it must cost less than R388.31 per hectare. Obviously, the greater the number of fruit that can be saved by a CrleGV-SA application, the more easily justifiable is any cost. It will, however, be important to determine what level of activity/infestation is too high for CrleGV-SA to be acceptably effective. Another factor that a CrleGV-SA application will have to be measured against, is the cost of applying Alsystin. Each application of Alsystin (10 ml/100 ℓ), if applied adequately on mature orange trees, would cost in excess of R2 000 per hectare. This is extremely expensive.

If CrleGV-SA is applied sufficiently close to harvest so as to be able to reduce the chance of undetectable infestation in harvested fruit occurring, the cost of spraying is far more easily justified. If infested fruit are packed, fruit can decay en route to the market causing more dire and costly losses. Fungal infection in one decaying fruit can spread rapidly and easily to the other fruit in the carton. Even more seriously though, would be the increased chance of interception of live *C. leucotreta* larvae on the market, which would not only scar the product reputation with consumers, but could cause trade repercussions. *C. leucotreta* is endemic to sub-Saharan Africa, and as such is regarded by foreign markets as a phytosanitary pest.

6.4 CONCLUSION

Half-life of CrleGV-SA on citrus trees was calculated to be less than 1 day on the northern aspect of trees, and between 3 and 6 days on the southern aspect. These values were determined from late winter to early spring. Persistence of CrleGV-SA should be determined at other times of the year, and a more definite half-life value of CrleGV-SA on the shady aspect of trees determined. More importantly, for purposes of practical application, OAR of the virus dropped below 50 % after 5 days on the northern aspect of trees and was still at 69 % on the southern aspect of trees when the trial was terminated after 3 weeks. This was considered to be satisfactorily efficacious.

CrleGV-SA reduced *C. leucotreta* infestation by almost 60 % over a 39 day period. This compared favourably with the chemical standard, Alsystin, which reduced infestation by 58.5 % over the same time period. Alsystin is, however, known to be effective for a period of around 56 days. Efficacy of CrleGV-SA may also have persisted at a low level for substantially longer than the 39 days measured. This should be examined in future field trials.

To reap maximum benefit from the use of CrleGV-SA, it should be integrated into a biointensive programme for the management of not only *C. leucotreta*, but of other members of the citrus pest complex too. This approach is discussed in the final chapter.

7

INTEGRATION OF CrleGV-SA INTO A SYSTEM FOR THE MANAGEMENT OF *C. LEUCOTRETA* ON CITRUS

7.1 INTRODUCTION

The pest management concept grew out of the discontent with the purely insecticidal approach to pest control of the 1950s (Pedigo, 1996). Development of resistance to plant protection products by target pests, and public pressure to reduce the environmental impact of pest management practices, are probably the two most significant factors which have motivated the adoption of integrated pest management (IPM) strategies internationally (Hattingh, 1994). In 1967 the FAO Panel of Experts on Integrated Pest Control defined "integrated control" as: "A pest management system that in the context of the associated environment and the population dynamics of the pest species, utilises all suitable techniques and methods in as compatible a manner as possible and maintains the pest populations at levels below those causing economic injury." Today, "integrated control" equals "pest management" which equals "integrated pest management" in most people's minds (Van Emden & Peakall, 1996). Literature abounds with definitions of the practice of IPM. The essence of most of these is the harmonious combination of various pest management techniques into a flexible, ecologically sound strategy which provides economical, long term control of pest populations and levels (Hattingh, 1994). The various techniques referred to, include biological, cultural and chemical control. Biological control is the pivotal aspect, with the efficacy thereof determining the extent of chemical intervention required to maintain quality standards (Hattingh, 1994).

More biological control projects have been successfully carried out in citrus agroecosystems around the world than in any other major cropping system. Formal IPM strategies for citriculture were widely formulated and actively promoted on an international scale in the late 1960s and early 1970s, although IPM techniques had been practised long before this (Knapp, 1984; Luck, 1981; Smith, 1990). Bedford (1968) actively promoted the philosophy and demonstrated its feasibility in southern Africa. There are a number of compelling reasons justifying the adoption of IPM practices in the southern African citrus industry (Moore, 2000). These are the sustainability of the approach, prevention of repercussion pest outbreaks, resistance management, avoidance of chemical residue problems, environmental responsibility, and compliance with market pressure. Key elements in the implementation of IPM are the use of reliable pest and natural enemy monitoring systems, the use of intervention thresholds, judicious use of plant protection products based on a knowledge of effects on non-target beneficial insects, natural enemy conservation strategies, and natural enemy augmentation (Moore, 2000).

The advantages and disadvantages of baculoviruses in pest management systems have often been reviewed (Deacon, 1983; Cunningham, 1988; Huber, 1990). Possibly the most frequently quoted drawback of a baculovirus is its slow speed of kill relative to a chemical insecticide. In order for baculoviruses to be able to compete with chemicals, their full range of potential advantages should be exploited. One of their most important advantages is their host specificity and consequently their safety to non-target beneficial insects, which is vital to the success of IPM.

IPM strategies have been fairly extensively proposed for the southern African citrus industry (Hattingh, 1994; Hattingh, 1996; Grout *et al.*, 1998; Bedford *et al.*, 1998), and specifically for the Eastern Cape Province (Willers *et al.*, 1981; Du Toit, 1996). However, only one scenario proposing the integration of microbial control and arthropod biocontrol into an IPM system has been made (Moore *et al.*, 1999b). In order to optimise and compliment the efficacy of CrleGV-SA and to maximise the possibility of it becoming a successful tool for commercial management of *C. leucotreta*, it cannot be proposed as a stand-alone weapon. It must be integrated into a holistic system for the sustainable management of *C. leucotreta*. This study proposes

to investigate the various aspects of an IPM system for *C. leucotreta* control, into which CrleGV-SA would need to fit. In particular, the compatibility CrleGV-SA and the egg parasitoid, *Trichogrammatoidea cryptophlebiae*, including a parasitoid augmentation programme, are investigated, as is the manner in which the virus and the parasitoid would complement one another.

7.2 MATERIALS AND METHODS

7.2.1 Monitoring *C. leucotreta* moths with pheromone traps

Plate 7.1 A Lorelei trap used for monitoring *C. leucotreta* moths in citrus orchards. A pheromone dispenser, containing a synthetic female pheromone, attracts male moths. The floor of the trap is lined with a poly-butene glue covered plastic sheet for capturing the moths.

One Lorelei pheromone trap (Reg. no. L4877 Act 36/1947) (Plate 7.1) was hung in a 2 ha orchard of 15 year-old Palmer navel orange trees on Rietfontein Farm, in the Sundays River Valley, Eastern Cape Province, on 25 October 2000. The trap was hung according to recommendations (Grout *et al.*, 1998), on the upwind (southern) side of the orchard. A sheet of stiff transparent plastic (A4 dimensions) was smeared with a layer of poly-butene glue and fixed to the floor of the trap with paper clips. Every seven days, the trap was checked and any male *C. leucotreta* moths entangled in the glue were removed and recorded. This was continued until fruit were harvested

during the last week of May 2001.

7.2.3 Parasitoid augmentation

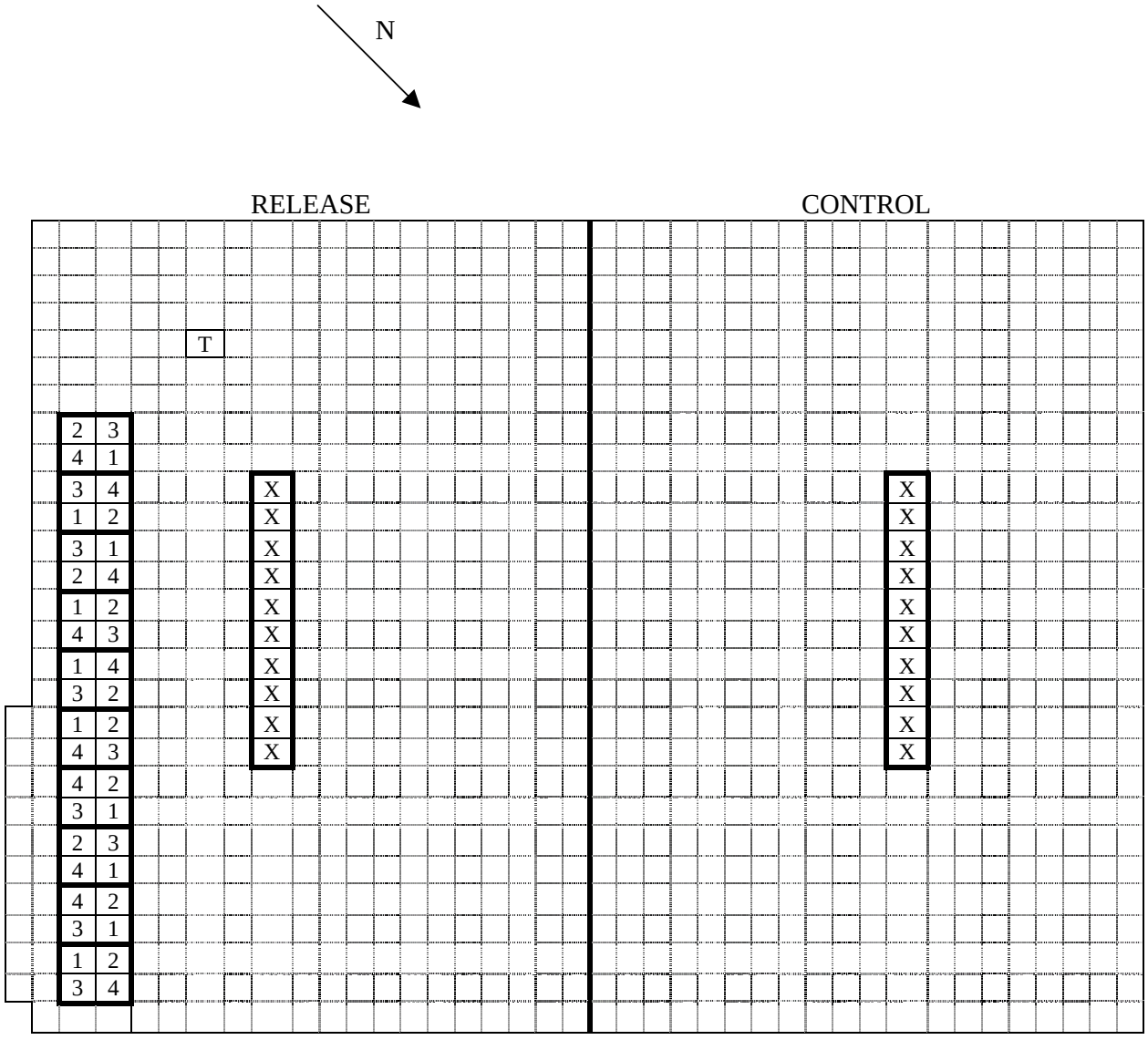

Fig. 7.1 Layout of *T. cryptophlebiae* augmentation and CrleGV-SA trials at Rietfontein Farm, Eastern Cape Province. Each block represents one tree; T = pheromone trap; X = data tree for evaluation of parasitism of *C. leucotreta* eggs and fruit infestation; 1 = untreated trees; 2 = Alsystin treated trees; 3 = CrleGV-SA (7.02 x 10^6 OBs/ml) treated trees; 4 = CrleGV-SA (7.02 x 10^7 OBs/ml) treated trees.

The orchard in which the pheromone trap was hung was divided into two equal portions (each ± 1 ha). The first half of the orchard was used as a parasitoid release block and the second half as a non-release control block (Fig. 7.1). The egg parasitoid, *T. cryptophlebiae* (Plate 7.2), was reared in the laboratory on sheets of *C. leucotreta* eggs, obtained according to the method described in sections 4.2.3 and 4.3.2. Parasitoids were liberated in the release block as parasitised *C. leucotreta* eggs. As the parasitoid life cycle took 10 days in the laboratory (at 27°C), parasitised eggs were placed in the orchard within eight days of being parasitised. An estimated 25000 parasitoids were released per hectare approximately every four weeks from 25 October 2000 (Table 7.1). During the last three weeks before harvest, 25000 parasitoids were released each week. It took about 15 sheets to provide a total of 25000 parasitised eggs. These were dispersed evenly throughout the orchard (Fig. 7.1). Each egg sheet was stapled to a leaf on the inside of a tree canopy. A total of 275000 parasitoids were therefore released per hectare.

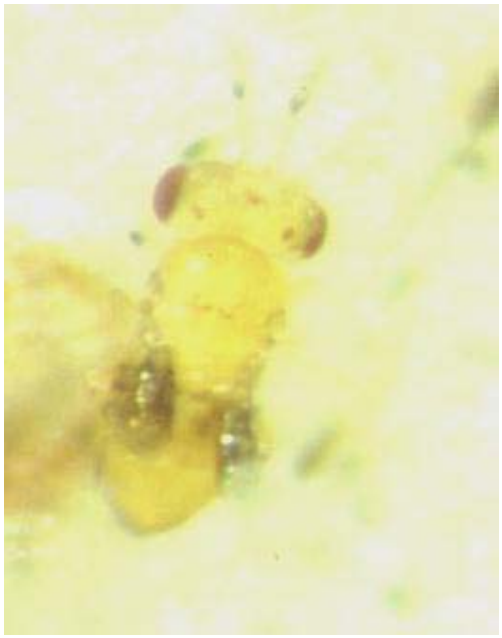

Plate 7.2 *Trichogrammatoidea cryptophlebiae*, the highly effective egg parasitoid of *C. leucotreta* (X 150).

Each week, from 25 October 2000, random surveys were conducted on fruit for *C. leucotreta* eggs. Eggs were first noted on 6 December 2000. Each week, from this date, release efficacy was determined by evaluating parasitism of *C. leucotreta* eggs on fruit and *C. leucotreta* infestation of fruit. These factors were evaluated on 10 data

trees (adjacent to one another in a row) in the middle of each of the two blocks (release and control) (Fig. 7.1). Ten fruit on each tree were randomly selected and inspected for eggs. Only live eggs or parasitised eggs from which the parasitoid had not yet emerged, were recorded. Simultaneously, all fruit which had dropped from these trees were collected and examined to determine the cause of drop. Any signs of *C. leucotreta* infestation, in particular, were recorded. Mean percentage of *C. leucotreta* eggs parasitised each week in release and control blocks and mean fruit infested with *C. leucotreta* larvae (per tree per week) were calculated using Statgraphics (Statistical Graphics Corporation, 1996). Means were compared using Students' T-tests.

Table 7.1 Releases of the *C. leucotreta* egg parasitoid, *T. cryptophlebiae*, at Rietfontein Farm, Eastern Cape Province, during 2000/2001.

Date	**Parasitoids released per ha**
25 October 2000	25000
21 November 2000	25000
19 December 2000	25000
16 January 2001	25000
20 February 2001	25000
13 March 2001	25000
10 April 2001	25000
2 May 2001	25000
9 May 2001	25000
15 May 2001	25000
22 May 2001	25000
Total	275000

7.2.4 Investigation of an egg parasitism threshold

Egg parasitism and fruit drop were monitored in the two blocks, to determine the effectiveness of parasitoid augmentation and the level of parasitism required to effectively control *C. leucotreta*. This level could then be used as a threshold value to assist in deciding whether chemical intervention was necessary. A relationship between level of parasitism and level of fruit infestation was investigated through visual interpretation of a graphic display of data.

7.2.5 CrleGV-SA application

A trial was conducted to test the combined efficacy of CrleGV-SA and parasitoid augmentation against *C. leucotreta*. The CrleGV-SA trial was laid out as single tree treatments replicated 10 times in a randomised block design within the parasitoid release block at Rietfontein Farm. Order of treatments within blocks was determined using random number tables. Application of treatments (on 11 April 2001) was commenced shortly after pheromone traps indicated an increase in moth activity (Fig. 7.2). The two concentrations of CrleGV-SA used were 7.02 x 10^6 OBs/ml at a rate of 1.15 x 10^{14} OBs/ha and 7.02 x 10^7 OBs/ml at a rate of 1.07 x 10^{15} OBs/ha. Bladbuff 5, at a concentration of 75 ml/100 ℓ, was added to each CrleGV-SA tank mix, in order to improve wetting and spreading. CrleGV-SA treatments were compared with an Alsystin (10 ml/100 ℓ) spray, as the chemical standard, and with untreated control trees. An average of 31.5 ℓ was applied per tree between 15h00 and 17h00.

A week before evaluation of trials began (three weeks after application), fruit lying on the ground underneath the trees, were removed. Each week thereafter until harvest, fruit that had dropped from each tree were collected in paper bags, which were labelled with the treatment (name or number). Fruit were inspected and dissected in the laboratory, and the cause of drop recorded. Before inspection, the labelled side of the bag was faced away from the inspector. Only once the inspection results for the fruit from a particular bag had been recorded, was the label noted. This was done to avoid any bias in the inspection process. Mean *C. leucotreta* larval infestation of the fruit from each treatment (fruit infested per tree per week) was compared by ANOVA and the Bonferroni LSD multiple range test using Statgraphics (Statistical Graphics Corporation, 1996). The combined impact of CrleGV-SA and parasitoid releases was calculated.

7.2.6 Natural impact of CrleGV-SA

Each week from 6 February until 9 May 2001 (the last evaluation before fruit were harvested) all larvae that were recovered from fruit were collected individually in microtubes and frozen at -40ºC. At the end of the trial (after 9 May 2001) larvae were screened for CrleGV by Shalene Singh of Rhodes University, using dot blot assays

(Singh, 2002). Infection levels of larvae on each collection date were plotted against moth catches and fruit infestation levels on the same dates.

7.3 RESULTS AND DISCUSSION

7.3.1 Monitoring *C. leucotreta* moths with pheromone traps

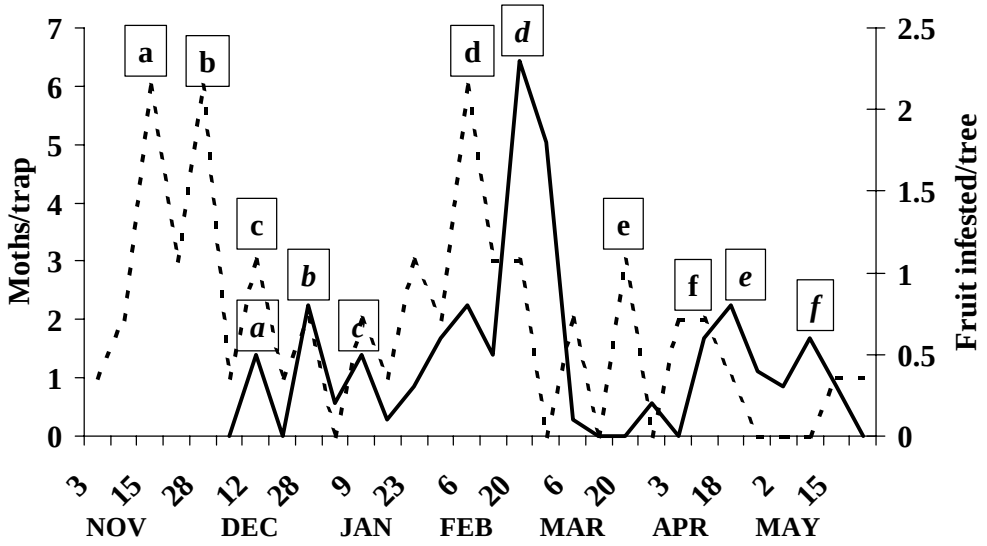

Fig. 7.2 Pheromone trap catches (· · · ·) (Y1 axis) and fruit infestation (——) (Y2 axis) at Rietfontein Farm, Eastern Cape Province, from 3 November 2000 to 22 May 2001. Normal font letters in blocks indicate the more prominent peaks in moth capture and corresponding letters in italics indicate the related peak in fruit infestation.

A trap threshold of 10 moths per trap is recommended (Grout *et al.*, 1998). At no stage during the 2000/2001 citrus season was the threshold surpassed in the trial orchard at Rietfontein Farm (Table 7.2). Despite this, the relationship between trap catches and fruit infestation (in dropped fruit) recorded three to four weeks later was apparent (Fig. 7.2).

7.3.2 Parasitoid augmentation

Percentage parasitism of eggs was not considered to be the most accurate way of

measuring the potential effectiveness of the parasitoids to reduce *C. leucotreta* infestation. If, for example, 10 eggs were laid on a fruit and nine of them were parasitised, percentage parasitism would be 90 %. This sounds like an extremely effective level of parasitism. However, in effect, a larva could successfully hatch from the one egg that escaped parasitism, and burrow into the fruit, causing abscission. This would render the level of 90 % parasitism meaningless. Therefore, a value termed "percentage effective parasitism" was used. This meant that only when all eggs on a fruit were parasitised, was parasitism considered to be effective. Mean percentage parasitism over a period of time was therefore higher than mean percentage effective parasitism.

