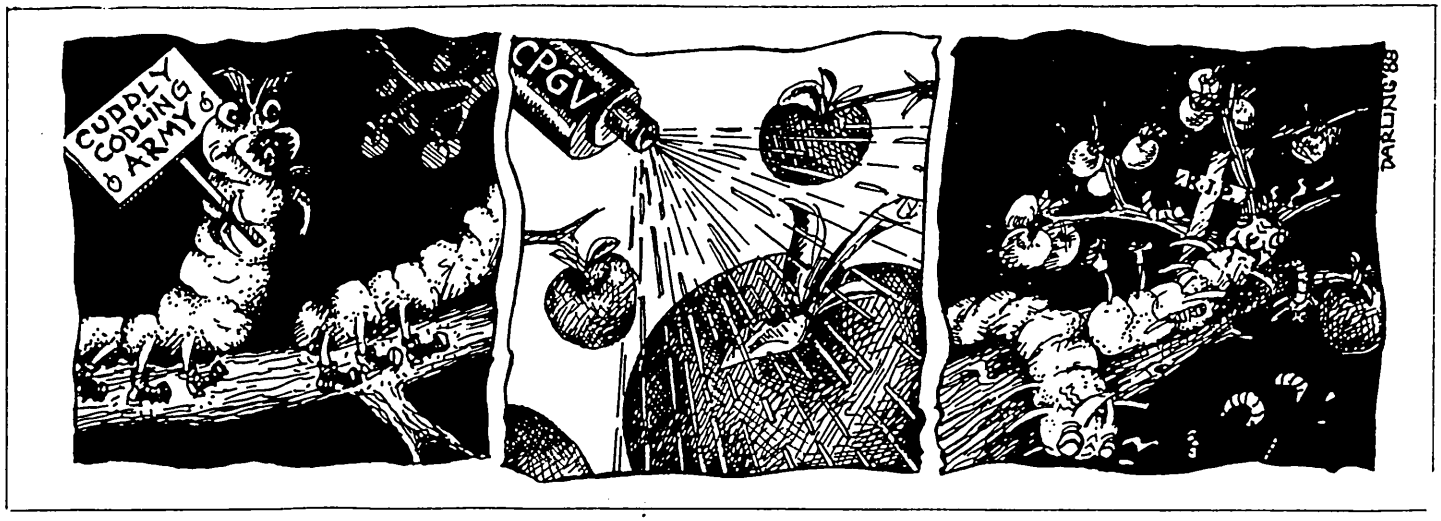
Development of *Cydia pomonella* granulosis virus for biological control in orchards

by

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ABSTRACT

The codling moth, *Cydia pomonella*, is an important worldwide pest of apples, pears and walnuts and can be controlled by the biological insecticide *Cydia pomonella* granulosis virus (CpGV). Laboratory and field experiments were performed to improve the production, formulation and application of CpGV; investigate its uptake by host larvae from plant surfaces; evaluate its impact on non-target arthropods and to assess its potential as part of an orchard integrated pest management (IPM) programme. CpGV was also tested in field trials in commercial orchards.

CpGV was compatible with a range of application machinery and was effective from ultra-low to high-volume rates. It was generally compatible with most pesticides commonly used in orchards. No spray additives tested were effective at extending the persistence of CpGV on foliage. However, increasing the frequency of sprays largely overcame this problem.

Codling moth larvae were shown to ingest CpGV from fruit and leaf surfaces and the rate of ingestion could be increased with spray additives believed to act as feeding stimulants. Such additives gave dramatic improvements in fruit damage control. The shelf-life of CpGV could also be improved by formulation.

CpGV application obviated the use of acaricides for the control of pest mites since, in the absence of deleterious effects of many conventional pesticides, predatory mites and insects were able to thrive. CpGV was shown to provide the basis for an IPM programme, although lack of effective control measures for leafrollers (Tortricidae) remains a drawback.

Procedures for codling moth rearing and virus production were evaluated and a number of impure and dry preparations were tested in laboratory and field trials. An enzyme-linked immunosorbent assay (ELISA) was developed to measure the amount of virus in impure preparations considered suitable for industrial production of CpGV.

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INTRODUCTION

Increasing costs of chemical pesticides, concerns over safety to the farm worker, the consumer and 'the environment' and the steady increase in incidence of pest resistance to conventional insecticides has prompted considerable research over the last three decades into judicious and reduced pesticide usage or 'integrated pest management' (IPM). Much emphasis has been placed on the development of selective control agents targeted only at pest species and which would not disrupt beneficial and non-pest insects and mites. All insects are susceptible to diseases, and many insect pathogens are highly infectious and relatively specific to their host species. Yet, in spite of extensive research effort, few insect pathogens have been successfully exploited as pest control agents. Various strains of the bacterial insecticide *Bacillus thuringiensis* (B.t.) have been widely used against agricultural and medical insect pests and have been developed commercially for world-wide use. Two insect viruses are currently produced commercially: *Neodiprion sertifer* nuclear polyhedrosis virus (NPV) for control of pine sawfly, and *Mamestra brassicae* NPV chiefly for the control of cabbage moth (Payne, 1988). Only one fungal insecticide, *Metarhizium anisopliae*, is available for use in field crops, against sugar cane pests in Brazil, whereas commercial products of another species, *Verticillium lecanii*, are restricted to the humid environment of glasshouses (favouring spore germination and growth) for aphid and whitefly control (Payne, 1988). The sparsity of available commercial insect pathogens as control agents may be attributed to a number of factors: lack of research (many agents are still at the 'developmental stage'); too costly to produce; or merely lack of efficacy in controlling their target pests in field situations.

One agent which may well prove suitable for commercial exploitation, is the granulosis virus for control of the codling moth, *Cydia pomonella*, the worldwide pest of apples, pears and walnuts. *Cydia pomonella* granulosis virus (CpGV) has been field tested on apples since the mid 1960's (Falcon *et al.*, 1968), and has stimulated research and commercial interest in Europe, North and South America, and Australia. Following earlier studies in collaboration with Long Ashton Research Station, research at the AFRC Institute of Horticultural Research (IHR) has been funded over three years

by the Agricultural Genetics Company, with the aim of developing CpGV for use in orchards as part of an integrated pest management (IPM) programme. Although adequate control of codling moth has been achieved with CpGV (eg. Glen and Payne, 1984), previous research has highlighted problems such as poor foliar persistence, mass production difficulties and its narrow host range, which, while having beneficial effects such as the preservation of beneficial insects and mites, also creates additional requirements for the control of non-host pests. Additional work was thus necessary to overcome problems which would otherwise limit the commercial development of CpGV, improve efficacy, and evaluate its performance over several seasons at different sites. Emphasis was placed on optimizing its use strategy, improving efficacy by formulation, developing a product suitable for mass production with adequate shelf-life, and evaluating its efficacy as a control agent and its potential for incorporation within a full IPM programme.