Mean effective parasitism in the release and control blocks over the trial period, was 42.40 ± 6.6 % and 37.48 ± 6.6 % respectively (Fig. 7.3).

The ultimate measurement of the effectiveness of parasitoid augmentation is in damage to fruit or infestation of fruit. Generally, infestation of fruit was low throughout the trial. As moth catches never exceeded the threshold of 10 moths per trap (Fig. 7.2), this is not surprising. An average of 0.47 ± 0.11 fruit were infested per tree per week in the non-release control block and the release block averaged 0.36 ± 0.07 fruit per tree per week (Fig. 7.3). (This difference was not statistically significant, according to the Students' T-test at the 95 % probability level). This lower level of fruit infestation in the release block was despite the higher level of eggs in this block. Over the trial period, *C. leucotreta* eggs were recorded on an average of 4.88 % of fruit per week in the release block. In the control block, this was 3.28 % of fruit.

It can therefore be stated that through parasitoid augmentation, a 12.6 % increase in egg parasitism resulted in a 23.4 % decrease in fruit infestation (Fig. 7.3). This is not a dramatic decline in infestation. (Difference in mean number of fruit infested per tree per week for the trial period was not statistically significant, according to the Students' T-test at the 95 % probability level). Reductions in infestation of 60 % (unpublished data; Newton & Odendaal, 1990) and 41 % (Moore *et al.*, 2000a) have previously been recorded.

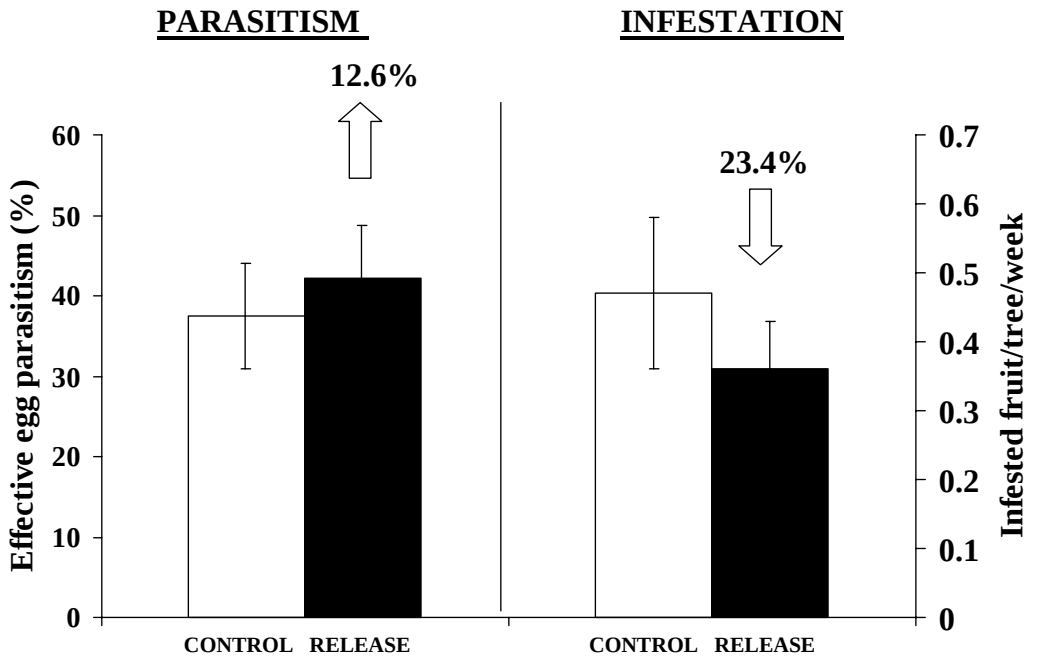

Fig. 7.3 Percentage effective parasitism (± SE) of *C. leucotreta* eggs and infestation of fruit (navel oranges) (± SE) with *C. leucotreta* larvae in a block in which *T. cryptophlebiae* parasitoids were released and in an adjoining non-release control block. Arrows indicate percentage change relative to the control.

7.3.3 Investigation of an egg parasitism threshold

Parasitism of *C. leucotreta* eggs at Rietfontein Farm was generally of a fairly low level, despite parasitoid augmentation. Previous studies have revealed mean levels of parasitism from December to May in non-release orchards of up to 79 % and in release orchards up to 83 % (Moore & Richards, 2000). Whilst this trial was being conducted at Rietfontein Farm, a similar *T. cryptophlebiae* augmentation trial was run concurrently at another farm (Moedskepvlakte Farm) in the same area. Here, mean parasitism from December to May was recorded at 66 % and 53 % in the release and control blocks respectively (unpublished data). There must have been some good reason for the low level of parasitism at Rietfontein Farm. It is possible that the spray regime, even from the previous season, had substantially suppressed natural enemy levels. Nevertheless, although no significant statistical correlation could be established, there appeared to be a relationship (negative correlation) between parasitism and infestation, particularly from mid-February to harvest (Fig. 7.4). In

previous trials in which parasitism reached substantially higher levels, the relationship between parasitism and infestation was eminent. In a trial conducted during the 1999/2000 season, egg parasitism was found to relate to fruit infestation detected five weeks later (Moore & Richards, 2000). If a five week time lag for parasitism was included, then a statistically significant negative relationship was found between parasitism and infestation.

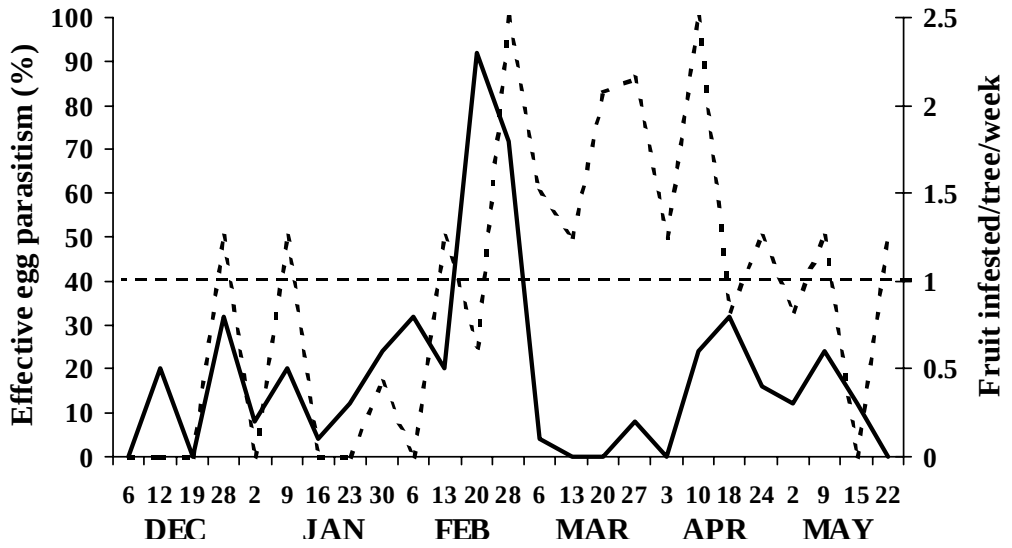

Fig. 7.4 Egg parasitism (- - - -) (Y1 axis) and fruit infestation (——) (Y2 axis) in the control block at Rietfontein Farm, Eastern Cape Province, from 6 December 2000 to 22 May 2001. An arbitrary infestation threshold of one fruit/tree/week is signified (– – –).

Hofmeyr & Hofmeyr (1991) suggested an infestation in one dropped fruit per tree per week for the season (Fig. 7.4) as being the level at which chemical intervention would have been justified. Because of the fluctuation in infestation and parasitism and the relatively low levels of both (particularly during the first portion of the trial) (Fig. 7.4), it is difficult to determine what level of parasitism was required to suppress infestation to below this threshold level. During February, infestation surpassed the threshold level for a period of two weeks. The crash in infestation which followed, seemed to relate to 50 % egg parasitism recorded three weeks previously. During the following eight weeks parasitism averaged 81.75 %, and infestation was maintained at its lowest level throughout the trial.

In previous trials, infestation began to decline at around 50 % parasitism. However, infestation only dropped below the level of one infested fruit per tree per week once parasitism exceeded 80 % (Moore & Richards, 2000). A low level of infestation was maintained by parasitism averaging 82.3 %. A level of 80 % parasitism is therefore recommended as a threshold. If the trap threshold is surpassed then a spray can be applied for *C. leucotreta* control, provided parasitism is consistently below 80 % and there are fewer than 16 weeks before harvest (a maximum of two applications of Alsystin at 10 ml/100 ℓ water are permissible per season; this will protect the fruit against *C. leucotreta* for no more than 16 weeks).

7.3.4 CrleGV-SA application

Infestation of dropped fruit was evaluated weekly from three to six weeks after application. The grower was repeatedly requested not to remove fruit from beneath trial trees. Despite this, before evaluations could be conducted on weeks four and six (after treatment), the grower collected and removed fruit from the trial block, whilst performing orchard sanitation. Fruit infestation results were therefore only available for the third and fifth weeks after application. This data was pooled and reduction in fruit infestation was calculated as

$$100 \times \left(1 - \frac{\text{number of infested fruit in treatment}}{\text{number of infested fruit in control.}}\right)$$

Alsystin reduced infestation by 15.4 %, and the two concentrations of CrleGV-SA by 61.5 % and 46.1 % (Fig. 7.5). The more concentrated virus treatment was the only one which significantly reduced *C. leucotreta* infestation. Similarities were noted in trends between this trial and the one conducted at Sun Orange Farm (section 6.3.2). Firstly, very little reduction in infestation could be detected for Alsystin up to and including five weeks post treatment. Secondly, the higher concentration of CrleGV-SA did not provide a greater reduction in infestation than did the lower concentration, up to and including five weeks after treatment. In fact, in both trials, the inverse (marginally) was true.

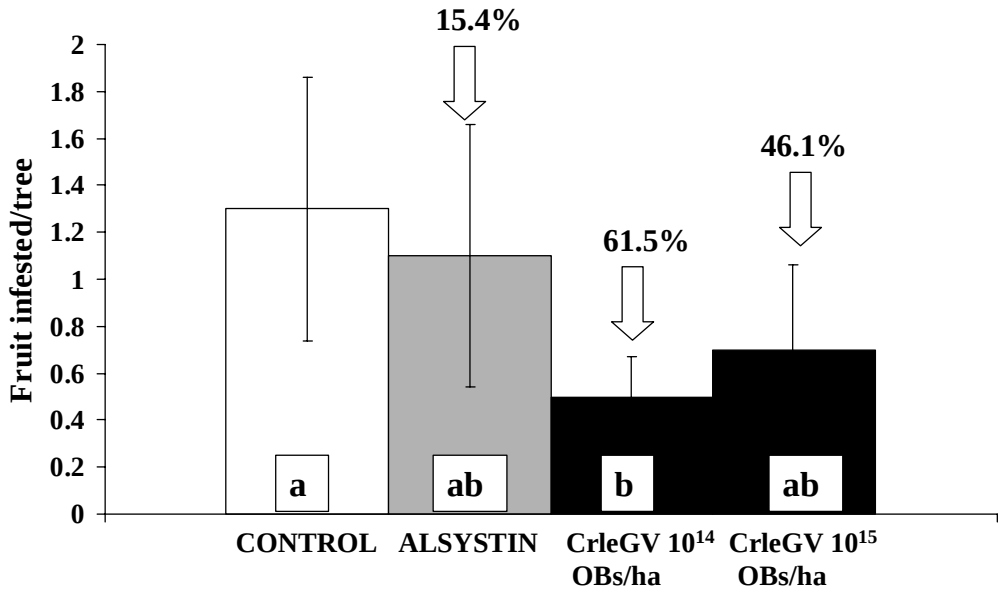

Fig. 7.5 Mean (± SE) infestation in dropped fruit (Palmer navel oranges) per tree for a period of 3 - 5 weeks after treatment, for treatments applied for control of *C. leucotreta* at Rietfontein Farm, Eastern Cape Province, on 11 April 2001. Arrows indicate reduction in infestation relative to untreated trees. Bars with the same letter are not significantly different (P > 0.05; Bonferroni multiple range test).

CrleGV-SA therefore succeeded in reducing *C. leucotreta* infestation more dramatically than could *T. cryptophlebiae* augmentation. However, the two control approaches, although both biological control strategies, are quite different. One would expect more of an immediate chemical type knockdown from CrleGV-SA and more of a gradual suppression from the parasitoids. Together, the two modes of control reduced infestation by 70.5 % (Fig. 7.6). The 23.4 % reduction in infestation caused by parasitoid augmentation (Fig. 7.6) was measured over a 25 week period, whereas the 61.5 % reduction caused by CrleGV-SA was measured over a two week period. Therefore, the total reduction in infestation of 70.5 % was only for a two week period. A CrleGV-SA application is capable of reducing infestation by around 60 % for up to 39 days, such as at Sun Orange Farm (see section 6.3.2). Therefore the 70.5 % reduction in infestation could have persisted for 39 days.

If parasitoid augmentation had reduced infestation by 60 %, as previously recorded (Newton & Odendaal, 1990; unpublished data), then combined with CrleGV-SA (61.5 %), infestation would be reduced by 84.6 %.

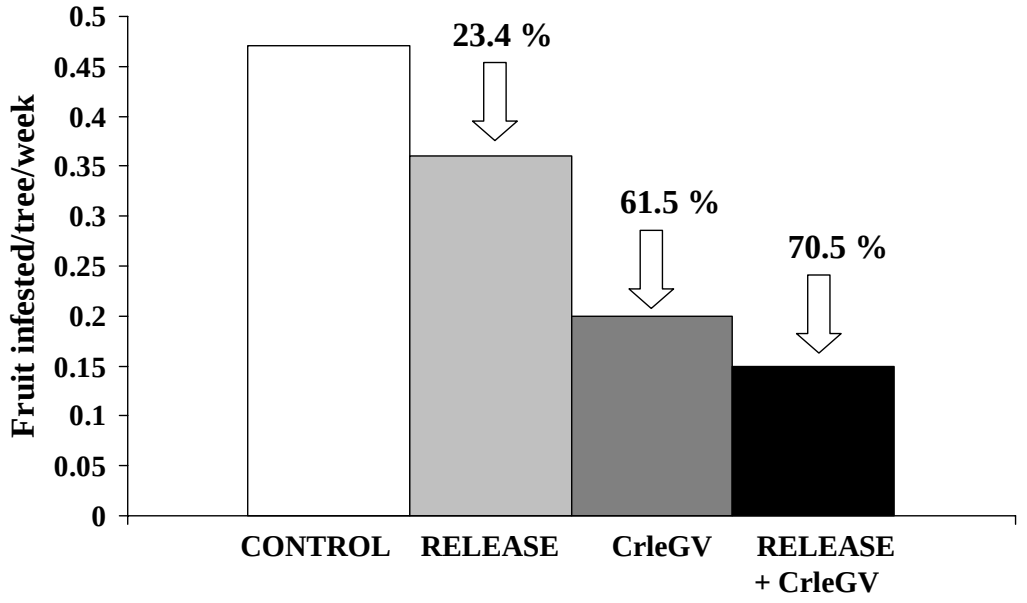

Fig. 7.6 Average *C. leucotreta* infested fruit (Palmer navel oranges) drop per tree per week from untreated trees, from trees in a block where *T. cryptophlebiae* parasitoids were released, from trees sprayed with CrleGV-SA (± 10^{14} OBs/ha) and from trees subjected to both of these treatments. Arrows indicate percentage reduction in infestation relative to untreated trees.

An average of 0.47 infested fruit dropped per tree per week from untreated control trees (Fig. 7.6). A 70.5 % reduction in infestation (from parasitoid augmentation and CrleGV-SA) would be a reduction of 0.33 infested fruit per tree per week. At a density of 555 trees per hectare (the usual number of trees planted per hectare), 183 fruit would be saved per hectare per week. Over 39 days this would be 1453 fruit, valued at R305.19 (Table 6.2). If CrleGV-SA was applied twice and each application of CrleGV-SA reduced infestation by 61.5 % for 39 days, then twice the number of fruit would be spared (valued at more than R600). This does not even take into consideration the additional reduction in infestation from parasitoid releases, before CrleGV-SA sprays would have been applied.

The cost of these treatments should not exceed the returns. However, as explained in section 6.3.2, if CrleGV-SA is applied sufficiently close to harvest so as to be able to reduce the chance of undetectable infestation in harvested fruit and thereby the chance of interception of the pest on the market, the cost of treating is far more easily justified.

7.3.5 Natural impact of CrleGV-SA

No CrleGV-SA was detected in larvae until 13 March 2001 (Fig. 7.7). It is possibly this lack of virus in the *C. leucotreta* population that allowed the highest level of infestation recorded during the entire trial period, on 20 February 2001. This peak in infestation was followed promptly by a CrleGV-SA outbreak detected in 25 % of the larvae collected, which rose to 50 % shortly thereafter (Fig. 7.7). CrleGV-SA incidence then dropped to 10 % of larvae. This related to an increase in infestation, which in turn may have lead to the rise in CrleGV-SA infection once again, which persisted until harvest. This rise in infection related to a decline in level of infestation. It is speculative, but it appears that the natural occurrence of CrleGV-SA may have had some regulatory effect on the *C. leucotreta* population. All larvae in which CrleGV-SA was detected, were alive. The virus detected may therefore have been at a sub-lethal level, and those larvae may have survived to produce another generation. However, it is presumed that the increase in infection detected in these live larvae was indicative of an increase in infection in the population leading to mortality i.e. an epizootic. It would not be possible to confirm this, as the natural behaviour of infected larvae is to exit the fruit, where they would probably disappear due to any number of environmental (e.g. wind or rain) or biological (e.g. predation) factors. Very rarely were virus-killed larvae found within fruit, even under trial conditions.

In a previous trial in which larvae were collected weekly and screened in the same manner (using dot blot assays), a plot of CrleGV-SA infection against *C. leucotreta* infestation indicated a similar relationship between the two parameters (Moore & Richards, 2000; 2001). Larvae from a total of four orchards in Sundays River Valley were screened over a two year period. CrleGV-SA was detected in larvae from three of the four orchards.

A central feature of epizootiological models is a threshold above which density dependent mortality occurs (Brown, 1987; Onstad & Carruthers, 1990). The threshold can change depending on the amount of primary inoculum and initial infection levels. However, the fundamental point is that the opportunity for an

epizootic to occur is greatly enhanced by an increase in host population density. An increase in host density improves the opportunity for horizontal transmission of the virus. Steineke & Jehle (2001) found horizontal transmission of CpGV between *C. pomonella* larvae to be as high as 40 %. Although Eastwell *et al.* (1999) found that 23 % of *C. pomonella* larvae in a wild population were infected with CpGV, they detected no indication of epizootics.

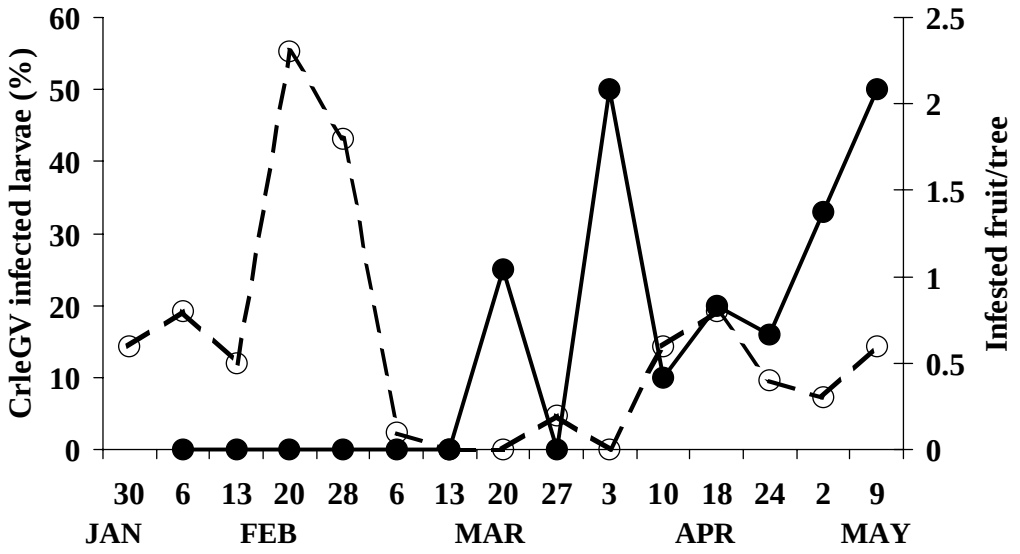

Fig. 7.7 *C. leucotreta* fruit infestation (– – ○– –) (Y2 axis) and CrleGV-SA detection in larvae (—●—) (Y1 axis), in an orchard of navel orange trees at Rietfontein Farm, Eastern Cape Province, from 6 February to 9 May 2001.