Chapter 1

Literature Review

I. Nature and biology of the pest

The codling moth, *Cydia pomonella* (Lepidoptera: Tortricidae) is a serious worldwide pest of apples, pears and walnuts. Fruit damage is caused by larvae boring into ripening fruits, usually rendering them unsaleable. Although codling moth is a pest in temperate and tropical regions, the severity of the pest problem varies with climate. In the relatively cool conditions of Northern Europe, codling moth is usually restricted to one generation (eg. Sweden; Subinprasert, 1988), although exceptionally warm spring and summer conditions can result in a second pest generation; as recorded in the UK in 1976 (Glen *et al.*, 1976). There are usually two generations in Southern Germany (Huber and Dickler, 1977), three in Chile (Gonzalez, 1982), whilst in Tunisia four generations occur in a single season, though the last one does not cause major fruit damage (Mussche and Garbous, 1986). The codling moth can cause considerable damage at low density, with only a few adult females able to lay large numbers of eggs close to fruits (Wearing, *et al.*, 1973); even relatively few damaged apples create extra problems for selection prior to storage if secondary problems such as fruit rotting are to be avoided.

The codling moth is a 'k-strategist' existing - under natural conditions - in a long-term close relationship with its k-strategist host (Way, 1978). The codling moth has relatively low fecundity and slow development rate, it is restricted to a few perennial host plant species and does not over-exploit its food resource, damaging only a fraction of the total fruit and, even when damaged, fruit are more often able to produce some viable seeds (Way, 1978). The codling moth is subject to a number of density-dependent and partially density-dependent mortality factors at various life stages (see below) and these, and its close non-exploitative host relationship, make the codling moth a regular and consistent pest not subject to the massive population fluctuations of r-strategist, exploitative pests such as cereal aphids.

In the UK, adults emerge from mid June to mid July, though in warm springs emergence may commence in late May with second-generation adults

emerging in August. Females lay between 40 and 50 eggs (Geier, 1963) with an oviposition preference for the upper tree canopy (Richardson and DuChanois, 1950a and 1950b). Oviposition occurs during warm evenings (Alford, 1984) and is adversely affected by both rainfall and low temperature (Hagley, 1976) but stimulated by apple odour (Wearing *et al.*, 1973). Eggs are laid singly (Geier, 1963; Subinprasert and Svensson, 1988) and, in field studies, seventy-five percent of eggs were laid on leaves with 18% on fruits and 7% on twigs - none were laid on the underside of leaves (Subinprasert and Svensson, 1988). Jackson (1979) found that only 57% of eggs were laid on the upper side of the leaves with 35% laid on lower leaves. Richards (1984) found no preference for upper or lower leaf surface, although his use of laboratory reared insects in cage studies may explain his observations. Eggs hatch in about ten to fourteen days in the UK, although this may be much longer in cold weather (Alford, 1984). In the warmer climate of the Australian Capital Territory, mean egg development is complete in seven days (Geier, 1963). The preference for oviposition on foliage rather than fruit is likely to result in reduced risks, from, for example, fruit drop and dislodgement from the expanding fruit (Jackson, 1979).

Larvae pass through five instars, mostly spent inside a single fruit. Larvae are attracted to moisture and apple skin odour (Sutherland, 1972 and 1975; Subinprasert, 1988). Using laboratory reared larvae, Glen and Clark (1985) found that 52% of larvae entered apples via the calyx, 26% within 10 mm of the stalk and only 22% entered through the sides. Subinprasert (1988) also found that the calyx was the preferred site of entry, whereas Geier (1963) found that only 18-19% of entries were via the calyx whilst 61-63% were via free surfaces; differences in apple varieties or climatic differences could perhaps explain these different findings. Although it is rare to find several larva in a fruit, more than one larvae do sometimes penetrate the fruit surface. In small fruits this often leads to immediate cannibalism with the "winner" of a confrontation suffering no disadvantage, whilst in larger fruits, competition may result in poor development of both surviving larvae (Geier, 1963). When fully grown, larvae vacate the fruit and find suitable overwintering sites, e.g. under bark and in cracks in the tree trunk (Alford, 1984). Codling moth overwinter as larvae, in silken cocoons, pupating the following spring.



Plate 1.1 Codling moth (*Cydia pomonella*) Adult

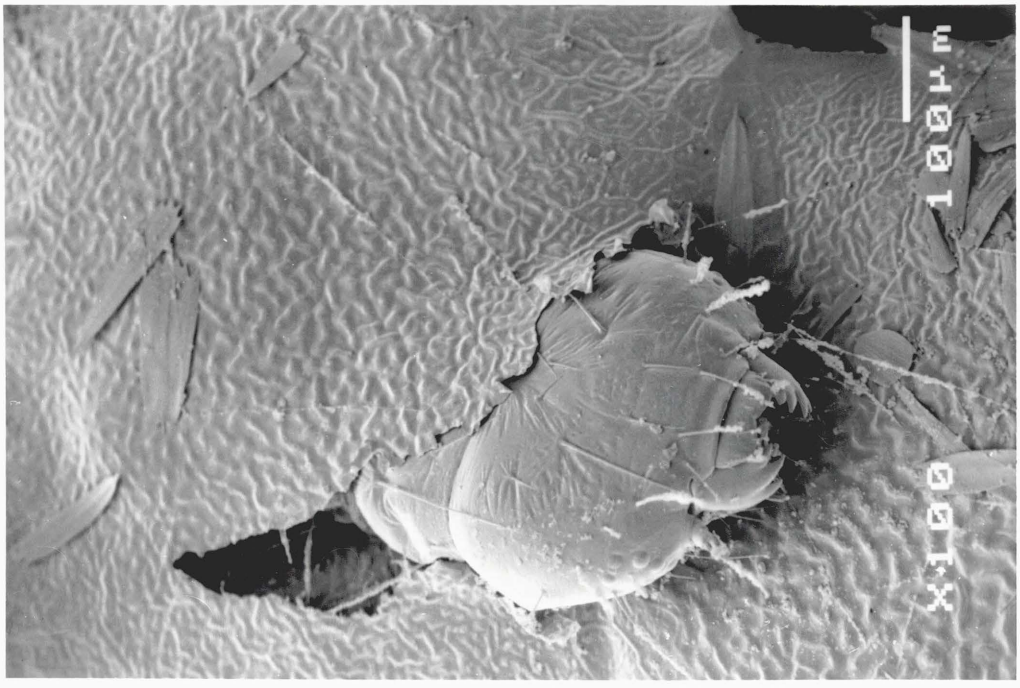


Plate 1.2 Codling moth larvae emerging from egg (Scanning electron micrograph)

II. Natural control

A number of mortality factors affect the different life stages of the codling moth. Survival of eggs is typically around 50% (Ferro *et al.*, 1975) with both physical (above) and biological factors contributing to egg mortality. In UK trials, predators took 28-62% of eggs, with earwigs, *Forficula auricularia*, killing 7-13% in the first generation and 33-34% in the second generation, and heteropteran predators responsible for 18-50% predation (Glen *et al.*, 1976). The seven-spot ladybird, *Coccinella septempunctata*, was found important in Sweden (Subinprasert, 1988). In studies in the UK, the number of eggs killed depended on the searching ability, size and number of earwig and heteropteran predators, the number of alternative prey (spider mites) and the weather conditions which affect predator searching ability (Glen and Brain, 1978). Egg predation is density-independent; high numbers of predators are thus likely to cause a significant reduction in the overall population, regardless of the initial egg density (Glen, 1982). By contrast, predation of larvae by birds such as blue tits, *Parus caeruleus* (below), is density-dependent and thus, to some extent, regulates the population from year to year (Glen, 1982, Subinprasert, 1987). Bird predation is, however, under-compensating and, as a consequence, a high level of egg predation has a carry-over effect into the next season (Glen, 1982).