Even if CrleGV-SA epizootics do occur and do effectively suppress *C. leucotreta* levels, natural outbreaks cannot be induced. However, it is possible that by spraying CrleGV-SA in an orchard, a reservoir of inoculum can be introduced, which can lead to outbreaks in the future (Hunter-Fujita *et al.*, 1998). Nevertheless, it is encouraging to know that CrleGV-SA does occur naturally in populations of *C. leucotreta* in citrus orchards, and is very possibly another factor contributing towards the integrated control of this pest.

7.3.6 General

7.3.6.1 Orchard sanitation

Until the 1980s the only method recommended for controlling *C. leucotreta* in citrus orchards, was sanitation. This meant that all infested fruit, both on the trees and on the ground, were to be collected regularly and destroyed (Hepburn, 1947). Weekly cleaning of orchards was recommended as a routine practice (Hepburn *et al.*, 1954). In this way a potential population of moths was believed to be effectively reduced. Stofberg (1954) claimed that the effectiveness of such a practice was unknown, until he conducted a series of trials between 1939 and 1953. However, similar trials previously conducted by Ulyett (1939), concluded that weekly orchard sanitation was successful in reducing the larval population in the crop, after the progeny of the first influx of moths had been destroyed. He also showed that orchard sanitation unfortunately had an adverse effect on the level of larval parasitoids. Stofberg (1954) found that a programme of regular sanitation could save between 24 and 60 fruit per tree from *C. leucotreta* infestation.

December was originally recommended as the time of year to begin with such a sanitation programme (Georgala, 1969), but Schwartz (1974) found this to be far too late to achieve the best results. Higher numbers of pupae were recovered from orchards from late October to mid December than at any time of the season thereafter. Strictly speaking, orchard sanitation should commence when fruit are pea sized (Grout *et al.*, 1998). This can help to limit the extent of the *C. leucotreta* generation, that develops from such fruit and becomes the source of moths responsible for high levels of fruit drop which can occur during December and January. Where removal of these small fruit is not feasible, sanitation should commence at the earliest practicable time. Recently, additional incentive to sanitise orchards from early in the season has been created by the cosmetic industry – it uses the oil from the small fruitlets. By selling these fruit to the cosmetic industry, growers are able to cover the labour costs of sanitising.

Once fruit have been removed from the orchard they must be destroyed either by burying under at least 30 cm of soil, sealing in strong plastic bags to rot for about four

weeks, or pulped with a hammermill and spread on the ground to dry (Grout *et al.*, 1998).

7.3.6.2 Larval parasitoids

At least five larval or larval-pupal parasitoids have been recorded from *C. leucotreta* on citrus in South Africa (Table 1.1). Three of these species must be considered as being fairly rare, and therefore not very effective. It is unclear whether two additional species, *Apanteles leucotretae* and *A. typhon*, have been recorded in South Africa (Prinsloo, 1984). There is a need for a concerted survey of the larval parasitoids of *C. leucotreta*. To date, the most commonly reported species of larval parasitoid is *Agathis bishopi* (Plate 7.3) which is particularly common in the Eastern Cape Province (Prinsloo, 1984).

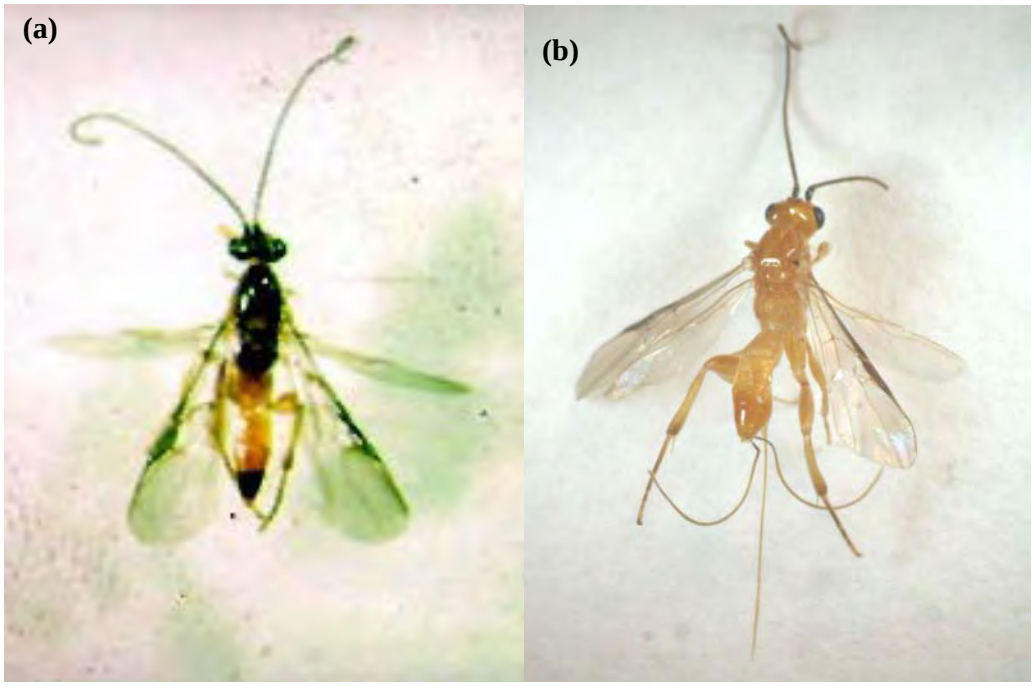

Plate 7.3 *Agathis bishopi* male **(a)** and female **(b)**: larval parasitoids of *C. leucotreta*.

Newton (1998) states that larval parasitoids do not seem to be important mortality factors. However, a survey of larval parasitoids and their seasonal prevalence, which is currently being conducted, revealed that 37 % of *C. leucotreta* larvae collected from the Gamtoos River Valley during December 2001, and 33 % of larvae collected from

the Sundays River Valley (both Eastern Cape Province) at the same time, were parasitised by *A. bishopi* (Noma Sishuba, personal communication). This level of parasitism at a relatively early stage in the season is extremely impressive, and appears to have caused a dramatic crash observed in the *C. leucotreta* population. In one particular orchard this crash in December appeared so dramatic that the normal *C. leucotreta* generational peak in January was absent (unpublished data).

The level of larval parasitism detected in Sundays River Valley in December was so effective that it became extremely difficult to establish a new laboratory culture of *C. leucotreta* collected from this area. However, this level of parasitism does not, however, seem to occur in all production regions of South Africa. Surveys simultaneously conducted at Citrusdal, Western Cape Province, and Nelspruit, Mpumalanga, did not reveal larval parasitism. Translocation of *C. leucotreta* larval parasitoids from one area to another, in which they do not yet occur, has been proposed (Ullyett, 1939: Moore & Richards, 2001) as a classical biological control strategy.

Larval parasitoids may not only be directly effective as natural enemies, but may serve as vectors for the transmission of CrleGV-SA (and other pathogens) from one larva to another (Levin *et al.*, 1983; Young & Yearian, 1990).

7.3.6.3 Chemical control and non-target effects

Currently four products, Alsystin, Nomolt, Penncap-M and Meothrin are registered for the control of *C. leucotreta* on citrus (see section 1.2.5.2 for active ingredients and other details). However, Penncap-M and Meothrin cannot even be considered in an IPM programme. Extensive non-target effect tests were conducted with a range of plant protection products, registered on citrus, against various natural enemy indicator species (Ware *et al.*, 1998; 1999). Products tested were applied to potted citrus seedlings according to strict and well-defined criteria. Seedlings were exposed to environmental conditions but moved under cover when rain was expected. Adult natural enemies were exposed to residues on leaves, within test arenas (modified Petri dishes) at various intervals after application. Penncap-M was the most detrimental product to the important California red scale parasitoid, *Aphytis lingnanensis*, causing

100 % mortality of adults when residues were eight weeks old (which is when the trial was terminated). Meothrin was the most detrimental product (of those that were applied according to registration) to the highly effective citrus mealybug parasitoid, *Coccidoxenoides peregrinus* – two week old residues causing 100 % mortality of adults, and six week old residues causing 44 % mortality.

If it is necessary to apply a chemical spray for *C. leucotreta* control, then Alsystin (being more effective than Nomolt (Hofmeyr & Hofmeyr, 1991)) is the best option within an IPM programme. However, Alsystin may also be detrimental to *T. cryptophlebiae* due to its capacity to render eggs unsuitable for development of the parasitoid (Hattingh & Tate, 1997). Therefore, once Alsystin residues expire (eight to 12 weeks after application, depending on concentration used) parasitoid numbers may be too depleted to maintain the chemically induced suppression of *C. leucotreta*. As the moths are active fliers, they should be able to reinfest an orchard well before the egg parasitoid is able to. It is therefore clear that the use of Alsystin does not facilitate sustainable management of the pest. Its use is also not compatible with a *T. cryptophlebiae* augmentative release programme. Added to this, both insect growth regulators – Alsystin and Nomolt – are extremely harmful to coccinellid (Coleoptera) predators (Greeff *et al.*, 1998), which are important natural enemies of a range of citrus pests.

7.3.6.4 Alternative control strategies

Myburgh (1963a), Schwartz (1979) and Du Toit (1981) investigated the lethal and sterilising effects of cobalt-60 gamma rays on *C. leucotreta*. Myburgh (1963a) discussed the feasibility of pest control by over flooding natural populations with insects rendered sterile by gamma irradiation. He believed that the method did not hold much promise of success due to the expansive areas in which the pest occurs. If *C. leucotreta* became established elsewhere in pockets of infestation, sterile male releases could be of value for eradication or curtailment of such outbreaks. Since Myburgh made this observation, a new establishment of *C. leucotreta* was reported in 1974 in the Citrusdal - Clanwilliam district of the Western Cape Province, although considered to be absent from the area three years earlier (Newton, 1998). Consequently, research towards a sterile male programme (SIT) is currently being

conducted in this area. SIT has often been recommended in combination with other biological control of biorational practices (Carpenter *et al.*, 1996; Carpenter, 2000) as it is believed to be benign to natural enemies.

From 1988 to 1998, 23 orchard trials were conducted with mating disruption of *C. leucotreta* (Hofmeyr & Hofmeyr, 1998). In all trials, except the few in which there was a product problem, good results from a commercial perspective, were obtained. Consequently, certain mating disruption products have been registered for use on citrus against *C. leucotreta*. Such a practice is highly compatible with IPM. However, growers who have subsequently used the method have reported very little success (Ballie Wahl; Isak Bruwer, personal communication). This may, to an extent, be a symptom of poor management of the product, possibly due to a lack of proper education on its use.

Research into attract-and-kill methods for control of *C. leucotreta* were initiated far more recently (Hofmeyr & Hofmeyr, 1999; Gerhard Booysen, personal communication). As yet results are inconclusive and no products have been registered.

7.4 CONCLUSION

Parasitism of *C. leucotreta* eggs was improved by 12.6 % where *T. cryptophlebiae* parasitoids were augmented. This resulted in a 23.4 % reduction in *C. leucotreta* infestation of navel oranges. This was not as impressive as the results achieved in other trials (Newton & Odendaal, 1990; unpublished data), where infestation was reduced by 60 %. CrleGV-SA applied at a rate of around 10^{14} OBs per hectare reduced infestation by a further 58.3 %.

An intervention threshold, which considers the level of parasitism of *C. leucotreta* eggs, is also recommended. If the trap threshold is surpassed then a spray can be applied for *C. leucotreta* control, provided parasitism is consistently below 80 %. Previously, decisions have been based on trap catches alone.

There are a number of other biological and biorational factors, which can work together to effectively suppress *C. leucotreta* to below an economic damage level. Some of these biological factors are naturally occurring, such as egg and larval parasitism and CrleGV-SA infection. Other factors are introduced: apart from augmentation of parasitoids and CrleGV-SA sprays, already mentioned, mating disruption, attract-and-kill systems, and sterile male overflooding (SIT) are biorational options.

Naturally occurring control factors can, to an extent, be enhanced by conservation of an environment conducive to natural enemy proliferation (e.g. selection and encouragement of appropriate inter-row vegetation (Hattingh, 1994)) and by judicious use of chemical insecticides. Chemical intervention should only be used as a last resort, and if considered to be necessary, the safest (to natural enemies), shortest residual product available, should be used.

Intervention, even with biological control agents and biorational systems, should only occur if their use can be specifically justified in a particular situation. Data to determine whether intervention is justifiable should be acquired by monitoring the pest and the level of its natural control. Pheromone trapping and scouting (for eggs and egg parasitism) must be employed. Fruit infestation should also be regularly monitored. This will reduce the chance to inaccurately ascribe pre-harvest fruit drop to *C. leucotreta*, when it may be attributable to some other factor such as *Alternaria citri* navel end rot (Schutte *et al.*, 1994) or citrus mealybug (Hattingh *et al.*, 1998).

Such a system should be sustainable and effective, and in the context of this study, will optimise the benefit that can be enjoyed from CrleGV-SA in the management of *C. leuctoreta*. This study indicates an important position for CrleGV-SA within an IPM system for the management of *C. leucotreta* on citrus.

8

FINAL DISCUSSION AND FUTURE PROSPECTS

8.1 INTRODUCTION

At the start of this study it was stated that the ultimate objective was to develop and evaluate CrleGV-SA as a biological control agent, and to integrate it into a system for the management of *C. leucotreta* on citrus. This entailed a number of steps or objectives: characterisation of the virus isolate; determination of the pathogenicity of the virus; establishment of an efficient, effective and affordable method for mass rearing the host for *in vivo* production of the virus; field testing CrleGV-SA in citrus orchards and examining its integration into a system for control of *C. leucotreta*. All of these objectives have to a large extent been met, although much work is still required, particularly on the more applied aspects of the project, before a commercially usable product can be made available.

8.2 DISCUSSION

8.2.1 Virus characterisation

The virus was satisfactorily characterised to fulfil five purposes (three of them having immediate value). Firstly, it was confirmed that the virus was a CrleGV isolate. This was established by determining commonality with the previously described CrleGV-CV3 (Jehle *et al.*, 1992a). Secondly, it was established that this CrleGV isolate is novel, being distinct from CrleGV-CV3. Thirdly, and most importantly within the context of this work, there is now a reference point for quality control purposes. It is now possible, at any stage, to establish whether work is still being conducted with the originally identified isolate, or whether a contaminant virus had taken over. Fourthly,

it will be possible to track the presence and spread of the virus in field studies. Lastly, if any other strains of CrleGV are isolated in future, and differences in potency or productivity between strains are identified, characterisation will enable distinction between the strains.

DNA profiles of CrleGV from three different sources were compared, using three different restriction enzymes. In one gel, there was an indication that there may be differences between the profiles of the three isolates. However, there also appeared to be some contamination of DNA. This study should be taken further. As previously mentioned, different isolates of the same virus may differ in their potency or productivity. Fritsch (1989) found three different isolates of CrleGV to differ in their infectivity (LC values) against *C. leucotreta* neonate larvae. This could have important implications for production and use of CrleGV on a commercial scale. If it is confirmed that there is more than one South African isolate of CrleGV, it will be necessary to conduct surface dose bioassays with the different isolates against neonate larvae. However, one problem is that a non-apparent CrleGV-SA infection exists in the laboratory culture of *C. leucotreta* used in this study. If a different isolate of CrleGV is ingested by one of these larvae (in a bioassay), it may act as a stressor, compromising the natural resistance of the larva and allowing the homologous isolate to manifest. The cause of mortality may incorrectly be ascribed to the introduced virus. In order to avoid such a problem, a virus-free *C. leucotreta* culture should be used for bioassays with heterologous isolates. Attempts to rid the culture of virus have thus far been unsuccessful (Michael Ludewig, personal communication). New hope has been placed in the discovery of an apparently virus-free field population of *C. leucotreta* (Singh, 2002). Large numbers of *C. leucotreta* larvae have been collected from this site in order to establish a virus-free culture.

8.2.2 Genetic modification of a granulovirus

Another potential advantage of characterising CrleGV-SA is that this is the first step towards sequencing of the CrleGV-SA genome. Sequencing of important sections of the genome will indicate the position of genes which are suitable for targeting for genetic modification of the virus. This can be done through insertion of insect toxin genes such as those obtained from the mite, *Pyemotes tritici* (Tomalski & Miller,

1991; 1992). Recombinant strains of AcMNPV expressing these toxins have been shown to kill significantly faster than the wild-type strain.

Another form of baculovirus genetic modification is the deletion of a gene. By deleting the *egt* (ecdysteroid-UDP glucosyl transferase) gene from CpGV, the recombinant strain (CpGV-*egt*$^{-}$) killed fifth instar *C. pomonella* larvae more quickly and affected the feeding behaviour of neonate larvae (Cross *et al.*, 1999; Winstanley *et al.*, 1998; 2000). (This is probably the first genetic modification of a GV). However, more interestingly and more dramatically, the median lethal time of CpGV-*egt*$^{-}$ against neonate *C. leucotreta* larvae was estimated to be 40 % shorter than the median lethal time with the homologous virus (CrleGV-CV3) (Keane & Winstanley, 2001). *C. leucotreta* neonate larvae were about 1000 times less susceptible to the wild-type CpGV than to CrleGV. This discovery of the pathogenicity of CpGV-*egt*$^{-}$ to *C. leucotreta* has exciting potential for control purposes. However, an impediment is the need to produce a recombinant virus in cell culture. *In vitro* production is not only expensive, but it is not possible to produce commercially viable quantities of virus. A permissive cell culture has been developed for CpGV (Winstanley & Crook, 1993), which can also be used for producing CpGV-*egt*$^{-}$. Small quantities of CpGV-*egt*$^{-}$ produced *in vitro* can be used to surface inoculate artifical diet. The recombinant virus can then be bulked up in fifth instar *C. leucotreta* larvae placed onto the diet.

Even if it is possible to develop, produce and use a genetically improved baculovirus for control of *C. leucotreta* on a commercial scale, the use of the wild-type virus will not be precluded. The same factor which is considered to be the main advantage of a genetically modified baculovirus i.e. improved speed of kill, actually limits its utility. Because of the greater speed of kill, fewer OBs are produced compared with the wild-type virus and their propensity to recycle in the environment is reduced (Bonning & Hammock, 1996). A genetically modified baculovirus would therefore be unlikely to persist in the environment at the same level or for as long as a wild-type virus (Richards *et al.*, 1998). An integrated use of both wild-type and recombinant virus may therefore be judicious.

8.2.3 Virus pathogenicity

Through bioassays the infectivity (dose-response relationship) and virulence (time-response relationship) of CrleGV-SA against both fifth instar and neonate larvae of *C. leucotreta* was determined. Not only was it confirmed that CrleGV-SA is sufficiently potent to warrant further investigation and development as a microbial control agent of *C. leucotreta*, but a norm for pathogenicity was established. In future, all batches of virus that are produced should be assayed alongside a sample of the virus for which lethal concentration and lethal time values have been established. Significant aberrations from the norm will indicate problems before a virus is tested in the field. Field concentrations can also be altered to compensate for any change (increase or decrease) in dose-response values.

The bioassay methodology established can also be used to test different formulations of CrleGV-SA. The effect of any formulation additive or tank additive which is to be tested in the field should first be assessed in bioassays in the laboratory. These could be surface dose assays or detached fruit assays, depending on which methodology is considered to be most appropriate. Surface dose assays are likely to be more sensitive to small influences but would be less reflective of a field situation than would detached fruit assays.

8.2.4 Virus production technique

The host production techniques that were developed for *C. leucotreta* are highly effective. Certain minor changes may be implemented, such as the use of pie dishes for rearing larvae, rather than honey jars. The aim would be twofold: to produce higher numbers of larvae per container and to be able to inoculate diet with virus without having to transfer the larvae. To achieve the second objective it may be necessary to somehow encourage larvae to move to the surface of the inoculated diet to improve their chance of ingesting OBs. This might be achieved through heating the base of the pie dish in a water bath, such as was done by Huber (1986a) when producing CpGV in *C. pomonella* larvae.

Production of virus, although already quite satisfactory, might be further improved by enlarging the fifth instar larvae in which the virus is produced, through treating the diet with a juvenile hormone mimic (Glen & Payne, 1984; Shieh, 1989). Impact of factors such as temperature, humidity and host density on virus production and pathogenicity can also be examined.

Microbial contamination in semi-purified CrleGV-SA preparations was generally low (section 5.3.5). However, it will be important to identify the species of contaminants so as to confirm that no unacceptable mammalian pathogens are present. This will also be necessary in order to register a CrleGV-SA product.

8.2.5 Field trials

Field trials on citrus indicated satisfactory persistence and efficacy with CrleGV-SA. However, more work is probably required on this applied aspect of the project than on any other aspect, before commercialisation can become a reality.

Firstly, a range of different concentrations of CrleGV-SA, from about 10^{12} - 10^{16} OBs per hectare, should be compared in the field.

Secondly, a range of different volumes of CrleGV-SA should be applied and compared. Citrus growers are accustomed to applying extremely high volume sprays, especially for the control of *C. leucotreta*. Well over 15000 ℓ per hectare is the norm for an application of Alsystin for *C. leucotreta* control (Grout *et al.*, 1998). Lower volumes per hectare have been shown to be less effective, particularly when applied with an automatic mist-blower rather than with hand held spray guns (Hofmeyr & Hofmeyr, 1991). The main reason for such thorough application is that Alsystin is exclusively an ovicide. If an egg is laid on a fruit, or part of a fruit, that has not been covered with Alsystin, then it will be unaffected, as will be the emerging larva. However, as CrleGV-SA is a larvicide it may not be necessary to apply it as thoroughly. There may therefore be some latitude for less than complete spray coverage, as a larva which may hatch from an egg which is not covered by virus, may still encounter and ingest OBs before or when it begins to burrow into the fruit.

Thirdly, both the persistence and efficacy of different preparations of CrleGV-SA should be compared. Different surfactants (spreaders, wetters and emulsifiers) and stickers should be tested in order to determine whether it is necessary and possible to improve the spreading and rainfastness of the virus. UV protectants should be tested, starting with simple tank additives such as milk powder, charcoal, Brewer's yeast and molasses. The last two could also serve as baits. Possibly the most important comparison would be that between a virus preparation produced from individually harvested larvae and a preparation derived from larvae with their diet (sections 5.3.2 - 5.3.4). It is possible that the small diet particles could provide substantial UV protection and therefore greater field persistence.

In future, field trials should be initiated far earlier in the citrus growing season than previously. Traditionally, sprays on navel orange tree varieties aimed at *C. leucotreta*, have not been applied before the middle of February, i.e. not more than 16 weeks before harvest. Local registration does not allow more than two applications of Alsystin per orchard per season (at 10 ml/100 ℓ water each). This will protect fruit against *C. leucotreta* for a maximum of 16 weeks. Therefore, an astute grower would not want to apply his first spray of Alsystin any longer than 16 weeks before harvest. However, it has been shown consistently that the highest level of *C. leucotreta* activity (both moth numbers and fruit infestation) in the Eastern Cape Province occurs between November and January (Moore & Fourie, 1999b; Moore & Richards, 2000; 2001; Moore *et al.*, 2000a; unpublished data). An application of CrleGV-SA against this peak may not only significantly reduce fruit loss during this critical period, but might sufficiently suppress the *C. leucotreta* population to prevent another build up in numbers during the season. To achieve such a dramatic effect, an area-wide application (possibly by air) may be necessary. An early application will also enable determination of the duration of residual efficacy of CrleGV-SA. In the trial conducted at Sun Orange Farm (section 6.3.2) CrleGV-SA seemed to substantially reduce *C. leucotreta* infestation for no longer than 39 days. However, it is possible that the effect of CrleGV-SA could continue at a low level for much longer. A low level of suppression over an extended period may have the same benefit, or even greater benefit, than a more dramatic suppression over a shorter period (such as with a chemical insecticide).

It must be remembered that citrus is not the only crop on which *C. leucotreta* is a pest. CrleGV-SA could be tested against *C. leucotreta* on macadamias, peaches, maize and cotton. It is even possible that CrleGV-SA could be effective against other *Cryptophlebia* species, such as *C. batrachopa* on macadamias (La Croix & Thindwa, 1986) and *C. peltastica* on litchis (Newton & Crause, 1990), both in southern Africa; *C. ombrodelta* on macadamias in Asia and Australasia (CIBC, 1976; Ironside, 1978); and *C. illepida* on Koa seed in Hawaii (Stein, 1983). However, legislation may preclude the use of CrleGV-SA in countries where it and its homologous host do not naturally occur.

8.2.6 Shelf formulation of a virus

Commercial practice for pesticides requires a shelf stability of at least 18 months, a period which allows adequate time for product distribution, marketing and reasonable retention by the end-user prior to actual application (Hunter-Fujita *et al.*, 1998). With baculoviruses this can be achieved either by retaining the active ingredient frozen until the time of use or by developing a formulation that confers stability under a reasonable range of shelf storage conditions. The first of these two options is of much less commercial convenience. The second option will invariably be a dry preparation, usually achieved through air-drying, freeze drying or spray drying. Formulations will be designed to retain biological activity of the active ingredient, to prevent the in-storage replication of any contaminant microorganisms and to ease handling of the product. Research on different shelf formulations of CrleGV-SA must still be conducted.

8.2.7 Commercial production of CrleGV-SA

The difficulty and expense of producing a shelf-stable formulation can be circumvented by adopting an entirely different approach to that proposed by most manuals. The citrus industry in South Africa is generally localised into tight knit production regions, often geographically distinguishable by being situated within valleys. Insectaries for the production of various natural enemies of key citrus pests have been established in at least five of these regions. Natural enemies are sold for augmentative mass releases to improve biological control. The bulk of each

insectary's production is sold to the growers within that region. A similar means of producing and selling a baculovirus product could be established. Growers could order, collect and spray the product within a day or so. Proper education of growers will guard against misuse of the product.

This regionalised and localised approach to production will have further advantages in labour creation for local communities, albeit not for large numbers of people; image enhancement for the production region, which can be used as a marketing tool; and close range education and influence of growers to adopt an IPM approach. A production plant can be more efficient and more cost effective, by being used for producing both CrleGV-SA and *T. cryptophlebiae* parasitoids. Ultimately, a single production plant might also produce sterile moths, for SIT, and larval parasitoids. Such multi-faceted production would facilitate and promote the IPM approach proposed in this study.

8.2.8 Non-target effect tests

One of the most commonly quoted advantages of baculoviruses as control agents is their host specificity and consequently their safety to non-target beneficial insects, which is vital to the success of IPM (e.g. Deacon, 1983; Cunningham, 1988; Huber, 1990). However, it cannot simply be assumed that CrleGV-SA will be innocuous to all beneficial insects occurring on citrus in southern Africa. A series of non-target tests should be conducted against a range of natural enemy indicator species. Not only have the protocols for such tests been well established, but the system has been adopted by the local agrochemical industry and regulatory body as a standard component for the registration of all new products for use on citrus in South Africa (Hattingh *et al.,* 2000). These tests are therefore mandatory for registration. Results of tests are sure to serve to promote a positive view of a CrleGV-SA product in comparison with the chemical alternatives.

8.3 CONCLUSION

Through this study it is believed that a great advance has been made towards the development of a commercial CrleGV-SA product for *C. leucotreta* control. Such a product is likely to find a fairly broad niche in the market. Growers are becoming more aware of the detrimental effects of chemical insecticides and the benefits of biological and biorational pest control. Simultaneously, pressure from lucrative export markets to reduce the use of chemical pesticides is mounting. A drawback experienced with microbial control products, particularly throughout the developed world, is their expense. This is unlikely to be a problem in this case, when one considers the extreme expense of the product which is used most frequently against *C. leucotreta* on citrus (i.e. Alsystin) in South Africa. One application of Alsystin will cost more than R2000 (± US$172 at the current exchange rate) per hectare.

However, in order for further progress to be made with the development of CrleGV-SA, substantial financial resources will be required. This is needed to complete the necessary trial work, erect and equip a production plant, assemble a mammalian toxicological package and apply for local registration.

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Appendix 1. Chemical reagents used in virus identification, virus purification, DNA extraction and DNA agarose gels of CrleGV-SA.

Light microscopy

Buffalo Black 12B (100 ml)

Buffalo Black	1.5 g
Glacial acetic acid	40 ml
Distilled water	60 ml

Electron microscopy

2.5 % Glutaraldeyde

8 % Glutaraldehyde	10 ml
0.1 M Phosphate buffer	16 ml
Double distilled water	6 ml
Calcium chloride ($CaCl_2$)	32 mg

4 % Osmium tetroxide (OsO_4) stock

OsO_4	1 g
Double distilled water	25 ml

Wrap in foil – dissolve at room temperature (usually 24 h)

Store in refrigerator

1 % Osmium tetroxide

4 % OsO_4	1 ml
0.3 M Sucrose	1 ml
0.1 M Phosphate buffer	2 ml

Wrap in foil during fixation

Reynolds lead citrate

Lead nitrate ($Pb(NO_3)_2$)	1.33 g
Sodium citrate ($Na_3(C_6H_5O_4)\cdot 2H_2O$	1.76 g
1 N NaOH (1 g/25 ml)	8 ml

Freshly boiled and cooled distilled water

Dissolve lead nitrate completely in 30 ml distilled water.

Add sodium citrate. (A heavy white precipitate will form).

Add NaOH and dilute to 50 ml with boiled then cooled water.

Mix until precipitate is dissolved.

pH should be 12

Store in refrigerator

Virus purification

1 M Tris-HCl (pH 7.6) buffer (1 ℓ)

Tris base	121.1 g

Distilled water

Dissolve tris base in 800 ml distilled water.

Adjust pH to 7.6 with HCl.

Make up to 1 ℓ and autoclave.

0.1 % SDS (100 ml)

SDS	0.1 g
Distilled water	100 ml

80 % glycerol (50 ml)

0.1 % SDS	10 ml
Glycerol	40 ml

0.5 M EDTA (100 ml)

EDTA	16.35 g
Distilled water	

Proteinase K (20 mg/ml) (1 ml)

Proteinase K	0.002 g
distilled water	1 ml
Store at -20ºC	

DNA extraction

0.05 M sodium carbonate (Na_2CO_3) (1 ℓ)

Sodium carbonate	5.5 g
Distilled water	1000 ml
Sterilise by autoclaving	

10 mM TE (pH 8.0) (1 ℓ)

Tris base	121.1 g
Distilled water	1000 ml
Adjust pH to 8.0 with concentrated HCl (before adding EDTA)	
0.5 M EDTA (pH 8.0)	1 ml
Sterilise by autoclaving	

Tris-saturated phenol

Melt 100 g of phenol at 68ºC.
Add an equal volume of 0.5 M Tris-HCl (pH 8) at room temperature.
Stir on magnetic stirrer for 15 minutes.
When the two phases have separated, aspirate the upper aqueous phase.
Add an equal volume of 0.1 M Tris-HCl (pH 8) to the phenol and stir.
Extract the upper aqueous phase.

Repeat the extraction procedure until the pH of the phenol is greater than 7.8.
Add 0.1 X volume of 0.1 M Tris-HCl (pH 8) containing 200 μl of β-mercaptoethanol.
Store the phenol at 4ºC in dark bottle.
The phenol should be made in a fume hood and pH checked using pH paper.

Cholorform:isoamyl alcohol 24:1 (100 ml)

Chloroform	96 ml
Isoamyl alcohol	4 ml

3 M Sodium acetate (1 ℓ)

Sodium acetate	408.1 g
Distilled water	800 ml

Adjust the pH to 7.0 with dilute acetic acid.
Make up to 1 ℓ with distilled water and sterilise by autoclaving.

95 % ethanol (alcohol) (100 ml)

Pure ethanol	95 ml
Distilled water	5 ml

Store at -20ºC

70 % ethanol (alcohol) (100 ml)

Pure ethanol	70 ml
Distilled water	30 ml

Store at -20ºC

DNA agarose gels

Loading buffer (6 X)

Xylene cyanol	0.25 g
Glycerol	30 ml

Make up to 100 ml with distilled water

10 X TBE buffer (1 ℓ)

Tris base	107.8 g
Boric acid	55 g
Di-sodium EDTA	7.44 g

Make up to 800 ml with distilled water and alter pH to 8.3 with boric acid.

Make up to 1 ℓ and autoclave.

0.1 % agarose in TBE gel

Agarose powder	0.7 g
1 X TBE	70 ml

Dissolve in microwave until fluid begins to boil.

Pour into a gel tray while hot.

10 X TAE buffer (1 ℓ)

Tris base	48.4 g
EDTA (0.5 M)	20 ml
Glacial acetic acid	11.42 ml

Make up to 1 ℓ with water and autoclave.

0.7 % agarose in TAE gel

Agarose powder	0.84 g
1 X TAE	120 ml

Dissolve in microwave until fluid begins to boil.

Pour into a gel tray while hot.

λ-DNA/*Hind*III marker

Digest 260 µl λ DNA (0.25 µg/µl) with 24 µl of 10 X buffer B and 10 µl of *Hind*III enzyme for 6 hours at 37ºC.

Check that the digest is successful by running 8 µl on a 1 % TAE gel.

To 200 µl of the digest add 550 µl TE buffer (10 mM, pH 8) and 150 µl loading buffer (6 X).

Dispense into microtubes (300 µl volumes).

λ-DNA/*Bste*II marker

Digest 260 µl λ DNA (0.25 µg/µl) with 24 µl of 10 X buffer D and 10 µl of *Bst*E II enzyme for 3 hours at 60ºC.

Check that the digest is successful by running 8 µl on a 1 % TAE gel.

To 200 µl of the digest add 550 µl TE buffer (10 mM, pH 8) and 150 µl loading buffer (6 X).

Dispense into microtubes (300 µl volumes).

λ-Mix 19 marker

Vortex gently just prior to use.

Prepare DNA Marker, 19, before loading as following:

DNA Marker, 19	1µl (0.5µg)
6X Loading buffer	1µl
Deionized water	4µl

Most of the fragments have 12 nt *cos* site of bacteriophage λ, therefore they may anneal and form the additional bands. These fragments can be separated by heating at 65°C for 5 min and then cooling on ice for 3 min.

Appendix 2 PROBAN (Van Ark, 1995) output of probit analysis of dose-response data (LC) with CrleGV-SA against neonate *C. leucotreta* larvae in surface dose and detached fruit bioassays.

```
*******************************************************************************
Dose-response surface dose (see section 3.3.1.2)
Rep1:
 *******************************************************************************

          GENERAL INPUT INFORMATION
          =========================

          DOSES TRANSFORMED TO LOG10

          NATURAL MORTALITY = 16%
          NON-RESPONSE      =  0%
```

DOSE	TRANSFORMED DOSE(X)	NUMBER EXPOSED	NUMBER RESPON.	%RESPONSE	CORRECTED %RESPONSE	EMPIRICAL PROBITS
76300.00000000	4.8825	50	46	92.00	90.48	6.309
15260.00000000	4.1836	50	36	72.00	66.67	5.431
3052.00000000	3.4846	50	22	44.00	33.33	4.569
610.40000000	2.7856	50	16	32.00	19.05	4.124
122.10000000	2.0867	50	18	36.00	23.81	4.288

```
          TEST FOR FIT OF LINE
          ====================

          CHI-SQUARED FOR DEVIATIONS =   6.919     D. F. =  3
          APPROXIMATE PROBABILITY    =    .0730    TEST LEVEL = 0,05
          TABLED CHI-SQUARED VALUE   =   7.815

          DEVIATIONS FROM LINE ARE HOMOGENEOUS


          FITTED PROBIT LINE
          ==================

          REGRESSION EQUATION IS Y = a + bX
          WHERE Y IS EMPIRICAL PROBITS AND X IS DOSE

          INTERCEPT (a)                  =     1.9306
          REGRESSION COEFFICIENT (b)     =      .8409
          STANDARD ERROR OF b            =      .1318
          MEAN FOR EMPIRICAL PROBITS (Y) =     5.0872
          MEAN FOR DOSE (X)              =     3.7537

            DOSE (X)         EXPECTED
                          EMP.PROBIT (Y)
            4.88252           6.036
            4.18355           5.449
            3.48458           4.861
            2.78561           4.273
            2.08672           3.685


          EXPECTED QUANTITIES FOR LINE
          ============================
```

G FOR FIDUCIAL LIMITS = .0943

EXPECTED % RESPONSE	DOSE	STANDARD ERROR	FIDUCIAL LIMITS UPPER	LOWER
50.0	3.650	.1315	3.910	3.368
90.0	5.174	.2580	5.874	4.770
95.0	5.606	.3183	6.482	5.116
99.0	6.416	.4372	7.636	5.752
99.9	7.325	.5746	8.937	6.456

QUANTITIES TRANSFORMED BACK TO ORIGINAL

EXPECTED % RESPONSE	DOSE	STANDARD ERROR	FIDUCIAL LIMITS UPPER	LOWER
50.0	.4466923E+04	.1350663E+04	.8131779E+04	.2334720E+04
90.0	.1492843E+06	.8858384E+05	.7473814E+06	.5893844E+05
95.0	.4036733E+06	.2954791E+06	.3035680E+07	.1305334E+06
99.0	.2608028E+07	.2622410E+07	.4323426E+08	.5643218E+06
99.9	.2112027E+08	.2791308E+08	.8654454E+09	.2858525E+07

```
 ********************************************************************************
Dose-response surface dose (see section 3.3.1.2)
Rep2:
 ********************************************************************************

          GENERAL INPUT INFORMATION
          =========================

          DOSES TRANSFORMED TO LOG10

          NATURAL MORTALITY =  8%
          NON-RESPONSE      =  0%
```

DOSE	TRANSFORMED DOSE(X)	NUMBER EXPOSED	NUMBER RESPON.	%RESPONSE	CORRECTED %RESPONSE	EMPIRICAL PROBITS
76300.00000000	4.8825	50	46	92.00	91.30	6.359
15260.00000000	4.1836	50	36	72.00	69.57	5.512
3052.00000000	3.4846	50	16	32.00	26.09	4.359
610.40000000	2.7856	50	20	40.00	34.78	4.609
122.10000000	2.0867	50	12	24.00	17.39	4.061

```
          TEST FOR FIT OF LINE
          ====================

          CHI-SQUARED FOR DEVIATIONS =  11.045     D. F. =  3
          APPROXIMATE PROBABILITY    =    .0120    TEST LEVEL = 0,05
          TABLED CHI-SQUARED VALUE   =   7.815

          DEVIATIONS FROM LINE ARE HETEROGENEOUS
          COMBINATION OF DOSES WILL BE ATTEMPTED
```

DOSE(X)	OBSERVED MORTALITY	EXPECTED MORTALITY	CHI-SQUARED	CHI-SQUARED COMBINED
4.8825	46	42.42	1.620	1.620
4.1836	36	34.19	.033	.033
3.4846	16	23.53	8.836	8.836
2.7856	20	13.30	1.715	2.972
2.0867	12	5.99	1.395	
			13.599	13.462

```
          COMBINED CHI-SQUARED     =  13.462     D. F. =  2
          APPROXIMATE PROBABILITY  =    .0010    TEST LEVEL = 0,05
          TABLED CHI-SQUARED VALUE =   5.991

          DEVIATIONS FROM LINE ARE NOW CONSIDERED HOMOGENEOUS

          FITTED PROBIT LINE
          ==================

          REGRESSION EQUATION IS Y = a + bX
          WHERE Y IS EMPIRICAL PROBITS AND X IS DOSE

          INTERCEPT (a)                 =     2.1763
```

REGRESSION COEFFICIENT (b) = .7892
STANDARD ERROR OF b = .1119
MEAN FOR EMPIRICAL PROBITS (Y) = 5.0436
MEAN FOR DOSE (X) = 3.6330

DOSE (X)	EXPECTED EMP.PROBIT (Y)
4.88252	6.030
4.18355	5.478
3.48458	4.926
2.78561	4.375
2.08672	3.823

EXPECTED QUANTITIES FOR LINE
==========

G FOR FIDUCIAL LIMITS = .0773

EXPECTED % RESPONSE	DOSE	STANDARD ERROR	FIDUCIAL LIMITS UPPER	LOWER
50.0	3.578	.1245	3.827	3.319
90.0	5.202	.2549	5.869	4.797
95.0	5.662	.3135	6.494	5.170
99.0	6.525	.4287	7.675	5.860
99.9	7.493	.5615	9.007	6.626

QUANTITIES TRANSFORMED BACK TO ORIGINAL
==========

EXPECTED % RESPONSE	DOSE	STANDARD ERROR	FIDUCIAL LIMITS UPPER	LOWER
50.0	.3782877E+04	.1083052E+04	.6717915E+04	.2085262E+04
90.0	.1591194E+06	.9326909E+05	.7404559E+06	.6262974E+05
95.0	.4592577E+06	.3311329E+06	.3118095E+07	.1479740E+06
99.0	.3353136E+07	.3306128E+07	.4736073E+08	.7246128E+06
99.9	.3114444E+08	.4022207E+08	.1016892E+10	.4229448E+07

```
 ********************************************************************************
Dose-response surface dose (see section 3.3.1.2)
Rep3:
 ********************************************************************************

          GENERAL INPUT INFORMATION
          =========================

          DOSES TRANSFORMED TO LOG10

          NATURAL MORTALITY = 12%
          NON-RESPONSE      =  0%
```