Of the five instars, mortality is highest in the first instar - particularly in the fruit searching period, with 65% of released larvae failing to get inside fruit (Ferro *et al.*, 1975). Losses increased with the distance from fruit and with brood size (Subinprasert, 1988). Laboratory reared larvae are highly susceptible to infection with a granulosis virus, and yet, although it was first isolated from larvae in a natural codling moth population in Chihuahua, Mexico (Tanada, 1964), it has never been observed in the wild as a significant mortality factor.

Bird predation causes up to 95% reduction in the number of overwintering larvae (Solomon and Glen, 1979) and also affects the sex ratio of surviving larvae. Despite an initially equal sex ratio, after 95% predation the ratio of males to females had changed to about 2:1. Females have larger head capsules than males and were found less able to squeeze into small holes; as a consequence males were able to find better hiding places from predators (Glen *et al.*, 1981a). Overwintering larvae are also

killed by parasitism (Zech, 1959), and fungal diseases (Glen, 1982). In the UK the pupal parasite, *Pimpla turionellae* accounted for 75% of the parasites emerging from pupae (Glen *et al.*, 1977), whereas in Sweden, the tachinid *Elodea morio* accounted for 50% of the parasitism with the braconid *Ascogaster quadridentatus* and the ichneumonid *Pristomerus vulnerator* also important (Subinprasert, 1987 and 1988). *E. morio* was less common in Germany, responsible for only 7.5% of the parasitism (Zech, 1959).

III. Baculoviruses and their use in pest control

The biology and use of baculoviruses in field control have been reviewed by, for example, Hunter *et al.*, (1984) and Payne (1986 and 1988). However, some fundamental details are included in this review for their relevance to the research reported here.

Baculoviruses are a group of viruses found exclusively in arthropods and are biochemically dissimilar to viruses of vertebrates, plants or microorganisms (Payne, 1988). They are often specific to a single family, genus or even (as far as is known) a single species. Baculoviruses are rod-shaped DNA viruses, categorised by their structure into three groups: granulosis and nuclear polyhedrosis viruses (occluded viruses) consisting of virus particles enclosed within a proteinaceous 'occlusion body'; and a third, 'non-occluded' group lacking this proteinaceous coat . The occlusion body is a stable structure produced late in the infection process and serves to protect virus infectivity when outside the host (Payne, 1988).

Infection with occluded viruses occurs when host insects consume virus-contaminated food. After ingestion, the occlusion body dissolves in the insect gut releasing the virus particle which passes through the midgut peritrophic membrane, and infects and multiplies in the gut epithelial cells. A lipoprotein envelope surrounding the virus particle, fuses with host cell membranes allowing the nucleocapsid to migrate to the nucleus where replication occurs. In Lepidoptera, the infection soon spreads to other host tissues; particularly fat body, haemocytes and hypodermis. The midguts of lepidopteran larvae, like other phytophagous insects, are generally alkaline - probably to aid degradation of tannins -

and the high pH causes the dissolution of the occlusion body (Hunter *et al*, 1984).

In most cases, successful pest control has only been achieved by regular application of insect viruses, and only in a few cases (eg. control of rhinoceros beetle, *Oryctes rhinoceros* with a non-occluded baculovirus (Bedford, 1980)) have classical biological control attempts been successful with an epizootic resulting from a single introduction of a baculovirus to a population (Payne, 1988). Unlike *Oryctes* virus, horizontal transmission to conspecifics is quite often poor - in the case of codling moth, this results from a solitary host life style, relatively rapid host death (with little chance of infected larvae contacting other larvae) and poor persistence outside the host (despite the occlusion body; see section VII). For most baculoviruses, an inundative application strategy is more likely to be successful; their ability to remain viable outside the host, and their high infectivity (a single CpGV granule is sufficient to kill a host larva, (Sheppard and Stairs, 1977) allow them to be sprayed on foliage like conventional insecticides. Ironically, this 'drawback' of the need to re-apply on a regular basis, is likely to generate commercial interest by providing a product with a continuous market. Unfortunately, death from naturally occurring viral 'insecticides' cannot match the activity of conventional insecticides - even with the most virulent baculoviruses, death directly from virus infection is unlikely to occur less than 3-4 days after ingestion (Payne, 1988). Their success depends on optimizing their application and, perhaps, on developing a use-philosophy different from conventional insecticides, where a slower death of the target pest is tolerated in return for the ecological benefits of reduced environmental impact, preservation of natural control of many 'secondary pests' and decreased pesticide residues.

Although a number of insect viruses have reached the stage of commercial trials, only two are currently available commercially: *Neodiprion sertifer* NPV, for pine sawfly control, and *Mamestra brassicae* NPV for the control of cabbage moth and other Noctuid pests. The likelihood of increasing commercial interest in baculoviruses should result if attempts to improve their host-range and toxicity by genetic manipulation are successful. Speed of kill could be increased, for example, by inserting genes that code for insecticidal toxins (eg. *Bacillus thuringiensis*

endotoxin) or insect neuropeptides. Improvements in production techniques - *in vivo*, or in cell-culture - are also essential for the commercialisation of natural, and any future genetically altered, viruses (Ballard *et al* 1986, Payne, 1988).

IV. *Cydia pomonella* granulosis virus (CpGV): isolation, structure and host range

CpGV is a member of the granulosis subgroup of baculoviruses, which are characterised by single virus particles within a double membrane inside a granular protein capsule or 'granule' (Plate 1.3). Each granule measures about 300x400 nm, and is usually smoothly cylindrical (though sometimes cuboidal and larger) (Stairs *et al*, 1968). The virus was first isolated in Mexico in 1963 (Tanada, 1964) and has since been found in laboratory cultures in North America and Europe and from crab apples in Hungary in 1974 and 1975 (Laszló and Maté, 1975) although the possibility of accidental spread by human activity cannot be ruled out in these later isolations. CpGV isolated from several regions of the world differ very little, genetically, from the original Mexican isolate with only minor changes in the so-called "English" and "Russian" strains. Crook (1986) suggests that this may be because each isolate has, knowingly or unknowingly, originated from the original Mexican strain. Payne et al (1984) tested three different isolates of CpGV by electrophoretic analysis of the DNA fragments after treatment with restriction enzymes; a "Reading" isolate differed in a single region of the genome and confirmed differences reported by Harvey and Volkman (1983). Claims by Harvey and Volkman (1983) of 100-fold reduction in infectivity were not supported by Payne et al (1984) in bioassay studies. The precise region of the granulin gene has been determined in relation to the whole genome (Crook, 1986).

In addition to codling moth, CpGV also infects a number of other tortricids: the pea moth, Cydia nigricana (Payne, 1981); the European pine shoot moth, *Rhyacionia buoliana* (Huber and Dickler, 1977 and Huber, 1978b); the oriental fruit moth, *Grapholitha molesta* (Falcon *et al.*, 1968); the plum fruit moth *Cydia funebrana* and *Lathronympha strigana* (Huber 1982).