DOSE	TRANSFORMED DOSE(X)	NUMBER EXPOSED	NUMBER RESPON.	%RESPONSE	CORRECTED %RESPONSE	EMPIRICAL PROBITS
76300.00000000	4.8825	50	46	92.00	90.91	6.335
15260.00000000	4.1836	50	42	84.00	81.82	5.909
3052.00000000	3.4846	50	26	52.00	45.45	4.886
610.40000000	2.7856	50	10	20.00	9.09	3.665
122.10000000	2.0867	50	10	20.00	9.09	3.665

```
          TEST FOR FIT OF LINE
          ====================

          CHI-SQUARED FOR DEVIATIONS =   3.574     D. F. =  3
          APPROXIMATE PROBABILITY    =    .3110    TEST LEVEL = 0,05
          TABLED CHI-SQUARED VALUE   =   7.815

          DEVIATIONS FROM LINE ARE HOMOGENEOUS


          FITTED PROBIT LINE
          ==================

          REGRESSION EQUATION IS Y = a + bX
          WHERE Y IS EMPIRICAL PROBITS AND X IS DOSE

          INTERCEPT (a)                  =      .6731
          REGRESSION COEFFICIENT (b)     =     1.2000
          STANDARD ERROR OF b            =      .1667
          MEAN FOR EMPIRICAL PROBITS (Y) =     5.1953
          MEAN FOR DOSE (X)              =     3.7685
```

DOSE (X)	EXPECTED EMP.PROBIT (Y)
4.88252	6.532
4.18355	5.693
3.48458	4.855
2.78561	4.016
2.08672	3.177

```
          EXPECTED QUANTITIES FOR LINE
          ============================

          G FOR FIDUCIAL LIMITS =  .0741
```

EXPECTED % RESPONSE	DOSE	STANDARD ERROR	FIDUCIAL LIMITS UPPER	LOWER
50.0	3.606	.1009	3.799	3.387
90.0	4.674	.1596	5.079	4.413
95.0	4.976	.1945	5.481	4.665
99.0	5.544	.2655	6.246	5.127
99.9	6.181	.3492	7.111	5.637

QUANTITIES TRANSFORMED BACK TO ORIGINAL

EXPECTED % RESPONSE	DOSE	STANDARD ERROR	FIDUCIAL LIMITS UPPER	LOWER
50.0	.4033902E+04	.9363273E+03	.6290371E+04	.2436234E+04
90.0	.4717581E+05	.1732098E+05	.1200383E+06	.2588452E+05
95.0	.9472566E+05	.4237301E+05	.3026581E+06	.4627771E+05
99.0	.3501851E+06	.2138746E+06	.1760772E+07	.1340252E+06
99.9	.1516623E+07	.1218131E+07	.1291356E+08	.4334253E+06

```
                          **************************
                          *   COMPARISON OF LINES   *
                          **************************


     ABRIDGED ANALYSIS OF VARIANCE
     =============================
                                                 DEVIATIONS FROM REGRESSION
                  INTERCEPT   REGR.COEF.  D.F.      SUM OF       RESIDUAL
                     (a)         (b)                SQUARES      VARIANCES
LINE: Rep1          1.931        .841      3   .691860E+01         2.306
LINE: Rep2          2.176        .789      3   .110450E+02         3.682
LINE: Rep3           .673       1.200      3   .357412E+01         1.191
-------------------------------------------------------------------------
                                            9   .215378E+02         2.393
-------------------------------------------------------------------------
    COMBINED                     .892      11   .259457E+02         2.359
-------------------------------------------------------------------------
           DIFFERENCE BETWEEN SLOPES        2   .440796E+01         2.204
-------------------------------------------------------------------------
    TOTAL           1.794        .891      13   .263172E+02         2.024
-------------------------------------------------------------------------
       DIFFERENCE BETWEEN ADJ. MEANS        2   .371466E+00          .186
-------------------------------------------------------------------------


     BARTLETTS TEST FOR HOMOGENEITY OF RESIDUAL VARIANCES
     ====================================================

     CHI-SQUARED VALUE        =    .482     D. F. =  2
     APPROXIMATE PROBABILITY  =    .7880    TEST LEVEL = .0100
     TABLED CHI-SQUARED VALUE =   9.210

     RESIDUAL VARIANCES ARE HOMOGENEOUS - SLOPES OF LINES MAY BE COMPARED


     COMPARISON OF SLOPES (PARALLELISM)
     ==================================

      SOURCE       D.F.    SUM OF SQ.    MEAN SQ.      F-VALUE
     ------------------------------------------------------------
     PARALLELISM     2    .440796E+01       2.204          .92
     HETEROGENEITY   9    .215378E+02       2.393
     --------------------------------
      TOTAL         11      .2595E+02

     CHI-SQUARED TEST FOR PARALLELISM
     --------------------------------
     CHI-SQUARED VALUE        =   4.408     D. F. =  2
     APPROXIMATE PROBABILITY  =    .1080    TEST LEVEL = 0,05
     TABLED CHI-SQUARED VALUE =   5.991


     LINES ARE PARALLEL AND ELEVATIONS MAY BE COMPARED


     REGRESSION COEFFICIENTS
     -----------------------

      Rep2            .7892
```

Rep1	.8409
Rep3	1.2000

COMPARISON OF ELEVATIONS (ADJUSTED MEANS)

F-VALUE FOR ELEVATIONS = .079 D.F. = 2 & 11
APPROXIMATE PROBABILITY = .9250 TEST LEVEL = 0,05
TABLED F-VALUE = 3.98

ELEVATIONS ARE NOT SIGNIFICANTLY DIFFERENT

MEANS OF EMPIRICAL PROBITS

Rep2	5.0436
Rep1	5.0872
Rep3	5.1953

INTERCEPTS OF LINES

Rep3	.673
Rep1	1.931
Rep2	2.176

****
Detached fruit infested (eggs on fruit) (see section 3.3.2)
****

GENERAL INPUT INFORMATION
==========

DOSES TAKEN AS READ IN

NATURAL MORTALITY = 0%
NON-RESPONSE = 0%

DOSE	TRANSFORMED DOSE(X)	NUMBER EXPOSED	NUMBER RESPON.	%RESPONSE	CORRECTED %RESPONSE	EMPIRICAL PROBITS
**********		26	8	30.77	30.77	4.498
**********		26	7	26.92	26.92	4.385
**********		26	6	23.08	23.08	4.264
432000.00000000	432000.0000	26	6	23.08	23.08	4.264
86400.00000000	86400.0000	26	1	3.85	3.85	3.232

TEST FOR FIT OF LINE
==========

CHI-SQUARED FOR DEVIATIONS = 4.952 D. F. = 3
APPROXIMATE PROBABILITY = .1740 TEST LEVEL = 0,05
TABLED CHI-SQUARED VALUE = 7.815

DEVIATIONS FROM LINE ARE HOMOGENEOUS

FITTED PROBIT LINE
==========

REGRESSION EQUATION IS Y = a + bX
WHERE Y IS EMPIRICAL PROBITS AND X IS DOSE

INTERCEPT (a) = 4.0873
REGRESSION COEFFICIENT (b) = .0000
STANDARD ERROR OF b = .0000
MEAN FOR EMPIRICAL PROBITS (Y) = 4.2165
MEAN FOR DOSE (X) =**********

DOSE (X)	EXPECTED EMP.PROBIT (Y)
**********	4.541
**********	4.178
2160000.00000	4.105
432000.00000	4.091
86400.00000	4.088

EXPECTED QUANTITIES FOR LINE
==========

G = 1 OR > 1 -- NO FIDUCIAL LIMITS CAN BE CALCULA TED
EXPERIMENTAL PROCEDURES PROBABLY NEED ATTENTION

EXPECTED % RESPONSE	DOSE	STANDARD ERROR
50.0	108689000.000	64796320.0000
90.0	261310300.000	166962200.0000
95.0	304574400.000	196122900.0000
99.0	385719900.000	250877300.0000
99.9	476690100.000	312311600.0000

****
Larvae per detached fruit (eggs on fruit) (see section 3.3.2)
****

GENERAL INPUT INFORMATION
==========

DOSES TRANSFORMED TO LOG10

NATURAL MORTALITY = 0%
NON-RESPONSE = 0%

DOSE	TRANSFORMED DOSE(X)	NUMBER EXPOSED	NUMBER RESPON.	%RESPONSE	CORRECTED %RESPONSE	EMPIRICAL PROBITS
**********	7.7324	80	59	73.75	73.75	5.636
**********	7.0334	80	45	56.25	56.25	5.157
**********	6.3345	80	27	33.75	33.75	4.581
432000.00000000	5.6355	80	30	37.50	37.50	4.681
86400.00000000	4.9365	80	7	8.75	8.75	3.644

TEST FOR FIT OF LINE
==========

CHI-SQUARED FOR DEVIATIONS = 9.990 D. F. = 3
APPROXIMATE PROBABILITY = .0190 TEST LEVEL = 0,05
TABLED CHI-SQUARED VALUE = 7.815

DEVIATIONS FROM LINE ARE HETEROGENEOUS
COMBINATION OF DOSES WILL BE ATTEMPTED

DOSE(X)	OBSERVED MORTALITY	EXPECTED MORTALITY	CHI-SQUARED	CHI-SQUARED COMBINED
7.7324	59	58.26	.035	.035
7.0334	45	45.86	.038	.038
6.3345	27	32.49	1.561	1.561
5.6355	30	20.38	6.099	1.175
4.9365	7	11.17	1.808	
			9.541	2.809

COMBINED CHI-SQUARED = 2.809 D. F. = 2
APPROXIMATE PROBABILITY = .2440 TEST LEVEL = 0,05
TABLED CHI-SQUARED VALUE = 5.991

DEVIATIONS FROM LINE ARE NOW CONSIDERED HOMOGENEOUS

FITTED PROBIT LINE
==========

REGRESSION EQUATION IS Y = a + bX
WHERE Y IS EMPIRICAL PROBITS AND X IS DOSE

INTERCEPT (a) = .9342
REGRESSION COEFFICIENT (b) = .6043

STANDARD ERROR OF b = .0716
MEAN FOR EMPIRICAL PROBITS (Y) = 4.8187
MEAN FOR DOSE (X) = 6.4277

DOSE (X)	EXPECTED EMP.PROBIT (Y)
7.73239	5.607
7.03342	5.185
6.33445	4.762
5.63548	4.340
4.93651	3.918

EXPECTED QUANTITIES FOR LINE
==========

G FOR FIDUCIAL LIMITS = .0539

EXPECTED % RESPONSE	DOSE	STANDARD ERROR	FIDUCIAL LIMITS UPPER	LOWER
50.0	6.728	.1167	6.981	6.509
90.0	8.848	.3076	9.621	8.351
95.0	9.449	.3749	10.396	8.847
99.0	10.577	.5040	11.856	9.771
99.9	11.841	.6509	13.497	10.802

QUANTITIES TRANSFORMED BACK TO ORIGINAL
==========

EXPECTED % RESPONSE	DOSE	STANDARD ERROR	FIDUCIAL LIMITS UPPER	LOWER
50.0	.5341615E+07	.1434006E+07	.9562927E+07	.3228089E+07
90.0	.7052373E+09	.4988892E+09	.4180454E+10	.2245665E+09
95.0	.2815054E+10	.2427043E+10	.2491361E+11	.7029953E+09
99.0	.3775719E+11	.4376628E+11	.7180985E+12	.5898511E+10
99.9	.6934648E+12	.1038126E+13	.3139631E+14	.6341134E+11

****
Detached fruit infested (larvae on fruit) (see section 3.3.2)
Rep1:
****

GENERAL INPUT INFORMATION
==========

DOSES TRANSFORMED TO LOG10

NATURAL MORTALITY = 0%
NON-RESPONSE = 0%

DOSE	TRANSFORMED DOSE(X)	NUMBER EXPOSED	NUMBER RESPON.	%RESPONSE	CORRECTED %RESPONSE	EMPIRICAL PROBITS
**********	9.0326	17	16	94.12	94.12	6.565
**********	8.7315	17	9	52.94	52.94	5.074
**********	8.4306	17	13	76.47	76.47	5.722
**********	8.1294	17	8	47.06	47.06	4.926
**********	7.8284	17	10	58.82	58.82	5.223

TEST FOR FIT OF LINE
==========

CHI-SQUARED FOR DEVIATIONS = 8.538 D. F. = 3
APPROXIMATE PROBABILITY = .0360 TEST LEVEL = 0,05
TABLED CHI-SQUARED VALUE = 7.815

DEVIATIONS FROM LINE ARE HETEROGENEOUS
COMBINATION OF DOSES WILL BE ATTEMPTED

COMBINATION NOT POSSIBLE - NO DOSES WITH > 85% OR 15% MORTALITY
EXAMINE DATA FOR POSSIBLE SYSTEMATIC DEVIATIONS

DOSE(X)	OBSERVED MORTALITY	EXPECTED MORTALITY	CHI-SQUARED
9.0326	16	13.71	1.974
8.7315	9	12.60	3.980
8.4306	13	11.32	.745
8.1294	8	9.92	.890
7.8284	10	8.45	.567

HETEROGENEITY FACTOR IS APPLIED FOR FIDUCIAL LIMITS

FITTED PROBIT LINE
==========

REGRESSION EQUATION IS Y = a + bX
WHERE Y IS EMPIRICAL PROBITS AND X IS DOSE

INTERCEPT (a) = -.6851
REGRESSION COEFFICIENT (b) = .7252
STANDARD ERROR OF b = .5733
MEAN FOR EMPIRICAL PROBITS (Y) = 5.3974

MEAN FOR DOSE (X) = 8.3872

HETEROGENEITY FACTOR APPLIED = 2.8458

DOSE (X)	EXPECTED EMP.PROBIT (Y)
9.03262	5.865
8.73151	5.647
8.43056	5.429
8.12937	5.210
7.82840	4.992

EXPECTED QUANTITIES FOR LINE
==========

G = 1 OR > 1 -- NO FIDUCIAL LIMITS CAN BE CALCULA TED
EXPERIMENTAL PROCEDURES PROBABLY NEED ATTENTION

EXPECTED % RESPONSE	DOSE	STANDARD ERROR
50.0	7.839	.5456
90.0	9.606	1.0193
95.0	10.107	1.3997
99.0	11.047	2.1285
99.9	12.100	2.9538

QUANTITIES TRANSFORMED BACK TO ORIGINAL
==========

EXPECTED % RESPONSE	DOSE	STANDARD ERROR	FIDUCIAL LIMITS UPPER	LOWER
50.0	.6906124E+08	.8666284E+08		
90.0	.4040559E+10	.9472760E+10		
95.0	.1280554E+11	.4122453E+11		
99.0	.1114258E+12	.5454957E+12		
99.9	.1259892E+13	.8559466E+13		

```
 ********************************************************************************
Detached fruit infested (larvae on fruit) (see section 3.3.2)
Rep2
 ********************************************************************************

          GENERAL INPUT INFORMATION
          =========================

          DOSES TRANSFORMED TO LOG10

          NATURAL MORTALITY =  0%
          NON-RESPONSE      =  0%
```

DOSE	TRANSFORMED DOSE(X)	NUMBER EXPOSED	NUMBER RESPON.	%RESPONSE	CORRECTED %RESPONSE	EMPIRICAL PROBITS
**********	9.0326	22	16	72.73	72.73	5.605
**********	8.7315	22	18	81.82	81.82	5.909
**********	8.4306	22	12	54.55	54.55	5.114
**********	8.1294	22	10	45.45	45.45	4.886
**********	7.8284	22	8	36.36	36.36	4.651

```
          TEST FOR FIT OF LINE
          ====================

          CHI-SQUARED FOR DEVIATIONS =   2.228     D. F. =  3
          APPROXIMATE PROBABILITY    =    .5300    TEST LEVEL = 0,05
          TABLED CHI-SQUARED VALUE   =   7.815

          DEVIATIONS FROM LINE ARE HOMOGENEOUS


          FITTED PROBIT LINE
          ==================

          REGRESSION EQUATION IS Y = a + bX
          WHERE Y IS EMPIRICAL PROBITS AND X IS DOSE

          INTERCEPT (a)                  =    -2.9357
          REGRESSION COEFFICIENT (b)     =      .9678
          STANDARD ERROR OF b            =      .2985
          MEAN FOR EMPIRICAL PROBITS (Y) =     5.1957
          MEAN FOR DOSE (X)              =     8.4018

            DOSE (X)         EXPECTED
                          EMP.PROBIT (Y)
            9.03262           5.806
            8.73151           5.515
            8.43056           5.224
            8.12937           4.932
            7.82840           4.641


          EXPECTED QUANTITIES FOR LINE
          ============================

          G FOR FIDUCIAL LIMITS =  .3655
          G IS RATHER LARGE: ACCURACY OF EXPERIMENTAL PROCEDURES MAY BE SUSPECT
```

EXPECTED % RESPONSE	DOSE	STANDARD ERROR	FIDUCIAL LIMITS UPPER	LOWER
50.0	8.200	.1426	8.453	7.713
90.0	9.524	.3691	11.285	9.055
95.0	9.899	.4793	12.223	9.300
99.0	10.603	.6910	13.992	9.750
99.9	11.392	.9314	15.982	10.248

QUANTITIES TRANSFORMED BACK TO ORIGINAL

EXPECTED % RESPONSE	DOSE	STANDARD ERROR	FIDUCIAL LIMITS UPPER	LOWER
50.0	.1583264E+09	.5193182E+08	.2836673E+09	.5168268E+08
90.0	.3340147E+10	.2835419E+10	.1926407E+12	.1136099E+10
95.0	.7927645E+10	.8740178E+10	.1670722E+13	.1997468E+10
99.0	.4010513E+11	.6374289E+11	.9828235E+14	.5625241E+10
99.9	.2468874E+12	.5288690E+12	.9600758E+16	.1771070E+11

****
Detached fruit infested (larvae on fruit) (see section 3.3.2)
Rep3:
****

GENERAL INPUT INFORMATION
==========

DOSES TRANSFORMED TO LOG10

NATURAL MORTALITY = 0%
NON-RESPONSE = 0%

DOSE	TRANSFORMED DOSE(X)	NUMBER EXPOSED	NUMBER RESPON.	%RESPONSE	CORRECTED %RESPONSE	EMPIRICAL PROBITS
**********	9.0326	20	18	90.00	90.00	6.282
**********	8.7315	20	12	60.00	60.00	5.253
**********	8.4306	20	10	50.00	50.00	5.000
**********	8.1294	20	14	70.00	70.00	5.524
**********	8.8284	20	10	50.00	50.00	5.000

TEST FOR FIT OF LINE
==========

CHI-SQUARED FOR DEVIATIONS = 9.116 D. F. = 3
APPROXIMATE PROBABILITY = .0270 TEST LEVEL = 0,05
TABLED CHI-SQUARED VALUE = 7.815

DEVIATIONS FROM LINE ARE HETEROGENEOUS
COMBINATION OF DOSES WILL BE ATTEMPTED

COMBINATION NOT POSSIBLE - NO DOSES WITH > 85% OR 15% MORTALITY
EXAMINE DATA FOR POSSIBLE SYSTEMATIC DEVIATIONS

DOSE(X)	OBSERVED MORTALITY	EXPECTED MORTALITY	CHI-SQUARED
9.0326	18	13.94	3.896
8.7315	12	13.11	.275
8.4306	10	12.25	1.064
8.1294	14	11.35	1.428
8.8284	10	13.39	2.590

HETEROGENEITY FACTOR IS APPLIED FOR FIDUCIAL LIMITS

FITTED PROBIT LINE
==========

REGRESSION EQUATION IS Y = a + bX
WHERE Y IS EMPIRICAL PROBITS AND X IS DOSE

INTERCEPT (a) = 2.0552
REGRESSION COEFFICIENT (b) = .3832
STANDARD ERROR OF b = .7062
MEAN FOR EMPIRICAL PROBITS (Y) = 5.3584

MEAN FOR DOSE (X) = 8.6209

HETEROGENEITY FACTOR APPLIED = 3.0385

DOSE (X)	EXPECTED EMP.PROBIT (Y)
9.03262	5.516
8.73151	5.401
8.43056	5.285
8.12937	5.170
8.82840	5.438

EXPECTED QUANTITIES FOR LINE
==========

G = 1 OR > 1 -- NO FIDUCIAL LIMITS CAN BE CALCULA TED
EXPERIMENTAL PROCEDURES PROBABLY NEED ATTENTION

EXPECTED % RESPONSE	DOSE	STANDARD ERROR
50.0	7.686	1.8205
90.0	11.030	4.4792
95.0	11.979	6.2159
99.0	13.757	9.4838
99.9	15.751	13.1530