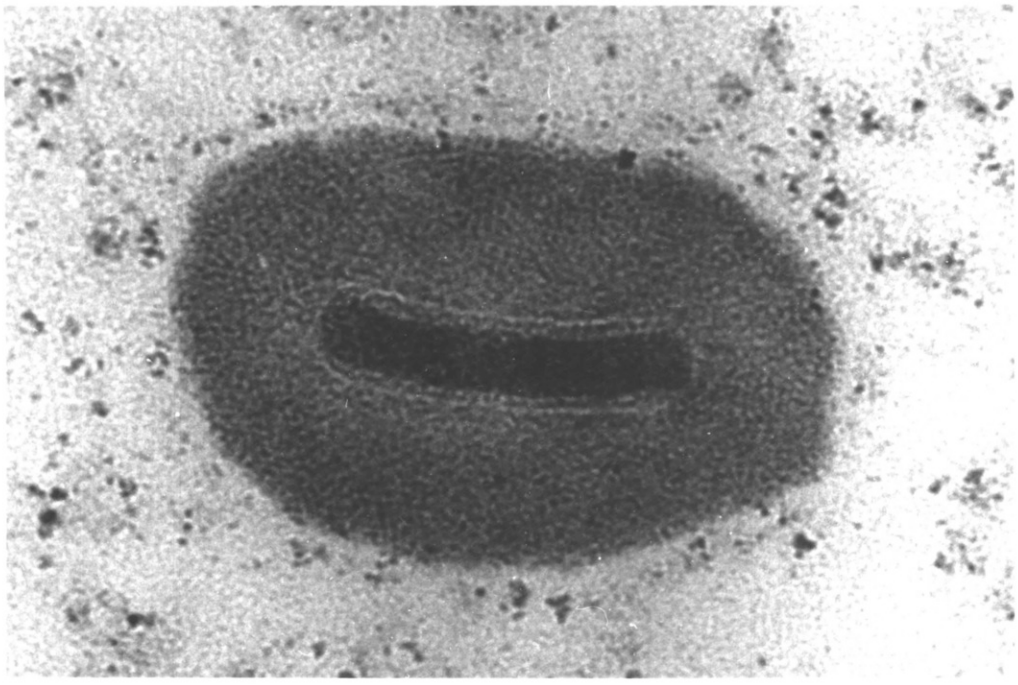


Plate 1.3 *Cydia pomonella* granulosis virus (Transmission electron micrograph)

V. Bioassay and virus infectivity

The infectivity of CpGV, or any insect pathogen is measured by bioassay, in which a number of insects are dosed with the pathogen and the response, ie death, is measured. The dose/mortality response can be converted effectively to a straight line by plotting log dose against probit mortality. The fiducial limits of the line are smallest at 50% mortality (ie the medial response). Hence, the most useful value for the comparison of different preparations, formulations, or isolates, is the dose giving 50% mortality (eg. the 50% log dose, LD_{50} or the 50% log concentration, LC_{50}). The slope of the probit mortality/dose response is typically low for insect pathogens - generally between 0.8 and 2.1 for insect viruses - meaning that a large increase in dose is required to achieve a small increase in mortality. The shallower the slope of the response also means that more larvae must be used at each dose in a bioassay to achieve comparable levels of precision (Burges and Thomson, 1971). Slopes of a number of different bioassay techniques for CpGV surveyed by Huber (1986) ranged from about 1.2-2.0. Discrimination of two CpGV samples by bioassay is, as a consequence, only possible to a factor of, at best, two-fold (Huber, 1986).

Sheppard and Stairs (1977) estimated the median dose responses for neonate and mature codling moth larvae; the response varied considerable with age:

LD_{50}: First instar = 5; Fifth instar = 49.
Slope; First instar = 1.35; Fifth instar = 0.35.

The shallow response slope for fifth instars indicates a less uniform response than for first instars; 1 granule should cause the death of 25% of both instars but, for 70% mortality, the calculated dose would be 12 granules for first instars and 1578 for fifth instars.

VI. Use of CpGV

CpGV was first tested as a biological insecticide in 1966, in Northern California (Falcon *et al.*, 1968). Since then it has been extensively field

tested in many countries: Australia (Morris *et al.*, 1971): Switzerland (Keller, 1973); West Germany (Huber and Dickler, 1975, 1976, 1977, 1978a, Dickler, 1977, 1984, Dickler and Huber, 1978a, 1978b, 1983, 1986, 1988); USA (Sheppard and Stairs, 1976, 1977); Canada (Jaques *et al.*, 1977, 1981, Jaques and Laing, 1982); Austria (Fischer-Colbrie, 1978); the United Kingdom (Glen *et al.*, 1980, 1981b, Payne *et al.*, 1983, 1984, Glen and Payne, 1984, Richards, 1984); France (Audemard *et al.*, 1983, Audemard and Burgerjon, 1984, 1988, Burgerjon, 1986, Burgerjon and Sureau, 1986, 1988); Chile - on pears and walnuts - (Ripa, 1982); Italy (Cravedi and Molinari, 1983); Greece (Yamvrias, 1984) and Spain (Esteban and Balduque, 1988).

Many field trials have been undertaken merely to test the feasibility of CpGV under different orchard and climatic conditions, often with little experimental replication or control, but in other trials, workers have considered the effects of changing the applied dosage (Huber and Dickler, 1978a, Glen and Payne, 1984), the inclusion of spray additives in an attempt to improve persistence (Keller, 1973, Richards, 1984), altering the spray volume (Richards, 1984) and the effect of altering the number of spray applications (Huber and Dickler, 1977, Jaques and Laing, 1982, Dickler and Huber, 1986, 1888). Their results are discussed in chapters 4 and 6, in relation to the findings of this research study.

Laboratory bioassays show that CpGV is highly infectious to the codling moth (Keller, 1973, Sheppard and Stairs, 1977, Payne, 1981) and yet field trials have produced variable levels of success. Falcon *et al.*, (1968) achieved 79-87% reduction in the numbers of overwintering larvae, whilst deep fruit damage was reduced by 83-90% in UK trials (Glen and Payne, 1984), 83-90% in Germany (Huber and Dickler, 1976, 1977, 1978) and 71-100% in Chile (Ripa, 1982). However, in several years trials in Spain, even frequent applications at high dose could only achieve 53% control (Esteban and Balduque, 1988). In Canada, CpGV was inferior to insecticidal treatment with azinphos-methyl, with 58-85% control of total fruit damage compared to 93-98% control with the chemical (Jaques *et al.*, 1981). A number of factors are likely to be responsible for the variable results, not least of which are those connected to the experimentation; CpGV has a different mode of action to chemical insecticides and many field experiments do not provide optimum conditions for its use. CpGV has a relatively poor field persistence compared to most insecticides (Jaques and Laing, 1982, Glen and Payne, 1984, Huber, 1988). If, following spray application, reinfestation of

codling moth from neighbouring orchards and unsprayed trees occurs after the virus has largely lost its activity (particularly in regions with a second, and subsequent pest generations), further damage will result, reducing the apparent efficacy of CpGV. CpGV may give adequate control of the initial orchard population but could then be subjected to continuing pest reinvasion requiring subsequent virus applications. Burgerjon (1986) considered that, for this reason, randomized block experiments with untreated control trees were inadequate to compare CpGV with chemicals. How then should CpGV be tested? Clearly, without replication and untreated controls, the efficacy of CpGV could never be truly assessed. Trials with CpGV have thus tended to evolve in two directions: small-scale, fully controlled, replicated trials where different virus treatments are compared and the overall level of control is unimportant (providing that treatment differences are discernible); and long-term, large scale unreplicated trials on whole orchards (eg. Burgerjon, 1986, Burgerjon and Sureau, 1986) where the 'commercial' efficacy of CpGV might be argued (although, never unequivocally) on the basis of continued low levels of damage over several seasons. Burgerjon (1986) was able to achieve low levels of fruit damage with only four virus applications against two codling moth generations; he accredited this success to the treatment of whole orchards, with surrounding orchards also treated with insecticides to prevent immigration. The evidence was strictly circumstantial, but such a strategy is a logical one, and one which might be highly feasible in regions where area-wide pest control is practised.