QUANTITIES TRANSFORMED BACK TO ORIGINAL
==========

EXPECTED % RESPONSE	DOSE	STANDARD ERROR	FIDUCIAL LIMITS UPPER	LOWER
50.0	.4848607E+08	.2030224E+09		
90.0	.1072510E+12	.1104923E+13		
95.0	.9518302E+12	.1360780E+14		
99.0	.5713631E+14	.1246295E+16		
99.9	.5630901E+16	.1703455E+18		

```
                          **************************
                          *   COMPARISON OF LINES   *
                          **************************


     ABRIDGED ANALYSIS OF VARIANCE
     =============================
                                                 DEVIATIONS FROM REGRESSION
                    INTERCEPT   REGR.COEF.  D.F.     SUM OF       RESIDUAL
                       (a)         (b)              SQUARES      VARIANCES
LINE: Fruit          -.685        .725      3   .853751E+01         2.846
LINE: Fruit         -2.936        .968      3   .222825E+01          .743
LINE: Fruit          2.055        .383      3   .911555E+01         3.039
-------------------------------------------------------------------------
                                               9   .198813E+02         2.209
-------------------------------------------------------------------------
    COMBINED                      .750     11   .212390E+02         1.931
-------------------------------------------------------------------------
           DIFFERENCE BETWEEN SLOPES          2   .135768E+01          .679
-------------------------------------------------------------------------
    TOTAL             -.767        .717     13   .228158E+02         1.755
-------------------------------------------------------------------------
       DIFFERENCE BETWEEN ADJ. MEANS          2   .157676E+01          .788
-------------------------------------------------------------------------


     BARTLETTS TEST FOR HOMOGENEITY OF RESIDUAL VARIANCES
     ====================================================

     CHI-SQUARED VALUE        =    .822     D. F. =  2
     APPROXIMATE PROBABILITY  =    .6680    TEST LEVEL = .0100
     TABLED CHI-SQUARED VALUE =   9.210

     RESIDUAL VARIANCES ARE HOMOGENEOUS - SLOPES OF LINES MAY BE COMPARED


     COMPARISON OF SLOPES (PARALLELISM)
     ==================================

      SOURCE       D.F.    SUM OF SQ.    MEAN SQ.      F-VALUE
     ----------------------------------------------------------
     PARALLELISM     2    .135768E+01        .679          .31
     HETEROGENEITY   9    .198813E+02       2.209
     -------------------------------
      TOTAL         11      .2124E+02

     CHI-SQUARED TEST FOR PARALLELISM
     --------------------------------
     CHI-SQUARED VALUE        =   1.358     D. F. =  2
     APPROXIMATE PROBABILITY  =    .5120    TEST LEVEL = 0,05
     TABLED CHI-SQUARED VALUE =   5.991


     LINES ARE PARALLEL AND ELEVATIONS MAY BE COMPARED


     REGRESSION COEFFICIENTS
     -----------------------

      Fruit           .3832
```

```
 Fruit           .7252
 Fruit           .9678


COMPARISON OF ELEVATIONS (ADJUSTED MEANS)
=========================================

F-VALUE FOR ELEVATIONS  =    .408     D.F. =  2 & 11
APPROXIMATE PROBABILITY =    .6740    TEST LEVEL = 0,05
TABLED F-VALUE          =   3.98

ELEVATIONS ARE NOT SIGNIFICANTLY DIFFERENT

MEANS OF EMPIRICAL PROBITS
--------------------------
 Fruit          5.1957
 Fruit          5.3584
 Fruit          5.3974

INTERCEPTS OF LINES
-------------------
 Fruit          -2.936
 Fruit           -.685
 Fruit           2.055
```

```
*******************************************************************************
Larvae per detached fruit (larvae on fruit) (see section 3.3.2)
Rep1:
 *******************************************************************************

          GENERAL INPUT INFORMATION
          =========================

          DOSES TRANSFORMED TO LOG10

          NATURAL MORTALITY =  0%
          NON-RESPONSE      =  0%


        DOSE      TRANSFORMED  NUMBER  NUMBER  %RESPONSE  CORRECTED  EMPIRICAL
                    DOSE(X)   EXPOSED  RESPON.            %RESPONSE   PROBITS

 ***************     9.0326      20       19     95.00      95.00      6.645
 ***************     8.7315      20       12     60.00      60.00      5.253
 ***************     8.4306      20       16     80.00      80.00      5.842
 ***************     8.1294      20        9     45.00      45.00      4.874
 ***************     7.8284      20       11     55.00      55.00      5.126



          TEST FOR FIT OF LINE
          ====================

          CHI-SQUARED FOR DEVIATIONS =   8.450     D. F. =  3
          APPROXIMATE PROBABILITY    =    .0370    TEST LEVEL = 0,05
          TABLED CHI-SQUARED VALUE   =   7.815

          DEVIATIONS FROM LINE ARE HETEROGENEOUS
          COMBINATION OF DOSES WILL BE ATTEMPTED


          COMBINATION NOT POSSIBLE - NO DOSES WITH > 85% OR  15% MORTALITY
          EXAMINE DATA FOR POSSIBLE SYSTEMATIC DEVIATIONS

            DOSE(X)  OBSERVED   EXPECTED   CHI-SQUARED
                     MORTALITY  MORTALITY

             9.0326      19       16.94         1.637
             8.7315      12       15.45         3.396
             8.4306      16       13.63         1.296
             8.1294       9       11.55         1.333
             7.8284      11        9.36          .541

          HETEROGENEITY FACTOR IS APPLIED FOR FIDUCIAL LIMITS


          FITTED PROBIT LINE
          ==================

          REGRESSION EQUATION IS Y = a + bX
          WHERE Y IS EMPIRICAL PROBITS AND X IS DOSE

          INTERCEPT (a)                 =    -2.2602
          REGRESSION COEFFICIENT (b)    =      .9171
          STANDARD ERROR OF b           =      .5405
          MEAN FOR EMPIRICAL PROBITS (Y) =     5.4200
```

MEAN FOR DOSE (X) = 8.3744

HETEROGENEITY FACTOR APPLIED = 2.8167

DOSE (X)	EXPECTED EMP.PROBIT (Y)
9.03262	6.024
8.73151	5.748
8.43056	5.472
8.12937	5.195
7.82840	4.919

EXPECTED QUANTITIES FOR LINE
==========

G = 1 OR > 1 -- NO FIDUCIAL LIMITS CAN BE CALCULA TED
EXPERIMENTAL PROCEDURES PROBABLY NEED ATTENTION

EXPECTED % RESPONSE	DOSE	STANDARD ERROR
50.0	7.916	.3643
90.0	9.314	.6053
95.0	9.710	.8242
99.0	10.453	1.2492
99.9	11.286	1.7332

QUANTITIES TRANSFORMED BACK TO ORIGINAL
==========

EXPECTED % RESPONSE	DOSE	STANDARD ERROR	FIDUCIAL LIMITS UPPER	LOWER
50.0	.8249409E+08	.6911446E+08		
90.0	.2059993E+10	.2867846E+10		
95.0	.5128659E+10	.9722742E+10		
99.0	.2837895E+11	.8153479E+11		
99.9	.1931723E+12	.7700572E+12		

```
 ********************************************************************************
Detached fruit infested (larvae on fruit) (see section 3.3.2)
Rep2:
 ********************************************************************************

          GENERAL INPUT INFORMATION
          =========================

          DOSES TRANSFORMED TO LOG10

          NATURAL MORTALITY =  0%
          NON-RESPONSE      =  0%


        DOSE      TRANSFORMED  NUMBER  NUMBER  %RESPONSE  CORRECTED  EMPIRICAL
                    DOSE(X)    EXPOSED  RESPON.            %RESPONSE   PROBITS

 ***************     9.0326      30       24     80.00      80.00      5.842
 ***************     8.7315      30       26     86.67      86.67      6.111
 ***************     8.4306      30       20     66.67      66.67      5.431
 ***************     8.1294      30       14     46.67      46.67      4.916
 ***************     7.8284      30       16     53.33      53.33      5.084



          TEST FOR FIT OF LINE
          ====================

          CHI-SQUARED FOR DEVIATIONS =   3.956     D. F. =  3
          APPROXIMATE PROBABILITY    =    .2650    TEST LEVEL = 0,05
          TABLED CHI-SQUARED VALUE   =   7.815

          DEVIATIONS FROM LINE ARE HOMOGENEOUS


          FITTED PROBIT LINE
          ==================

          REGRESSION EQUATION IS Y = a + bX
          WHERE Y IS EMPIRICAL PROBITS AND X IS DOSE

          INTERCEPT (a)                  =    -1.9996
          REGRESSION COEFFICIENT (b)     =      .8849
          STANDARD ERROR OF b            =      .2614
          MEAN FOR EMPIRICAL PROBITS (Y) =     5.4134
          MEAN FOR DOSE (X)              =     8.3773

            DOSE (X)         EXPECTED
                          EMP.PROBIT (Y)
            9.03262           5.993
            8.73151           5.727
            8.43056           5.461
            8.12937           5.194
            7.82840           4.928


          EXPECTED QUANTITIES FOR LINE
          ============================

          G FOR FIDUCIAL LIMITS =  .3353
          G IS RATHER LARGE: ACCURACY OF EXPERIMENTAL PROCEDURES MAY BE SUSPECT
```

EXPECTED % RESPONSE	DOSE	STANDARD ERROR	FIDUCIAL LIMITS UPPER	LOWER
50.0	7.910	.1848	8.177	7.171
90.0	9.358	.3149	10.758	8.949
95.0	9.769	.4292	11.719	9.223
99.0	10.539	.6504	13.536	9.723
99.9	11.402	.9022	15.580	10.276

QUANTITIES TRANSFORMED BACK TO ORIGINAL

EXPECTED % RESPONSE	DOSE	STANDARD ERROR	FIDUCIAL LIMITS UPPER	LOWER
50.0	.8130686E+08	.3456249E+08	.1504549E+09	.1484122E+08
90.0	.2282656E+10	.1653069E+10	.5726569E+11	.8891921E+09
95.0	.5874882E+10	.5798888E+10	.5237601E+12	.1671636E+10
99.0	.3459694E+11	.5175480E+11	.3436374E+14	.5287372E+10
99.9	.2525268E+12	.5239801E+12	.3806145E+16	.1889982E+11

```
 ********************************************************************************
Detached fruit infested (larvae on fruit) (see section 3.3.2)
Rep3:
 ********************************************************************************

          GENERAL INPUT INFORMATION
          =========================

          DOSES TRANSFORMED TO LOG10

          NATURAL MORTALITY =  0%
          NON-RESPONSE      =  0%


        DOSE      TRANSFORMED  NUMBER  NUMBER  %RESPONSE  CORRECTED  EMPIRICAL
                    DOSE(X)   EXPOSED  RESPON.            %RESPONSE   PROBITS

 ***************     9.0326      30       28     93.33      93.33      6.501
 ***************     8.7315      30       22     73.33      73.33      5.623
 ***************     8.4306      30       18     60.00      60.00      5.253
 ***************     8.1294      30       22     73.33      73.33      5.623
 ***************     7.8284      30       16     53.33      53.33      5.084


          TEST FOR FIT OF LINE
          ====================

          CHI-SQUARED FOR DEVIATIONS =   5.623     D. F. =  3
          APPROXIMATE PROBABILITY    =    .1300    TEST LEVEL = 0,05
          TABLED CHI-SQUARED VALUE   =   7.815

          DEVIATIONS FROM LINE ARE HOMOGENEOUS


          FITTED PROBIT LINE
          ==================

          REGRESSION EQUATION IS Y = a + bX
          WHERE Y IS EMPIRICAL PROBITS AND X IS DOSE

          INTERCEPT (a)                  =    -1.4068
          REGRESSION COEFFICIENT (b)     =      .8287
          STANDARD ERROR OF b            =      .2668
          MEAN FOR EMPIRICAL PROBITS (Y) =     5.5273
          MEAN FOR DOSE (X)              =     8.3679

            DOSE (X)         EXPECTED
                          EMP.PROBIT (Y)
            9.03262           6.078
            8.73151           5.829
            8.43056           5.579
            8.12937           5.330
            7.82840           5.080


          EXPECTED QUANTITIES FOR LINE
          ============================

          G FOR FIDUCIAL LIMITS =  .3982
          G IS RATHER LARGE: ACCURACY OF EXPERIMENTAL PROCEDURES MAY BE SUSPECT
```

EXPECTED % RESPONSE	DOSE	STANDARD ERROR	FIDUCIAL LIMITS UPPER	LOWER
50.0	7.732	.2447	8.058	6.563
90.0	9.278	.3222	10.893	8.868
95.0	9.717	.4544	12.063	9.155
99.0	10.539	.7116	14.276	9.674
99.9	11.461	1.0047	16.767	10.247

QUANTITIES TRANSFORMED BACK TO ORIGINAL

EXPECTED % RESPONSE	DOSE	STANDARD ERROR	FIDUCIAL LIMITS UPPER	LOWER
50.0	.5389226E+08	.3033155E+08	.1144181E+09	.3651757E+07
90.0	.1897189E+10	.1405757E+10	.7814315E+11	.7376191E+09
95.0	.5206274E+10	.5440616E+10	.1155609E+13	.1428539E+10
99.0	.3457909E+11	.5659712E+11	.1889974E+15	.4719892E+10
99.9	.2888405E+12	.6674359E+12	.5853402E+17	.1764095E+11

****
* COMPARISON OF LINES *
****

ABRIDGED ANALYSIS OF VARIANCE

	INTERCEPT (a)	REGR.COEF. (b)	D.F.	DEVIATIONS FROM REGRESSION SUM OF SQUARES	RESIDUAL VARIANCES
LINE: Larvae	-2.260	.917	3	.844998E+01	2.817
LINE: Larvae	-2.000	.885	3	.395581E+01	1.319
LINE: Larvae	-1.407	.829	3	.562296E+01	1.874
			9	.180287E+02	2.003
COMBINED		.872	11	.180772E+02	1.643
DIFFERENCE BETWEEN SLOPES			2	.484257E-01	.024
TOTAL	-1.836	.871	13	.188032E+02	1.446
DIFFERENCE BETWEEN ADJ. MEANS			2	.726074E+00	.363

BARTLETTS TEST FOR HOMOGENEITY OF RESIDUAL VARIANCES

CHI-SQUARED VALUE = .228 D. F. = 2
APPROXIMATE PROBABILITY = .8870 TEST LEVEL = .0100
TABLED CHI-SQUARED VALUE = 9.210

RESIDUAL VARIANCES ARE HOMOGENEOUS - SLOPES OF LINES MAY BE COMPARED

COMPARISON OF SLOPES (PARALLELISM)

SOURCE	D.F.	SUM OF SQ.	MEAN SQ.	F-VALUE
PARALLELISM	2	.484257E-01	.024	.01
HETEROGENEITY	9	.180287E+02	2.003	
TOTAL	11	.1808E+02		

CHI-SQUARED TEST FOR PARALLELISM

CHI-SQUARED VALUE = .048 D. F. = 2
APPROXIMATE PROBABILITY = .9640 TEST LEVEL = 0,05
TABLED CHI-SQUARED VALUE = 5.991

LINES ARE PARALLEL AND ELEVATIONS MAY BE COMPARED

REGRESSION COEFFICIENTS

Larvae .8287

```
 Larvae          .8849
 Larvae          .9171


COMPARISON OF ELEVATIONS (ADJUSTED MEANS)
=========================================

F-VALUE FOR ELEVATIONS  =    .221     D.F. =  2 & 11
APPROXIMATE PROBABILITY =    .8050    TEST LEVEL = 0,05
TABLED F-VALUE          =   3.98

ELEVATIONS ARE NOT SIGNIFICANTLY DIFFERENT

MEANS OF EMPIRICAL PROBITS
--------------------------
 Larvae         5.4134
 Larvae         5.4200
 Larvae         5.5273

INTERCEPTS OF LINES
-------------------
 Larvae         -2.260
 Larvae         -2.000
 Larvae         -1.407
```

Appendix 3 GenStat (2002) output of a logit transformation of a probit analysis of time-response data (LT) with the LC_{90} of CrleGV-SA against neonate *C. leucotreta* larvae in surface dose bioassays.

```
GenStat Release 6.1  (PC/Windows 98)              10 March 2003 12:58:06
Copyright 2002, Lawes Agricultural Trust (Rothamsted Experimental Station)

        ________________________________________________

        GenStat Sixth Edition
        GenStat Procedure Library Release PL14
        ________________________________________________

 1  %CD 'C:/WINDOWS'
 2  "Data taken from unsaved spreadsheet: New Data;1"
 3  "Data taken from unsaved spreadsheet: New Data;1"
 4  DELETE [redefine=yes] Time
 5  UNITS
 6  VARIATE [nvalues=25] Time
 7    READ Time

Data imported from GenStat Spreadsheet: C:\Home\MOORES\Stats\Genstat\CrleGV
L1 LT.gsh   on: 8-Mar-2003 10:20:41

  Identifier   Minimum      Mean   Maximum    Values   Missing
        Time     16.00     112.0     208.0        25         0

10  RESTRICT Time
11
12  "Data taken from unsaved spreadsheet: New Data;1"
13  DELETE [redefine=yes] Time
14  UNITS
15  VARIATE [nvalues=47] Time
16  READ Time

  Identifier   Minimum      Mean   Maximum    Values   Missing
        Time     16.00     106.4     208.0        47         0

20  RESTRICT Time
21
22  "Data taken from unsaved spreadsheet: New Data;1"
23  DELETE [redefine=yes] Time,Rep,Number
24  UNITS
25  VARIATE [nvalues=66] Time
26  READ Time

  Identifier   Minimum      Mean   Maximum    Values   Missing
        Time     16.00     101.1     208.0        66         0

30  VARIATE [nvalues=66] Rep
```

```
31  READ Rep

 Identifier  Minimum     Mean  Maximum   Values  Missing
        Rep    1.000    1.909    3.000       66        0

34  VARIATE [nvalues=66] Number
35  READ Number

 Identifier  Minimum     Mean  Maximum   Values  Missing
     Number    31.00    35.95    39.00       66        0

39  RESTRICT Time,Rep,Number
40
41  "Data taken from unsaved spreadsheet: New Data;1"
42  DELETE [redefine=yes] Mortality
43  UNITS
44  VARIATE [nvalues=66] Time
45  READ Time

 Identifier  Minimum     Mean  Maximum   Values  Missing
       Time    16.00    101.1    208.0       66        0

49  VARIATE [nvalues=66] Rep
50  READ Rep

 Identifier  Minimum     Mean  Maximum   Values  Missing
        Rep    1.000    1.909    3.000       66        0

53  VARIATE [nvalues=66] Number
54  READ Number

 Identifier  Minimum     Mean  Maximum   Values  Missing
     Number    31.00    35.95    39.00       66        0

58  VARIATE [nvalues=66] Mortality
59  READ Mortality

 Identifier  Minimum     Mean  Maximum   Values  Missing
  Mortality   0.0000    12.76    38.00       66       41

62  RESTRICT Time,Rep,Number,Mortality
63
64  "Data taken from unsaved spreadsheet: New Data;1"
65  UNITS
66  VARIATE [nvalues=66] Time
67  READ Time

 Identifier  Minimum     Mean  Maximum   Values  Missing
       Time    16.00    101.1    208.0       66        0
```

```
 71  VARIATE [nvalues=66] Rep
 72  READ Rep

  Identifier   Minimum      Mean   Maximum    Values   Missing
         Rep     1.000     1.909     3.000        66         0

 75  VARIATE [nvalues=66] Number
 76  READ Number

  Identifier   Minimum      Mean   Maximum    Values   Missing
      Number     31.00     35.95     39.00        66         0

 80  VARIATE [nvalues=66] Mortality
 81  READ Mortality

  Identifier   Minimum      Mean   Maximum    Values   Missing
   Mortality    0.0000     12.66     38.00        66        19

 85  RESTRICT Time,Rep,Number,Mortality
 86
 87  "Data taken from File: C:/Home/MOORES/Stats/Genstat/CrleGV L1 LT.gsh"
 88  DELETE [Redefine=yes] _stitle_: TEXT _stitle_
 89  READ [print=*;SETNVALUES=yes] _stitle_
 92  PRINT [IPrint=*] _stitle_; Just=Left

 Data imported from GenStat Spreadsheet: C:\Home\MOORES\Stats\Genstat\CrleGV
L1
LT.gsh
 on: 10-Mar-2003 13:09:03


 93  DELETE [redefine=yes] Rep
 94  UNITS
 95  VARIATE [nvalues=66] Time
 96  READ Time

  Identifier   Minimum      Mean   Maximum    Values   Missing
        Time     16.00     101.1     208.0        66         0

100  FACTOR [modify=yes;nvalues=66;levels=3;reference=1] Rep
101  READ Rep; frepresentation=ordinal

  Identifier    Values   Missing    Levels
         Rep        66         0         3

104  VARIATE [nvalues=66] Number
105  READ Number

  Identifier   Minimum      Mean   Maximum    Values   Missing
      Number     31.00     35.95     39.00        66         0
```

```
 109  VARIATE [nvalues=66] Mortality
 110  READ Mortality

   Identifier   Minimum      Mean   Maximum    Values   Missing
    Mortality    0.0000     13.79     39.00        66         0

 114  RESTRICT Time,Rep,Number,Mortality
 115
 116  "Probit analysis."
 117  CALCULATE logExpnVar=LOG10(Time)
 118  PROBITANALYSIS [PRINT=model,summary,estimates;
TRANSFORMATION=logit; MORTALITY=estimate;\
 119   GROUPS=Rep; SEPARATE=slope,mortality; DISP=1; LD=!(50)] Mortality;
DOSE=logExpnVar;\
 120   NBINOMIAL=Number
120.....................................................................


***** Nonlinear regression analysis *****

 Response variate: Mortality
  Binomial totals: Number
    Distribution: Binomial
   Link function: Calculated from: Lc[1], Lc[2], CalcLinpred,
                  CalcLogitFitted, CalcLogitDerivative
Nonlinear parameters: PrMortality['1'], PrMortality['2'], PrMortality['3']
  Model calculations: !E(...)
    Fitted terms: Rep + logExpnVar.Rep


*** Summary of analysis ***

                                mean  deviance
             d.f.     deviance     deviance     ratio
Regression      8      1420.68     177.5846    177.58
Residual       57        53.14       0.9323
Total          65      1473.82      22.6741
* MESSAGE: ratios are based on dispersion parameter with value 1

Dispersion parameter is fixed at 1.00
* MESSAGE: The following units have large standardized residuals:
         Unit     Response    Residual
           25        38.00        2.81
           66        39.00        2.42
* MESSAGE: The residuals do not appear to be random;
          for example, fitted values in the range 0.84 to 2.63
          are consistently smaller than observed values
          and fitted values in the range 20.05 to 25.64
          are consistently larger  than observed values
```