Inefficient application may be another factor contributing to poor trial results. Application machinery giving adequate control with contact-acting chemical insecticides may give unacceptable coverage for CpGV application where the virus must be ingested. It is likely that, for a stomach acting control agent, coverage would need to be superior to ensure spray droplets are present on *ingested* plant material; whereas for a contact-acting chemical, even poorly distributed spray may be adequate, since sufficient contact could occur as the pest searches for feeding sites. By far the most variable and uncontrollable factor is, however, the effect of climate; CpGV is rapidly inactivated by sunlight (Keller, 1973, Fritsch and Huber, 1985) and higher levels of inactivation could explain differences between trials where virus dose and application frequency ought to give good results. Inactivation of CpGV is discussed in more detail in the following section.

One disadvantage which CpGV has over conventional insecticides, is that host death does not occur until the virus infection process is virtually complete and that larvae may continue to feed after infection. Jaques *et al.*, (1977, 1981) reported *increased* superficial damage caused by larval feeding prior to death, though Glen *et al.*, (1980) found that most larvae died in the first instar and that any damage caused was often hidden; most larvae entered fruit via the calyx, where no visible damage was caused by first instars (Glen and Clark, 1985). The possibility of increasing the speed of kill by genetic manipulation of the virus has already been discussed (section III).

Spray application of CpGV has a purely 'insecticidal' effect with no evidence of any epizootics developing; horizontal transmission is unlikely owing to the solitary life style of codling moth larvae and no virus could be found persisting in the year following application (Huber and Dickler, 1977). Longworth (1976) claimed that virus had 'spread' to larvae 200 m from the sprayed area, though spray drift was probably the real cause. There is no evidence of any vertical transmission (Etzel and Falcon, 1976). Although CpGV cannot be used as a classical biological control measure, continued application over several seasons - particularly in orchards isolated from untreated trees - does produce benefits from consistently lower codling moth populations. In consecutive field trials, Falcon *et al.*, (1968) found continually lower damage levels, and in Greece, an unacceptable level of damage - 5.6% deep fruit damage - was followed the next season by much improved control with only 0.8% deep damage, comparable to chemically sprayed plots (Yamvrias, 1984).

Spray timing is critical since larvae are unlikely to ingest virus once protected inside the apple. Application of CpGV, and chemical larvicides, should be timed to coincide with the period of greatest larval emergence; this is doubly critical for CpGV owing to its poor persistence. On the basis of the threshold temperature for larval development, Hagley (1973) predicted peak emergence as occurring 137-144 day-degrees (above the 10°C threshold) after the first moth was caught in a pheromone trap. A more advanced model by Glen and Brain (1982) recommended a delay of 140 day-degrees (above 10°C) after the start of a 'reference week' in which more than five adults were trapped. In countries where there is more than one generation per season, the time over which larvae ingest virus is likely to be greater in the first generation since fruits are less

developed and larvae apparently spend more time feeding on foliage; consequently treatment of the first generation is more effective (Burgerjon and Sureau, 1986).

Successful control has been achieved with doses of between 10^{13} (Glen and Payne, 1984) and 1.5×10^{14} granules/ha/spray (Falcon *et al.*, 1968). Reducing the dose would reduce the costs of application and much effort has been made in the current study to achieve this. However, Burgerjon and Sureau (1986) have suggested that smaller-than-normal doses might be used in commercial orchards where the population is already low; CpGV has thus been applied on a regular basis on 5 ha of orchard in Israel, despite the actual codling moth population being below the threshold level for economic damage (Burgerjon and Sureau, 1986). Routine application of any pest control agent is likely to increase the pressure for resistance to develop in the host, although, as yet, there is no evidence of any host resistance to CpGV (Dickler and Huber, 1984).

VII. Foliar persistence of CpGV and potential for improved formulation

Although CpGV is relatively rainfast (Keller, 1973; Charmillot *et al.*, 1984; Richards, 1984; Burgerjon and Sureau, 1986), its persistence on foliage is poor, losing 96% activity in 21 days in Southern England, with a half-life of around three days (Glen and Payne, 1984). Jaques and Laing (1982) recorded a similar loss of 50% infectivity in just two days in Ontario. Inactivation of CpGV is caused by ultra-violet (UV) irradiation in sunlight (Keller, 1973; Fritsch and Huber, 1985).

Poor persistence is a common feature of insect virus insecticides, with UV irradiation - particularly between 280 and 320 nm wavelength (Shapiro *et al.*, 1983) - being the main cause of inactivation. On cotton in Egypt, *Spodoptera littoralis* NPV lost 50% activity in four days - 99.5% loss from the upper leaf surface - with ultra-violet inactivation and physical abrasion by wind and sand accounting for 99%. Leaf temperature at environmental conditions was unimportant in the deactivation process (Jones and McKinley, 1987). Ali and Sikorowski (1986) found that the high pH of cotton leaf surfaces caused inactivation of *Heliothis virescens* NPV. However, 90% of the total loss was still attributed to sunlight.

Many agents have been used to improve the persistence of insect viruses by acting as UV-protectants. Keller (1973) claimed that skimmed milk acted as a UV-protectant for CpGV, and a compound marketed as 'Shade', containing the sulphonated co-polymer of catechin and leucocyanidin, was used in a formulation of *Orgyia pseudotsugata* NPV for tussock moth control (Martignoni and Iwai, 1985). Attempts have been made to rationalise the choice of chemicals likely to confer UV-protection on the basis of their structure and physical properties. For example, Shapiro (1985) found that folic acid and riboflavin absorbed UV in the critical 280-320 nm region and, in laboratory studies, conferred some protection to *Lymantria dispar* NPV. However, p-amino benzoic acid also absorbed well in this region but gave no protection. Success with protectants has been somewhat hit-and-miss, with protection gained, in some cases, just from the impurities in a virus preparation (eg. Huber, 1988). Clearly, there would be a major advantage if UV protectants could be used with insect viruses but, as yet, success of certain agents in laboratory studies has not been followed by substantially improved control in field trials. Richards (1984) tested the spray additives 'Shade' and sulisobenzone which, although giving slight improvements in persistence, gave no improvement in the control of codling moth damage when formulated with CpGV; even with the additives, all virus half-lives were of less than one day, and no virus could be detected on foliage after nine days.

Claims about the efficacy of spray additives as UV protectants have often been misleading. Many measurements of virus persistence (eg. Ignoffo and Batzer, 1971) were based on a false assumption that the 'percent original activity remaining' (%OAR) was proportional to the mortality of larvae in bioassays before and after virus inactivation. Richards and Payne (1982) pointed out that the dose-mortality response to insect viruses was not linear; without first linearising the response by plotting probit mortality against log dose, such comparisons are invalid. Claims of 3.56 fold improvements with benzilidine sulfonic acid and 3.25 fold improvements with 'Shade' (Shapiro, *et al.*, 1983) are likely to be over-estimations of their true protectant ability. Furthermore, tests claiming UV protection with molasses, sucrose, 'Coax' (containing cottonseed extracts) (Shapiro, *et al.*, 1983), 'Shade' and extracts of wood and bark (Martignoni and Iwai, 1985), were inadequately controlled and could have masked feeding stimulant properties in these compounds (see Chapter 6). Unformulated virus was bioassayed before *and* after UV irradiation, but formulated virus

was only bioassayed *after* irradiation; any direct feeding stimulant effect of the additive would not have been tested. Even in field trials, UV protectants have not always been rigorously tested. Burgerjon and Sureau (1986) found that skimmed milk gave no improvement in the control of codling moth by CpGV; whether or not skimmed milk *did* have any beneficial effect may have been overshadowed by the high virus dose used in the field trial which probably gave such high levels of control that differences between treatments were undiscernible.

Another approach to improve persistence has been to select for UV-resistant virus strains. Shapiro and Bell (1984) selected a "UV-tolerant" strain of *Lymantria dispar* NPV, and claimed a 2.5 fold improvement in UV-resistance. However, their results were, once again, based on the false %OAR concept. Brassel and Benz (1979) selected a UV-resistant strain of CpGV by successively irradiating dry CpGV deposits and then feeding larvae on the remaining active virus. A series of six passages resulted in two parallel dose-mortality bioassay responses with an LD_{50} for the selected strain 5.6 times lower, after irradiation, than the non-selected strain; confidence limits were, unfortunately, not quoted. In a field test, the mortality of larvae fed on leaves sprayed with the standard preparation fell to 50% six to seven days after application, compared to sixteen days with the selected strain. However, the assumption that the starting levels for each virus were identical was unsubstantiated - based only on the identical mortality of larvae fed on leaves immediately after spraying; in each case this was 98%. Larval mortalities close to 100% cannot be used to discriminate sensitively between doses because of the sigmoid dose-response curve (Richards, 1984) and the possibility of a small percentage of larvae surviving even very high doses simply through lack of ingestion of virus on non-uniformly sprayed leaves. The trial was unreplicated, and no indication of the observed differences being significant were given. Even assuming that the experiments were valid, the non-linear field-inactivation curve of CpGV (Richards, 1984) means that doubling the time to reach 50% mortality of test larvae does not mean that persistence has been doubled.

In conclusion, although some improvements have been made with UV-protectants, in many cases claimed efficiencies have been exaggerated by poor experimentation, and others have failed to show benefits in field trials. Tests of spray additives should be adequately controlled using

replicated tests at virus doses liable to show differences between treatments. Suitable additives must also be economically viable; which means inexpensive to produce and used at relatively low levels. Success with benzilidine sulfonic acid was only achieved at 5% w/w (Shapiro *et al.*, 1983) whilst Martignoni and Iwai (1985) tested molasses at 25% v/v, levels more suited, perhaps, to ultra-low volume, than high volume spraying at 1000 l/ha.

VIII. Orchard Integrated Pest Management

"Integrated pest management (IPM) is a technology of pest control that has, as its goal, the control of agricultural pests to benefit society as a whole" (Pimentel, 1982). It is a system which combines and integrates biological and chemical pest management, and where chemicals are used only when necessary and in a manner least disruptive to biological control (Stern, et al., 1959).

The benefit "to society as a whole" is unlikely to be the principal driving force behind the deployment of IPM strategies by growers. Nevertheless, the less philanthropic benefits of selective use of control agents, including the potential of overall cost savings, promotes its use, particularly where the type of crop system gives a high level of reliability to such practice. The relatively stable nature of the orchard ecosystem (see section I) and the lack of crop processing prior to consumption, promote the use of IPM schemes with an emphasis on reduced pesticide useage. In orchards, the most obvious cost benefits seen with a successful IPM scheme, are the savings on acaricides; one of the biggest and most consistent orchard pest problems is that of the fruit tree red spider mite, *Panonychus ulmi*, which can be kept below economically damaging levels entirely by predators, chiefly the predatory phytoseiid mite *Typhlodromus pyri*. Resistance of key pests - particularly spider mites - to broad spectrum insecticides has also favoured a reduction in pesticide use in an attempt to delay the onset of resistance in pest populations. With the exception of a report concerning DDT-resistance in USA in the early 1950's (Cutright, 1954), codling moth has not developed resistance to chemical insecticides, but use of broad spectrum chemicals has selected for resistant strains of other pests which would otherwise be

controlled naturally by predators. The development of CpGV as a biological control agent has occurred against a background of research into orchard IPM. The suitability of CpGV for orchard IPM is discussed in greater depth in chapter 7. It is therefore sufficient now to cover some of the relevant points emerging from a wealth of literature reporting advances in orchard IPM throughout the world; an excellent recent review of which has been written by Solomon (1987).

There are c. 27,000 ha of apple in the UK comprising 14,000 ha desert, 9000 ha culinary and 4000 ha cider varieties. The crop is of high value and sold, in most cases, without any further processing; there is a risk, therefore, that a slight level of damage resulting from inadequate pest control would result in a cost penalty (Solomon 1987). Choice of IPM strategy is thus constrained by both the nature of the pest complex, and the economics of fruit production; growers are unlikely to tolerate cost increases without tangible economic benefits. Orchards are subject to a number of pest and disease problems: early season pests such as caterpillars (eg. winter moth, *Operophtera brumata*) aphids; summer fruit damaging pests including sawfly (*Hoplocampa testudinea*), codling moth and leafrollers (Tortricidae); 'secondary' pests, particularly red spider mites (*Panonychus ulmi*) and rust mites (Eriophyidae); and a range of foliar diseases such as apple scab (*Venturia inaequalis*) and powdery mildew (*Podosphaera leucotricha*). Damage varies from species to species, with caterpillars and some leafroller species causing superficial fruit damage, whilst codling moth causes damage through to the fruit core. *P. ulmi* does not damage fruit but can cause severe leaf bronzing which can effect yield in the current and subsequent seasons (Alford, 1984). The rust mite, *Aculus schlechtendali*, can cause fruit russetting when in high numbers (Solomon, 1987). Foliar diseases pose a constant threat requiring, on average, seventeen fungicide applications in a single UK season, compared to only five insecticides and acaricides (Solomon, 1987). With so many pest and disease problems, integrated control agents must be carefully chosen to complement each other so that the selective nature of one is not overshadowed by the broad spectrum properties of another. Many fungicides, for example, are toxic to predatory mites, spoiling the benefits of selective insecticides. Even chemicals which are toxic to both pest and predatory mites would not be IPM-compatible since *P. ulmi* recovers more rapidly than its predator *T. pyri* (Solomon, 1987).

In an IPM regime, pesticides are only applied as necessary; not just when pests are sighted, but when pest numbers have reached - or are likely to reach - economically damaging threshold levels. A monitoring scheme has been developed for UK orchards, with thresholds calculated for each pest species (Anon, 1979). Pheromone traps are used to monitor leafrollers, with treatments shown to be unnecessary below a threshold of five codling moths per trap, per week, and below twenty/trap/week for the fruit tree tortrix moth, *Archips podana* (Alford *et al.*, 1979).