*** Estimates of parameters ***

	estimate	s.e.
PrMortality['1']	0.01424	0.00862
PrMortality['2']	0.0247	0.0137
PrMortality['3']	0.0003344	*
* Linear		
Rep 1	-29.62	2.61
Rep 2	-26.77	2.77
Rep 3	-22.50	1.74
logExpnVar.Rep 1	13.73	1.20
logExpnVar.Rep 2	12.97	1.32
logExpnVar.Rep 3	11.364	0.871

* MESSAGE: some standard errors not available due to singularity
*MESSAGE: s.e.s are based on dispersion parameter with value 1

*** Effective doses ***

Group	LD	estimate	s.e.	lower 95%	upper 95%
1	50.00	2.158	0.008113	2.142	2.173
2	50.00	2.064	0.010841	2.041	2.083
3	50.00	1.980	0.009707	1.961	1.998

*** Effective doses ***

Group	LD	estimate	s.e.	lower 95%	upper 95%
1	90.00	2.318	0.01551	2.292	2.353
2	90.00	2.233	0.01782	2.204	2.273
3	90.00	2.173	0.01727	2.144	2.211

Appendix 4 PROBAN (Van Ark, 1995) output of probit analysis of dose-response data (LC) data with CrleGV-SA against fifth instar *C. leucotreta* larvae in surface dose bioassays.

```
*******************************************************************************
(See section 3.3.3.2)
Rep 1:
*******************************************************************************


          GENERAL INPUT INFORMATION
          =========================

          DOSES TRANSFORMED TO LOG10

          NATURAL MORTALITY =  4%
          NON-RESPONSE      =  0%


        DOSE      TRANSFORMED  NUMBER  NUMBER  %RESPONSE  CORRECTED  EMPIRICAL
                    DOSE(X)   EXPOSED  RESPON.            %RESPONSE   PROBITS

 ***************     8.5844      50       40     80.00      79.17      5.812
 ***************     7.8854      50       30     60.00      58.33      5.210
 ***************     7.1864      50       22     44.00      41.67      4.790
 ***************     6.4874      50       20     40.00      37.50      4.681
 614500.00000000     5.7885      50       12     24.00      20.83      4.188


          TEST FOR FIT OF LINE
          ====================

          CHI-SQUARED FOR DEVIATIONS =   1.681     D. F. =  3
          APPROXIMATE PROBABILITY    =    .6450    TEST LEVEL = 0,05
          TABLED CHI-SQUARED VALUE   =   7.815

          DEVIATIONS FROM LINE ARE HOMOGENEOUS


          FITTED PROBIT LINE
          ==================

          REGRESSION EQUATION IS Y = a + bX
          WHERE Y IS EMPIRICAL PROBITS AND X IS DOSE

          INTERCEPT (a)                  =     1.0717
          REGRESSION COEFFICIENT (b)     =      .5368
          STANDARD ERROR OF b            =      .0925
          MEAN FOR EMPIRICAL PROBITS (Y) =     4.9629
          MEAN FOR DOSE (X)              =     7.2489

            DOSE (X)         EXPECTED
                          EMP.PROBIT (Y)
            8.58444           5.680
            7.88542           5.305
            7.18639           4.929
            6.48742           4.554
            5.78852           4.179


          EXPECTED QUANTITIES FOR LINE
```

==========

G FOR FIDUCIAL LIMITS = .1142

EXPECTED % RESPONSE	DOSE	STANDARD ERROR	FIDUCIAL LIMITS UPPER	LOWER
50.0	7.318	.1634	7.667	6.987
90.0	9.706	.4537	11.019	9.026
95.0	10.382	.5642	12.028	9.544
99.0	11.652	.7763	13.932	10.506
99.9	13.075	1.0174	16.073	11.578

QUANTITIES TRANSFORMED BACK TO ORIGINAL

==========

EXPECTED % RESPONSE	DOSE	STANDARD ERROR	FIDUCIAL LIMITS UPPER	LOWER
50.0	.2079817E+08	.7813989E+07	.4647457E+08	.9697058E+07
90.0	.5076122E+10	.5297340E+10	.1043773E+12	.1060733E+10
95.0	.2411721E+11	.3129459E+11	.1067693E+13	.3497751E+10
99.0	.4484513E+12	.8006542E+12	.8556212E+14	.3205499E+11
99.9	.1187936E+14	.2779805E+14	.1183982E+17	.3782358E+12

```
********************************************************************************
(See section 3.3.3.2):
Rep2
********************************************************************************

          GENERAL INPUT INFORMATION
          =========================

          DOSES TRANSFORMED TO LOG10

          NATURAL MORTALITY =  4%
          NON-RESPONSE      =  0%
```

DOSE	TRANSFORMED DOSE(X)	NUMBER EXPOSED	NUMBER RESPON.	%RESPONSE	CORRECTED %RESPONSE	EMPIRICAL PROBITS
**********	8.5844	50	36	72.00	70.83	5.548
**********	7.8854	50	24	48.00	45.83	4.895
**********	7.1864	50	18	36.00	33.33	4.569
**********	6.4874	50	20	40.00	37.50	4.681
614500.00000000	5.7885	50	8	16.00	12.50	3.850

```
          TEST FOR FIT OF LINE
          ====================

          CHI-SQUARED FOR DEVIATIONS =   5.036     D. F. =  3
          APPROXIMATE PROBABILITY    =    .1670    TEST LEVEL = 0,05
          TABLED CHI-SQUARED VALUE   =   7.815

          DEVIATIONS FROM LINE ARE HOMOGENEOUS


          FITTED PROBIT LINE
          ==================

          REGRESSION EQUATION IS Y = a + bX
          WHERE Y IS EMPIRICAL PROBITS AND X IS DOSE

          INTERCEPT (a)                  =     1.1332
          REGRESSION COEFFICIENT (b)     =      .4984
          STANDARD ERROR OF b            =      .0940
          MEAN FOR EMPIRICAL PROBITS (Y) =     4.7868
          MEAN FOR DOSE (X)              =     7.3303

            DOSE (X)         EXPECTED
                          EMP.PROBIT (Y)
            8.58444           5.412
            7.88542           5.064
            7.18639           4.715
            6.48742           4.367
            5.78852           4.018


          EXPECTED QUANTITIES FOR LINE
          ============================

          G FOR FIDUCIAL LIMITS =  .1367
```

EXPECTED % RESPONSE	DOSE	STANDARD ERROR	FIDUCIAL LIMITS UPPER	LOWER
50.0	7.758	.1954	8.243	7.408
90.0	10.329	.5930	12.142	9.466
95.0	11.058	.7254	13.289	10.008
99.0	12.425	.9774	15.446	11.018
99.9	13.958	1.2628	17.870	12.144

QUANTITIES TRANSFORMED BACK TO ORIGINAL

EXPECTED % RESPONSE	DOSE	STANDARD ERROR	FIDUCIAL LIMITS UPPER	LOWER
50.0	.5727399E+08	.2573744E+08	.1751273E+09	.2558591E+08
90.0	.2134322E+11	.2911220E+11	.1387533E+13	.2924297E+10
95.0	.1143289E+12	.1907433E+12	.1943245E+14	.1019443E+11
99.0	.2662345E+13	.5985229E+13	.2793342E+16	.1042154E+12
99.9	.9075997E+14	.2636096E+15	.7421359E+18	.1393800E+13

```
********************************************************************************
(See section 3.3.3.2):
Rep3
********************************************************************************

          GENERAL INPUT INFORMATION
          =========================

          DOSES TRANSFORMED TO LOG10

          NATURAL MORTALITY =  4%
          NON-RESPONSE      =  0%
```

DOSE	TRANSFORMED DOSE(X)	NUMBER EXPOSED	NUMBER RESPON.	%RESPONSE	CORRECTED %RESPONSE	EMPIRICAL PROBITS
**********	8.5844	50	48	96.00	95.83	6.731
**********	7.8854	50	34	68.00	66.67	5.431
**********	7.1864	50	32	64.00	62.50	5.319
**********	6.4874	50	26	52.00	50.00	5.000
614500.00000000	5.7885	50	24	48.00	45.83	4.895

```
          TEST FOR FIT OF LINE
          ====================

          CHI-SQUARED FOR DEVIATIONS =   8.273     D. F. =  3
          APPROXIMATE PROBABILITY    =    .0400    TEST LEVEL = 0,05
          TABLED CHI-SQUARED VALUE   =   7.815

          DEVIATIONS FROM LINE ARE HETEROGENEOUS
          COMBINATION OF DOSES WILL BE ATTEMPTED
```

DOSE(X)	OBSERVED MORTALITY	EXPECTED MORTALITY	CHI-SQUARED	CHI-SQUARED COMBINED
8.5844	48	43.13	3.866	
7.8854	34	38.67	3.243	.020
7.1864	32	32.91	.245	.245
6.4874	26	26.30	.136	.136
5.7885	24	19.55	.955	.955
			8.445	1.355

```
          COMBINED CHI-SQUARED     =   1.355     D. F. =  2
          APPROXIMATE PROBABILITY  =    .5130    TEST LEVEL = 0,05
          TABLED CHI-SQUARED VALUE =   5.991

          DEVIATIONS FROM LINE ARE NOW CONSIDERED HOMOGENEOUS

          FITTED PROBIT LINE
          ==================

          REGRESSION EQUATION IS Y = a + bX
          WHERE Y IS EMPIRICAL PROBITS AND X IS DOSE

          INTERCEPT (a)                 =     1.8891
```

REGRESSION COEFFICIENT (b) = .4896
STANDARD ERROR OF b = .0925
MEAN FOR EMPIRICAL PROBITS (Y) = 5.3480
MEAN FOR DOSE (X) = 7.0651

DOSE (X)	EXPECTED EMP.PROBIT (Y)
8.58444	6.092
7.88542	5.750
7.18639	5.407
6.48742	5.065
5.78852	4.723

EXPECTED QUANTITIES FOR LINE
==========

G FOR FIDUCIAL LIMITS = .1372

EXPECTED % RESPONSE	DOSE	STANDARD ERROR	FIDUCIAL LIMITS UPPER	LOWER
50.0	6.354	.2244	6.728	5.755
90.0	8.972	.4027	10.178	8.373
95.0	9.714	.5319	11.334	8.937
99.0	11.106	.7845	13.524	9.973
99.9	12.666	1.0737	15.992	11.123

QUANTITIES TRANSFORMED BACK TO ORIGINAL
==========

EXPECTED % RESPONSE	DOSE	STANDARD ERROR	FIDUCIAL LIMITS UPPER	LOWER
50.0	.2261256E+07	.1166829E+07	.5346104E+07	.5683643E+06
90.0	.9379494E+09	.8687774E+09	.1505530E+11	.2361412E+09
95.0	.5179226E+10	.6336293E+10	.2159496E+12	.8643343E+09
99.0	.1276764E+12	.2303817E+12	.3345410E+14	.9395511E+10
99.9	.4639524E+13	.1145729E+14	.9807279E+16	.1326728E+12

```
                          **************************
                          *   COMPARISON OF LINES   *
                          **************************


     ABRIDGED ANALYSIS OF VARIANCE
     =============================
                                                DEVIATIONS FROM REGRESSION
                    INTERCEPT   REGR.COEF.  D.F.     SUM OF       RESIDUAL
                       (a)         (b)              SQUARES      VARIANCES
LINE: Rep1            1.072        .537      3   .168137E+01         .560
LINE: Rep2            1.133        .498      3   .503585E+01        1.679
LINE: Rep3            1.889        .490      3   .827280E+01        2.758
-------------------------------------------------------------------------
                                               9   .149900E+02        1.666
-------------------------------------------------------------------------
    COMBINED                        .508     11   .151369E+02        1.376
-------------------------------------------------------------------------
           DIFFERENCE BETWEEN SLOPES          2   .146857E+00         .073
-------------------------------------------------------------------------
    TOTAL             1.621         .473     13   .472703E+02        3.636
-------------------------------------------------------------------------
       DIFFERENCE BETWEEN ADJ. MEANS          2   .321334E+02       16.067
-------------------------------------------------------------------------


     BARTLETTS TEST FOR HOMOGENEITY OF RESIDUAL VARIANCES
     ====================================================

     CHI-SQUARED VALUE        =    .917     D. F. =  2
     APPROXIMATE PROBABILITY  =    .6380    TEST LEVEL = .0100
     TABLED CHI-SQUARED VALUE =   9.210

     RESIDUAL VARIANCES ARE HOMOGENEOUS - SLOPES OF LINES MAY BE COMPARED



     COMPARISON OF SLOPES (PARALLELISM)
     ==================================

      SOURCE       D.F.    SUM OF SQ.    MEAN SQ.      F-VALUE
     ------------------------------------------------------------
     PARALLELISM     2    .146857E+00        .073         .04
     HETEROGENEITY   9    .149900E+02       1.666
     --------------------------------
      TOTAL         11      .1514E+02

     CHI-SQUARED TEST FOR PARALLELISM
     --------------------------------
     CHI-SQUARED VALUE        =    .147     D. F. =  2
     APPROXIMATE PROBABILITY  =    .9210    TEST LEVEL = 0,05
     TABLED CHI-SQUARED VALUE =   5.991


     LINES ARE PARALLEL AND ELEVATIONS MAY BE COMPARED


     REGRESSION COEFFICIENTS
     -----------------------

      Rep3            .4896
```

```
 Rep2            .4984
 Rep1            .5368


COMPARISON OF ELEVATIONS (ADJUSTED MEANS)
=========================================

F-VALUE FOR ELEVATIONS  =  11.676     D.F. =  2 & 11
APPROXIMATE PROBABILITY =    .0020    TEST LEVEL = 0,05
TABLED F-VALUE          =   3.98

ELEVATIONS ARE SIGNIFICANTLY DIFFERENT

MEANS OF EMPIRICAL PROBITS
--------------------------
 Rep2           4.7868
 Rep1           4.9629
 Rep3           5.3480

INTERCEPTS OF LINES
-------------------
 Rep1            1.072
 Rep2            1.133
 Rep3            1.889

MULTIPLE COMPARISON OF ELEVATIONS WITH BONFERRONI METHOD
-------------------------------------------------------

NUMBER OF COMPARISONS   =  3
TEST LEVEL = (0,05/ 3)  =   .01667

* = SIGNIFICANTLY DIFFERENT

LINE:       Rep1   Rep3
  Rep2                *
  Rep1


RELATIVE POTENCY COMPARISONS
============================
                                    LD-50         LD-50
 LINE    REGRESSION EQUATION    TRANSFORMED     ORIGINAL
 ----    -------------------    -----------     --------
Rep1    Y =  1.2778 +  .5084X     7.3219    .2098401E+08
Rep2    Y =  1.0604 +  .5084X     7.7496    .5618186E+08
Rep3    Y =  1.7563 +  .5084X     6.3806    .2402318E+07

 LINE:     Rep1   Rep2   Rep3
   Rep1     1.000   .374  8.735
   Rep2     2.677  1.000 23.387
   Rep3      .114   .043  1.000
```

Appendix 5 GenStat (2002) output of a logit transformation of a probit analysis of time-response data (LT) with the LC_{90} of CrleGV-SA against fifth instar *C. leucotreta* larvae in surface dose bioassays.

```
GenStat Release 6.1  (PC/Windows 98)              10 March 2003 14:11:52
Copyright 2002, Lawes Agricultural Trust (Rothamsted Experimental Station)

                    ____________________________________________

                    GenStat Sixth Edition
                    GenStat Procedure Library Release PL14
                    ____________________________________________

 1  %CD 'C:/WINDOWS'
 2  "Data taken from File: C:/Home/MOORES/Stats/Genstat/CrleGV L5 LT.gsh"
 3  DELETE [Redefine=yes] _stitle_: TEXT _stitle_
 4  READ [print=*;SETNVALUES=yes] _stitle_
 7  PRINT [IPrint=*] _stitle_; Just=Left

 Data imported from GenStat Spreadsheet: C:\Home\MOORES\Stats\Genstat\CrleGV
L5
LT.gsh
  on: 10-Mar-2003 13:21:36


 8  DELETE [redefine=yes] Time,Rep,Number,Mortality
 9  UNITS
10  VARIATE [nvalues=47] Time
11  READ Time

  Identifier   Minimum      Mean   Maximum    Values   Missing
        Time     144.0     203.4     280.0        47         0

15  FACTOR [modify=yes;nvalues=47;levels=3;reference=1] Rep
16  READ Rep; frepresentation=ordinal

  Identifier    Values   Missing    Levels
         Rep        47         0         3

19  VARIATE [nvalues=47] Number
20  READ Number

  Identifier   Minimum      Mean   Maximum    Values   Missing
      Number     36.00     37.23     38.00        47         0

23  VARIATE [nvalues=47] Mortality
24  READ Mortality

  Identifier   Minimum      Mean   Maximum    Values   Missing
   Mortality    0.0000     22.74     38.00        47         0
```

```
  27  RESTRICT Time,Rep,Number,Mortality
  28
  29  "Probit analysis."
  30  CALCULATE logExpnVar=LOG10(Time)
  31  PROBITANALYSIS [PRINT=model,summary,estimates;
TRANSFORMATION=logit; MORTALITY=estimate;\
  32   GROUPS=Rep; SEPARATE=slope,mortality; DISP=1; LD=!(50)] Mortality;
DOSE=logExpnVar;\
  33   NBINOMIAL=Number

**** G5W0001 **** Warning (Code OP 2). Statement 234 in Procedure
PROBITANALYSIS

Command:  FIT [PRINT=#PRINT; CONSTANT=#Constop;
CALCULATION=!e(Params[]=Params[
Unsuccessful optimization: a parameter has gone out of bounds
Parameter PrMortality['1'] is out of bounds, at cycle number 6

45.....


***** Regression Analysis *****

 Response variate: Mortality
  Binomial totals: Number
    Distribution: Binomial
   Link function: Logit
    Fitted terms: Rep + logExpnVar.Rep


*** Summary of analysis ***

                              mean  deviance
           d.f.     deviance     deviance     ratio
Regression      5      1045.45     209.0906    209.09
Residual       41        22.20       0.5415
Total          46      1067.66      23.2099
* MESSAGE: ratios are based on dispersion parameter with value 1

Dispersion parameter is fixed at 1.00


*** Estimates of parameters ***

                                        antilog of
                  estimate        s.e.      t(*)   estimate
Rep 1                -98.11        8.22    -11.94      *
Rep 2                -73.95        5.85    -12.64      *
Rep 3                -38.82        3.24    -12.00      *
```

logExpnVar.Rep 1	42.89	3.59	11.95	*
logExpnVar.Rep 2	32.62	2.57	12.68	*
logExpnVar.Rep 3	17.25	1.42	12.17	31088045.

* MESSAGE: s.e.s are based on dispersion parameter with value 1

*** Effective doses ***

Group	LD	estimate	s.e.	lower 95%	upper 95%
1	50.00	2.288	0.003428	2.281	2.294
2	50.00	2.267	0.004062	2.259	2.275
3	50.00	2.250	0.006684	2.236	2.262

*** Effective doses ***

Group	LD	estimate	s.e.	lower 95%	upper 95%
1	90.00	2.339	0.005374	2.330	2.351
2	90.00	2.335	0.006313	2.324	2.348
3	90.00	2.378	0.010168	2.360	2.400

Appendix 6 Weather data, obtained from the South African Weather Service, for areas in which field trials were conducted.