In orchards, the main economic aims of an IPM scheme are to minimize overall pesticide usage and to avoid the application of acaricides for the control of *P. ulmi*. One strategy would be to use selective agents such as CpGV, accepting the possible need to apply additional selective agents for the control of other pests if and when they occur (see chapter 7). An alternative strategy has been developed using conventional chemicals but exploiting naturally occurring resistance in predatory mites. *T. pyri* is the most effective predator of both *P. ulmi* and the apple rust mite *A. schlechtendali*, and is capable of maintaining their populations at low, non-damaging levels (Cranham and Solomon, 1981). Unfortunately, ever since winter washes were used for orchard pest control in the 1920's, *P. ulmi* has been a serious pest problem owing to the development of resistance to most insecticides, whereas *T. pyri* has remained susceptible. Almost without exception, by the late 1970's *P. ulmi* had developed resistance to every major acaricide within four or five years of its widespread use (Way, 1978). More recently, however, organophosphorus-resistant *T. pyri* have been found in a number of orchards in England (Kapetanakis and Cranham, 1983; Solomon and Fitzgerald, 1984) and this has allowed the application of organophosphorus (OP) insecticides for the control of lepidopteran pests whilst *P. ulmi* is controlled naturally. It has been possible to transfer *T. pyri* from mass-culturing sites (on apple rootstock plants) by distributing infested shoots amongst orchard trees (Solomon and Fitzgerald, 1984). Selective agents are still required for control of other pests and diseases but a number of chemicals, such as pirimicarb for aphid control and bupirimate, dithianon, fenarimol and triadimefon for foliar disease control, are non-toxic to predatory mites (Cranham and Solomon, 1981). Although effective, this strategy does not reduce, *per se*, the level of pesticide residues on harvested fruit, nor does it reduce the risk of OP resistance developing in target pest species.

IPM schemes need not rely simply on the judicious use of pesticides; a number of cultural practices, such as the removal of overwintering sites for codling moth by grubbing out old trees, and removing split stakes could be integrated into the programme (Way, 1978). Over a longer time-scale, apple varieties could be planted which were avoided by certain pest species; the varieties Golden Delicious and Greensleeves, for example, appear to be virtually "immune" to the fruit damaging moth *Blastobasis decolorella* (Cross, 1985). A regional approach coordinating all growers would help prevent migration of lepidopteran pests from one orchard to another (Way, 1978), although disused orchards and garden trees could never be incorporated in such a scheme, and these might be important sources of pests.

Integrated pest management can only be truly effective as a long-term strategy. It may take more than one season for predator populations to reach sufficient levels to exert natural control. Furthermore, once IPM is established, the species complex may change. *The mussel scale, Lepidosaphes ulmi*, has increased in numbers on IPM orchards in the UK (Solomon, 1987), whilst in Holland, the major leafroller pest, *Adoxophyes orana* became only a minor pest in the absence of conventional insecticides, with several other leafroller species achieving pest status. Other species, including apple sawfly, *H. testudinea, the apple blossom weevil, Anthonomus pomorum* and the brown leaf weevil *Phyllobius oblongatus* also became pests (Gruys, 1982). Phytophagous insects previously thought to be non-pest, may require additional control measures and new damage threshold estimates. In short, IPM requires an intelligent approach to pest control, accepting the dynamic nature of the ecosystem and the need for continuous pest and predator monitoring.

A reduction in the number of pesticides used can result in reduced environmental impact and lower residues in fruit; clearly IPM *can* "benefit society as a whole". Nevertheless, except where premiums are paid for 'organic' produce, ecological benefits alone are insufficient to compensate any financial losses incurred through IPM. In UK trials, even with no acaricides sprayed, an orchard IPM system based on selective control agents did not reduce costs as compared to the routine chemical programme (Easterbrook *et al.*, 1985). In order to encourage growers to switch to an IPM programme, undertaking additional pest and disease monitoring, long-term benefits, such as decreased risk of pest resistance to common

insecticides, should be included in any cost-benefit equations. For CpGV to be successful, it must, not only be effective, but also have demonstrable short and long-term benefits of decreased usage of broad-spectrum insecticides. The aims of this research study were to optimize the use and efficacy of CpGV and to demonstrate its potential for incorporation in an IPM programme. However, its future as a commercial biological insecticide must additionally depend on the economics of production and the cost of its use to the grower; factors outside the scope of this research.

Chapter 2

General materials and methods

I. Rearing of stock codling moth culture

A continuous culture of codling moth was reared in a building isolated from the CpGV experimental area in controlled environment cabinets. Access to the unit was restricted to workers not involved with insect pathogens and hygiene was maintained by regular disinfection of work surfaces with a dilute sodium hypochlorite solution (Chloros, ICI Ltd). Equipment such as paint brushes for insect handling were fumigated overnight in a gas jar containing 5% formaldehyde solution.

Larvae were reared on artificial diet (Guennelon, *et al*, 1981) composed of: maize meal (190g); yeast powder (50g); Wheat germ (46g); agar (26.6g); ascorbic acid (7.0g); benzoic acid (3.0g); methyl para-hydroxybenzoate (2.3g); 30% formaldehyde (1.6 ml); and 1 litre of water. Diet was prepared by dissolving the agar in 600 ml water at 70°C before blending the remaining ingredients and water. The diet was allowed to set in re-useable plastic trays. Diet could be stored for 3-4 months at 4°C prior to use.

First instar (neonate) larvae were reared individually on blocks of diet, approximately 1.5 cm^3, in disposable plastic pots (N° 10 Polypots, Metal Box plc, London). Pots were kept at 24°C with an 18 hour photoperiod. Larval and pupal development took an average 31 days. Adults were removed from pots and transferred in groups of fifty to oviposition chambers composed of polythene bags (ca. 20 x 15 cm) stretched over 10 cm diameter cardboard rings covered with fine nylon gauze. Water was provided by a wet sponge at the bottom of the chamber. Eggs were laid on the polythene bags which were changed every two days. Adults were kept for about five days after emergence.

Polythene bags with eggs were cut open and fumigated for six hours in 500 ml jars with ca. 100 µl 5% formaldehyde on a filter paper. Fumigation was designed to inactivate any viral contamination of the egg surfaces (Payne, 1981). Eggs were maintained at 25°C until hatch.

A second codling moth culture was started using eggs imported from I.N.R.A., France (Station de Zoologie et d'Apidologie, 84140 Montfavet). These insects were reared as above, and used for comparative rearing trials with the existing stock.