HOURLY DATA : Wind

0034763 X UITENHAGE e 2001/08

DAY	h01	h02	h03	h04	h05	h06	h07	h08	h09	h10	h11	h12	h13	h14	h15	h16	h17	h18	h19	h20	h21	h22	h23	h24	tot	avg	mx	mxtm	mn	mntm
1	35091	35095	35100	35101	35091	35088	35090	35088	35106	34099	35102	35089	34085	34087	33076	33049	34047	31019	32021	33017	27016	27018	36012	6013	780600	32525.0	35193	23	0	2
2	3014	5018	35014	3011	1014	36012	1015	1016	0	3016	0	9025	11017	17038	20041	21044	21045	22032	24026	26019	26016	28011	0	35012	348456	14519.0	22086	16	04	3
3	0	28013	0	0	0	31013	0	0	27012	28033	27037	24033	24039	27045	26046	27050	28049	30050	31085	30070	29069	29062	28053	27049	501808	20908.7	32143	18	39	4
26	26037	29032	28024	24018	28022	28018	27018	29024	25016	27016	0	30013	28017	0	0	23013	25017	26021	28021	28022	29017	27019	2014	27014	544413	22683.9	17081	21	30	5
5	18013	11016	0	0	10020	8022	6016	8020	9018	11044	11056	11057	12052	12047	13054	13054	12047	11037	8018	8018	6022	0	4016	4021	206668	8611.2	11108	11	15	6
4014	0	0	0	0	15013	26011	28014	5015	1019	26024	34027	2057	2043	36049	36038	2022	2019	1019	36015	29018	30024	36016	29016	380473	15853.0	3111	1203	7	29	7
3	35035	30028	12015	3015	34016	26012	28014	0	0	0	17013	19014	17022	19031	20031	21038	21035	20020	25012	2017	0	3024	7021	7018	360431	15018.0	17074	13	16	8
2012	0	0	2012	4011	2013	35014	0	0	0	0	19013	23015	24018	26014	0	0	3024	3024	16011	0	0	2013	0	161194	6716.4	4046	1752	9	10014	
5018	11015	28012	2013	34028	35051	35078	36076	36081	36093	36087	36087	35055	36078	36080	36046	36049	36046	36045	36047	36050	36052	35043	735244	30635.2	36157	1453	10	34054		
31043	28058	28039	25021	26027	26047	26026	30022	29042	30058	31074	31089	32085	31092	28095	29083	30072	30079	30077	29074	30061	29062	30046	704426	29351.1	28193	1539	11	30056		
29053	29054	29052	30051	29033	30034	30050	30052	30049	29061	28057	28053	25053	28051	27044	25044	28034	31036	30030	30029	33014	0	26013	665003	27708.5	29118	117	12	0	0	2012
8013	3026	26013	0	0	0	7018	5035	7038	7038	7040	11055	12068	11056	10030	7013	4015	3015	1016	1016	2013	134530	5605.4	12103	1451	13	0	2013	36014	36015	0
1012	2012	0	0	0	19014	19016	20025	20019	17042	18039	19032	22019	30013	31018	28021	29028	27028	24020	400400	16683.3	17082	1418	14	26020						
24020	24019	26019	30036	31044	31047	29035	29057	29069	30061	29058	28064	28065	27074	27082	29077	29068	29060	29057	30041	30030	32045	31036	688184	28674.3	28150	1620	15	32036		
34032	34024	34027	34018	35034	34035	33031	28018	26025	25022	20016	24025	27040	22055	24048	26051	26044	28039	26026	36015	1021	3021	3021	615724	25655.2	20100	1500	16	0	0	2015
0	3012	3014	36013	1017	35035	32044	34058	34069	32062	33053	32059	33059	33045	28023	30031	32031	32033	30025	20017	3017	518732	21613.8	30135	1252	17	2018	3022	2019	36022	
35017	35034	36043	35021	34026	35049	35064	34071	34077	34076	34066	34049	33044	27025	33013	35015	26032	29052	30036	33027	704918	29371.6	34140	1305	18	32032	31012	0	28016	14015	
0	29046	32052	31049	30053	29074	30075	28053	26052	26055	24059	25063	27054	28033	29019	33014	2011	3024	2031	539892	22495.5	29131	1046	19	3017	0	0	0	4012	15013	12014
4038	3033	29025	26025	33038	35053	35052	35066	34042	26035	19038	20026	28014	29019	29023	30018	36017	485618	20234.1	36126	1142	20	1017	29054	28069	28061	29057	29053	27033	25018	29028
29039	28022	21015	19020	21032	23031	19036	21036	23025	30013	1015	2018	36019	36014	0	534725	22280.2	29132	205	21	0	4012	0	34012	32013	0	0	0	25015	25020	22016
16020	31032	32036	32043	22038	22024	23023	30017	36019	36020	32048	33021	34028	521457	21727.4	33124	2127	22	35029	35026	35025	36040	36046	33039	33045	34046	36050	32043	35052	34065	35067
34056	33059	33053	34044	4033	8031	4013	36017	3014	3014	33017	674924	28121.8	36128	1241	23	1023	34015	36015	1019	34032	33035	1024	1023	15014	35084	36078	35062	34060	34052	32040
29027	12049	15021	35015	36018	35016	36015	36016	0	596753	24864.7	36161	949	24	0	0	0	29042	30057	30048	27048	27057	29059	31056	30052	29072	28064	26055	26062	26064	27066
28059	29050	29043	30044	31045	30057	29060	602160	25090.0	30156	1125	25	31047	32042	2023	33020	0	12015	0	8016	0	13014	4027	33050	35044	33043	27026	16040	21042	21034	25021
28013	0	36013	0	1013	41543	17147.6	34104	1136	26	1013	34012	0	1016	0	1013	0	3012	0	21017	22016	22019	22032	21049	22052	22056	24055	27060	30049	30063	30051
31048	30058	27049	421740	17572.5	27122	1743	27	29054	30040	32024	1014	36025	36021	3013	0	0	5015	8020	6023	6027	9029	13060	12059	11054	12036	0	36019	36019	36019	0
0	357571	14898.8	26099	4	28	0	36013	35011	0	2012	0	1017	36012	7013	2023	2049	34057	33065	33060	33039	29027	15046	20027	27020	28022	25019	26016	33012	35015	492575
20524.0	32125	1336	29	7019	7019	30020	26021	0	29017	28030	29041	29045	27042	31018	9022	18027	19032	23032	23048	23049	25044	27032	27038	28035	30042	33050	28024	556747	23197.8	25093
1555	30	25037	26020	28015	29022	27021	26014	27015	25018	18021	17011	15041	16047	16049	16048	16049	16035	15043	16042	16039	14034	13039	14044	14050	15045	460799	19200.0	14110	2232	31
16056	16054	16057	15055	15040	15030	24029	21041	24043	25045	25031	25032	25036	26033	28039	28036	29021	31018	28025	31034	36014	2015	2016	34022	537822	22409.3	25112	934			
avg	13960	18733	16795	17861	17216	21314	19380	17284	18381	20971	21717	24624	24434	23949	25243	24112	22270	21713	23869	25480	24703	22833	18606	19248	504696	21029.0	25508	0	0	0

HOURLY DATA : Wind

0034763 X UITENHAGE e 2001/09

DAY h01 h02 h03 h04 h05 h06 h07 h08 h09 h10 h11 h12 h13 h14 h15 h16 h17 h18 h19 h20 h21 h22 h23 h24 tot avg mx mxtm mn mntm

132031 28025 30020 29029 35024 36016 36017 36017 33019 31037 28039 27037 27032 22041 22035 21040 21036 20027 23018 29011 34013 36017 36017 2014 674612 28108.8 26079 1110
2 2027 3016 28015 36012 0 3013 7013 27018 25016 24017 22016 15014 18024 20035 21042 22049 23043 23032 24026 29013 35016 0 2014 36022 445493 18562.2 22087 1534
3 3013 0 3017 2034 2033 2034 3033 3029 4027 2051 2059 36042 36047 36060 35039 30042 22039 20033 23037 26029 27045 28045 29039 33020 407847 16993.6 36122 1349
432015 30046 29051 28058 29064 30044 29040 30018 26018 29034 26039 26048 26052 26045 25044 22047 24045 24034 25015 0 2018 4024 3027 4035 529861 22077.5 29117 253
5 6023 9022 6026 5012 3024 3042 1030 33033 3030 2038 35056 34102 34117 33119 26077 25056 23025 23022 31013 3013 2013 34022 32040 31043 437998 18249.9 31230 1145
629029 31048 30081 30097 30077 30075 30088 30083 30080 30089 30087 29077 28083 28080 28082 28071 29062 27062 27050 27048 28046 28041 29048 29047 696631 29026.3 31197 310
730042 31037 33026 3014 2015 2014 2015 0 14013 24017 18019 8018 13017 13023 13048 13051 14042 12038 8012 0 7016 5023 5024 0 270524 11271.8 13107 1428
8 7017 7015 7012 7014 6020 5036 4024 18012 25020 25017 26021 25019 31027 33051 17036 15043 14021 18015 28018 36015 36020 2015 4011 35013 431512 17979.7 36109 1314
9 1012 0 0 36015 0 0 29022 31020 24018 24033 24042 24039 24050 20044 21047 21042 21040 19023 0 2017 4012 3032 2025 27016 357549 14897.9 22087 1441
10 1012 0 3015 2013 2012 0 36016 5013 0 21015 15016 19021 25022 21019 14028 13039 19040 19031 21025 25022 27028 27024 29035 28035 372481 15520.0 15078 1557
1126048 27045 25038 27046 28060 30068 31068 30064 30068 30077 30074 29075 29077 29080 27073 27064 27075 28067 29049 29054 27042 27059 29050 31050 683471 28478.0 27143 1117
1231059 30057 30033 32018 0 27014 30036 30048 29046 25040 26039 25033 19028 18033 23028 21037 20037 21025 25025 26023 27021 24029 27023 27018 593750 24739.6 30099 1
1326018 26015 27018 26021 27022 27022 27020 0 27019 27018 19033 17048 17038 15039 15048 15053 17038 17018 15022 16012 3016 8022 4017 29014 447591 18649.6 18112 1636
14 1017 1012 36014 4017 6021 6022 7023 8020 9029 10054 11072 11082 12096 13103 13103 13094 12097 12078 11061 11064 11062 10072 10050 8051 247314 10304.8 14157 1325
15 9038 8030 8030 7024 5022 6019 8017 6019 7025 8033 8034 9035 8033 9036 14040 16034 17016 34035 5018 5026 3026 36023 6028 6018 248659 10360.8 35151 1750
16 2027 34012 2017 2018 4014 34033 33049 32039 31027 30039 28054 27041 27047 26037 24043 23041 22038 22030 22022 28018 29017 28017 25019 0 535699 22320.8 27106 1051
17 0 0 36014 0 0 4012 2012 0 0 10023 12026 12030 12025 14028 6014 10020 8022 7021 8021 9018 0 11029 12032 11036 184383 7682.6 11088 2301
1811028 9029 8020 3011 0 5012 9017 3026 5026 8028 9016 10028 5020 36017 28021 27016 0 23014 9027 31011 27019 29021 25012 29012 349431 14559.6 10072 1824
1929012 26016 28015 0 5016 1016 0 0 12012 26018 28028 26024 25027 26019 21029 19028 23035 25041 27029 28023 28021 24011 30012 3012 460444 19185.2 26085 1723
2035011 3012 0 36012 36014 32012 31016 26025 25023 26028 24032 21027 21031 21028 20023 20022 18020 19018 21021 25012 0 0 0 0 460387 19182.8 21080 1031
21 0 27017 27019 23013 0 0 0 27011 27011 28017 25014 14021 11030 11031 13035 13039 13043 12035 12018 12025 11021 11035 12029 11024 340488 14187.0 12079 1635
2210019 11015 0 12026 13035 13035 14030 13031 12034 13049 12056 12048 13033 13033 13040 13045 13055 13054 12054 9037 8016 15015 13013 12020 282793 11783.0 14104 1708
23 9017 0 0 0 31012 0 0 7014 11017 9029 5025 6028 6033 5030 7034 13048 12053 13025 20020 11038 1043 2019 3033 4020 175538 7314.1 8119 1954
2436033 35066 26019 97118 32372.7
25 0
26 0
27 23049 24034 23033 23031 22031 18021 12037 11016 4015 4022 4026 4028 4031 176374 13567.2
28 4031 4031 5026 4036 11016 12015 0 25012 24013 35038 36075 36069 33053 31036 24029 21026 17037 14025 6013 2018 2018 1017 2015 36016 385665 16069.4 36137 1004
29 0 0 0 2011 0 0 0 0 0 7024 7030 8030 10035 13063 12058 13048 13038 14033 13014 34013 35015 36016 0 35013 252441 10518.4 14094 1252
3019016 0 0 0 2012 0 0 0 0 3045 2051 1059 36054 34051 31039 25033 16035 21036 24042 28051 29053 29055 28059 27057 355748 14822.8 28117 2255

avg14504 14095 15834 13714 10674 11867 14215 16175 16677 19535 19579 19672 21154 21860 19895 19339 17669 18997 17803 17986 16579 17137 14878 18468 408306 17012.8 22883 0 0 0

CODES USED:
A = No observation. Part of an accumulation period.
B = Rainfall occured but amount unknown.
Part of an accumulation period.
C = Total of accumulated rainperiod
# = Prelimanary data
--- = Data not requested
*** = Data not received yet or missing.

Rain Data for station [0034763 X] - UITENHAGE e 2001 08H00

Day	JAN	FEB	MAR	APR	MAY	JUN	JUL	AUG	SEP	OCT	NOV	DEC
01	---	---	---	---	---	---	---	#	#	---	---	---
02	---	---	---	---	---	---	---	#	#	---	---	---
03	---	---	---	---	---	---	---	8.8#	#	---	---	---
04	---	---	---	---	---	---	---	6.2#	#	---	---	---
05	---	---	---	---	---	---	---	0.8#	#	---	---	---
06	---	---	---	---	---	---	---	#	#	---	---	---
07	---	---	---	---	---	---	---	#	#	---	---	---
08	---	---	---	---	---	---	---	#	#	---	---	---
09	---	---	---	---	---	---	---	0.4#	0.4#	---	---	---
10	---	---	---	---	---	---	---	3.0#	#	---	---	---
11	---	---	---	---	---	---	---	#	#	---	---	---
12	---	---	---	---	---	---	---	#	2.8#	---	---	---
13	---	---	---	---	---	---	---	#	3.6#	---	---	---
14	---	---	---	---	---	---	---	#	2.8#	---	---	---
15	---	---	---	---	---	---	---	#	3.6#	---	---	---
16	---	---	---	---	---	---	---	#	#	---	---	---
17	---	---	---	---	---	---	---	#	2.4#	---	---	---
18	---	---	---	---	---	---	---	#	8.8#	---	---	---
19	---	---	---	---	---	---	---	#	#	---	---	---
20	---	---	---	---	---	---	---	#	#	---	---	---
21	---	---	---	---	---	---	---	#	4.6#	---	---	---
22	---	---	---	---	---	---	---	#	5.0#	---	---	---
23	---	---	---	---	---	---	---	#	#	---	---	---
24	---	---	---	---	---	---	---	#	#	---	---	---
25	---	---	---	---	---	---	---	#	#	---	---	---
26	---	---	---	---	---	---	---	#	#	---	---	---
27	---	---	---	---	---	---	---	#	#	---	---	---
28	---	---	---	---	---	---	---	3.6#	#	---	---	---
29	---	---	---	---	---	---	---	3.6#	#	---	---	---
30	---	---	---	---	---	---	---	23.6#	#	---	---	---
31	---	---	---	---	---	---	---	15.8#		---	---	---

SUNRISE(SR) AND SUNSET(SS) AT: UITENHAGE LAT -33.7 S LONG 25.4 E TIME ZONE 30.0 E MEAN YEAR

DATE	01	03	05	07	09	11	13	15	17	19	21	23	25	27	29	31
JAN/SR	05:12	05:13	05:15	05:16	05:18	05:20	05:22	05:23	05:25	05:27	05:29	05:31	05:33	05:35	05:37	05:39
JAN/SS	19:31	19:31	19:31	19:31	19:31	19:31	19:31	19:30	19:30	19:29	19:28	19:28	19:27	19:25	19:24	19:23
FEB/SR	05:40	05:42	05:44	05:45	05:47	05:49	05:51	05:53	05:55	05:57	05:58	06:00	06:02	06:04		
FEB/SS	19:22	19:21	19:19	19:17	19:16	19:14	19:12	19:10	19:08	19:06	19:03	19:01	18:59	18:56		
MAR/SR	06:05	06:07	06:09	06:10	06:12	06:14	06:15	06:17	06:18	06:20	06:22	06:23	06:25	06:26	06:28	06:29
MAR/SS	18:54	18:51	18:49	18:46	18:44	18:41	18:38	18:36	18:33	18:30	18:28	18:26	18:23	18:20	18:18	18:15
APR/SR	06:30	06:31	06:33	06:34	06:36	06:37	06:39	06:40	06:42	06:43	06:45	06:46	06:48	06:49	06:50	
APR/SS	18:14	18:11	18:08	18:06	18:03	18:01	17:58	17:56	17:53	17:51	17:48	17:46	17:44	17:42	17:40	
MAY/SR	06:52	06:53	06:55	06:56	06:58	06:59	07:01	07:02	07:04	07:05	07:06	07:08	07:09	07:10	07:11	07:13
MAY/SS	17:38	17:36	17:34	17:32	17:31	17:29	17:27	17:26	17:25	17:24	17:22	17:21	17:20	17:20	17:19	17:18
JUN/SR	07:13	07:14	07:15	07:16	07:17	07:18	07:19	07:20	07:20	07:21	07:21	07:22	07:22	07:22	07:22	
JUN/SS	17:18	17:17	17:17	17:17	17:17	17:17	17:17	17:17	17:17	17:17	17:17	17:18	17:18	17:19	17:19	17:20
JUL/SR	07:22	07:22	07:22	07:22	07:21	07:21	07:20	07:20	07:19	07:18	07:17	07:16	07:15	07:13	07:12	07:10
JUL/SS	17:21	17:22	17:22	17:23	17:24	17:25	17:27	17:28	17:29	17:30	17:31	17:33	17:34	17:35	17:37	17:38
AUG/SR	07:10	07:08	07:06	07:04	07:03	07:01	06:59	06:56	06:54	06:52	06:50	06:47	06:45	06:42	06:40	06:37
AUG/SS	17:39	17:40	17:41	17:43	17:44	17:45	17:47	17:48	17:50	17:51	17:52	17:54	17:55	17:56	17:58	17:59
SEP/SR	06:36	06:33	06:31	06:28	06:25	06:23	06:20	06:17	06:14	06:12	06:09	06:06	06:04	06:01	05:58	
SEP/SS	18:00	18:01	18:02	18:04	18:05	18:06	18:08	18:09	18:10	18:12	18:13	18:14	18:16	18:18	18:19	
OCT/SR	05:56	05:53	05:50	05:48	05:45	05:42	05:40	05:37	05:35	05:32	05:30	05:28	05:26	05:23	05:21	05:19
OCT/SS	18:20	18:22	18:23	18:25	18:26	18:28	18:29	18:31	18:33	18:34	18:36	18:37	18:39	18:41	18:43	18:44
NOV/SR	05:18	05:17	05:15	05:13	05:11	05:10	05:09	05:07	05:06	05:05	05:04	05:03	05:03	05:02	05:02	
NOV/SS	18:45	18:47	18:49	18:51	18:53	18:54	18:56	18:58	19:00	19:02	19:04	19:05	19:07	19:09	19:11	
DEC/SR	05:01	05:01	05:01	05:01	05:01	05:02	05:02	05:03	05:03	05:04	05:05	05:06	05:07	05:08	05:09	05:11
DEC/SS	19:13	19:14	19:16	19:17	19:19	19:20	19:22	19:23	19:24	19:26	19:27	19:28	19:28	19:29	19:30	19:30

Rain Data for station [0035334 3] - ADDO 2001 08H00

Day	JAN	FEB	MAR	APR	MAY	JUN	JUL	AUG	SEP	OCT	NOV	DEC	
01													#
02				0.4									#
03				2.9				3.4			28.0		#
04					7.2			6.1			4.0		#
05								0.5					#
06			2.5	5.0							0.3		#
07				2.5									#
08				4.0							1.7		#
09				6.0				2.0					#
10								5.4					#
11													#
12		1.2							7.0				#
13	65.5	0.4		2.3					2.5				#
14	20.0								4.5	0.3	5.4		#
15									3.0	3.5	0.5		#
16			4.0	35.0									#
17			22.5	12.7		0.4			2.2		2.5		#
18	2.5								9.8		4.0		#
19		0.4									14.5		#
20							0.4				2.8		#
21				2.3					0.3				#
22				7.2			20.2		2.5				#
23		0.7					5.4		23.5				#
24													#
25						0.6					0.3		#
26									11.6		0.6		#
27	2.5							0.0	2.1	0.8			#
28	0.2						1.1	1.0		4.5			#
29					1.4	0.6		1.9			3.5		#
30			0.2					5.5			0.1		#
31			11.5					17.2					#