II. Production, purification and quantification of CpGV

a) Mass production and infection

CpGV for laboratory and field trials was produced in vivo in larvae of *Cydia pomonella*. Larvae were reared on the semi-synthetic diet described above, but without formaldehyde. Larvae were housed individually in square cells in a 25-compartment square Petri dish, 10 x 10 x 2 cm (Sterilin, UK). A slice of diet 2-3 mm thick was pushed to the bottom of the cells. Slicing the diet gave a rough surface which proved essential for larval feeding. Larvae were sealed into each chamber with layers of clingfilm and polythene with the Petri dish lid padded with tissue paper and clamped firmly in place with bulldog clips. Huber (1988) found that thin layers dried up quickly, or that increasing the humidity to prevent this induced the growth of moulds; neither problem was experienced here. Huber's method of rearing fourth instar larvae in rolled layers of diet on aluminium foil (Huber, 1988) was unsatisfactory since this resulted in mortality of some larvae prior to infection and involved extra steps of larval transfer and their subsequent separation from the foil. The juvenile hormone analogue methoprene (Altosid 5E, Zoecon Corp.) was added to the diet at 2 ppm which had the effect of increasing the final weight of fully grown larvae, prolonging the period of larval maturity and preventing diapause (Glen and Payne, 1984).

Larvae were reared at 25-26°C in darkness. After 17 days, when larvae had reached fifth instar, two drops (ca. 60µl) of CpGV suspension, at 10^7 granules/ml, were applied to the diet in each cell from a disposable pipette. After 23-24 days when larvae were fully infected the plates were deep frozen for later extraction of the virus.

b) Purification of CpGV

CpGV was purified following a method similar to that described by Glen and Payne (1984). Infected dead insects were macerated in a Colworth 80 Stomacher and filtered through muslin to remove large particles. The muslin was squeezed to extract most of the liquid and 0.1% sodium dodecyl sulphate (SDS) added to wash as much virus through as possible. The filtrate was clarified by low speed centrifugation: 2000 rpm, 4°C for 10 minutes (Sorvall RC5B centrifuge with angle head rotor). The pellet was resuspended and the process repeated. The supernatants from both centrifugation were pelleted by centrifugation at 15,000 rpm, 4°C for 20 minutes and resuspended in a minimum volume of 0.1% SDS. The material was partially purified on a 30-80% (v/v) continuous glycerol gradient in 0.1% SDS in 45 ml centrifuge tubes, created by using an MSE gradient former (Measuring and Scientific Equipment Ltd, Crawley, UK) and peristaltic pump, adding 30% and 80% glycerol in water to the columns. Larger volume gradients were formed by layering equal volumes of 30, 40, 50, 60, 70 and 80% glycerol into centrifuge tubes and lightly mixing by raising and lowering a glass rod through the layers. The resuspended pellets were loaded on the gradients to give a 0.5 cm layer of the material resuspended from the pellets, and then centrifuged at 7000 rpm, 4°C, for 60 minutes (HS4 swinging bucket rotor).

A white band of semi-pure CpGV was collected approximately half way down the gradient and pelleted by centrifugation at 15,000 rpm for 20 minutes (angle head rotor) after diluting 1:1 in 0.1% SDS. Aggregated virus which had precipitated in the glycerol gradient was pelleted, resuspended in 0.1% SDS, sonicated for 5 seconds (power setting 3: Soniprobe, Dawe Instruments, London) and run on a second glycerol gradient. Even more virus could be extracted by precipitating all residues from the first two gradients, resuspending in 0.1% SDS and performing a low speed spin, 2000 rpm for 10 minutes, to remove waste material. The supernatant was pelleted and run on a glycerol gradient as before. This stage was most effective if residues from several preparations were pooled.

The virus was finally purified on a 45-60% (w/v) discontinuous sucrose gradient prepared by adding 6 ml layers of 60% and 45% sucrose to 16.5 ml centrifuge tubes and adding a 0.5 cm layer of semi-purified virus before centrifuging at 20,000 rpm for 90 minutes (MSE Prepspin 50

centrifuge). CpGV formed a white band at the gradient interface which was removed by pipetting the 45% sucrose layer and then adding 1-2 ml water to the virus band allowing easy extraction by pipette from above the heavier sucrose layer beneath. By adding a further 1-2 ml water, any last traces of virus could be removed without extracting too much sucrose. The virus was pelleted by centrifugation at 15,000 rpm for 20 minutes (angle head rotor) and resuspended in water. This last process was repeated twice to remove traces of SDS from the preparation.

c) Quantification

i) Capsule count.

The purified CpGV was counted in a 0.01 mm depth bacterial counting chamber (Hawksley, Lancing, W. Sussex). CpGV granules could be seen using a compound microscope fitted with dark-ground illumination. Estimates were based on the mean of three counts (ca. 150-200 granules per count) from each of two separate dilutions taken from each batch (Burges and Thomson, 1971; Payne, 1981).

ii) Protein determination by Folin Test.

Batches of purified CpGV were assessed for protein content by the Folin test (Lowry *et al*, 1951), comparing optical densities recorded for known dilutions of CpGV with dilutions of a standard protein, bovine serum albumin (Sigma Chemicals, Poole).

iii) Measurement of virus infectivity by bioassay.

The infectivity of each batch was checked by bioassaying against a standard sample of purified CpGV. Neonate larvae were individually fed on artificial diet with virus incorporated at 3×10^2, 9×10^2, 3×10^3, 9×10^3 and 3×10^4 granules/ml. Diet was prepared as above, but without formaldehyde or hormone and with the addition of an extra 330ml water to each batch. Just prior to each bioassay, diet was heat sterilized under 1 atmosphere pressure for 20 minutes. The addition of extra water allowed the diet to remain molten if kept at 50°C in a water bath. Diet was prepared in 7.5 ml aliquots and to each batch, after cooling to 50°C, 1 ml of the appropriate dilution of the CpGV sample and 1.5 ml of de-ionized water was added and

vortex-mixed. Whilst still molten, the diet was dispensed into round-bottom 96-well microtitre plates (Alpha Ltd, UK), half filling each well (ca. 150 µl). After allowing the diet to set, a single larva was placed in each well and the plates sealed with clingfilm and glass microscope slides clamped down with bulldog clips. Young codling moth larvae are quite capable of boring through thin plastic and the additional glass layer was essential to prevent their escape.

The virus induced mortality was scored after six days in a dark incubator at 26°C. CpGV infected larvae were readily identified by their characteristic milky-white colour and tendency for the epidermis to disintegrate after prodding with a needle. In cases of uncertainty, infection was verified by light microscope examination under dark-ground illumination where the virus granules could be seen. The concentration/mortality response was analysed by maximum likelihood probit (MLP). Virus-induced mortality in control larvae was incorporated in the analysis, though rarely exceeded 1-2%. Assays were discarded if control mortality exceeded 7.5%. Larvae dying from other causes, or missing from the plates, were removed from the totals in the analysis. Dilutions producing virus mortalities of 0 or 100% were not included in the analysis. Active CpGV typically produced LC_{50} values of around 2.4 ± 1.0 x 10^3 granules/ml diet with a probit mortality/$\log_{10}$ dose response slope of 1.6 ± 0.27 (based on the mean of twenty bioassays of the standard virus preparation).

d) Preparation of impure samples

i) Field trials:

For the 1987 field trials, the purification stage was omitted in the production of an impure CpGV preparation, designed to be of low cost, and thus suitable for use on commercial orchards. The entire contents of the insect production trays - diet, infected insects and frass - were homogenised in a household blender, and then in a Silverson laboratory blender, adding 1.5 volumes of water to allow processing. Remaining large particles and insect silk were removed by filtration through muslin. A typical batch (before dilution to final virus concentration) contained ca. 2000-2500 larvae + diet (totalling 2 litres) and 3 litres water. The

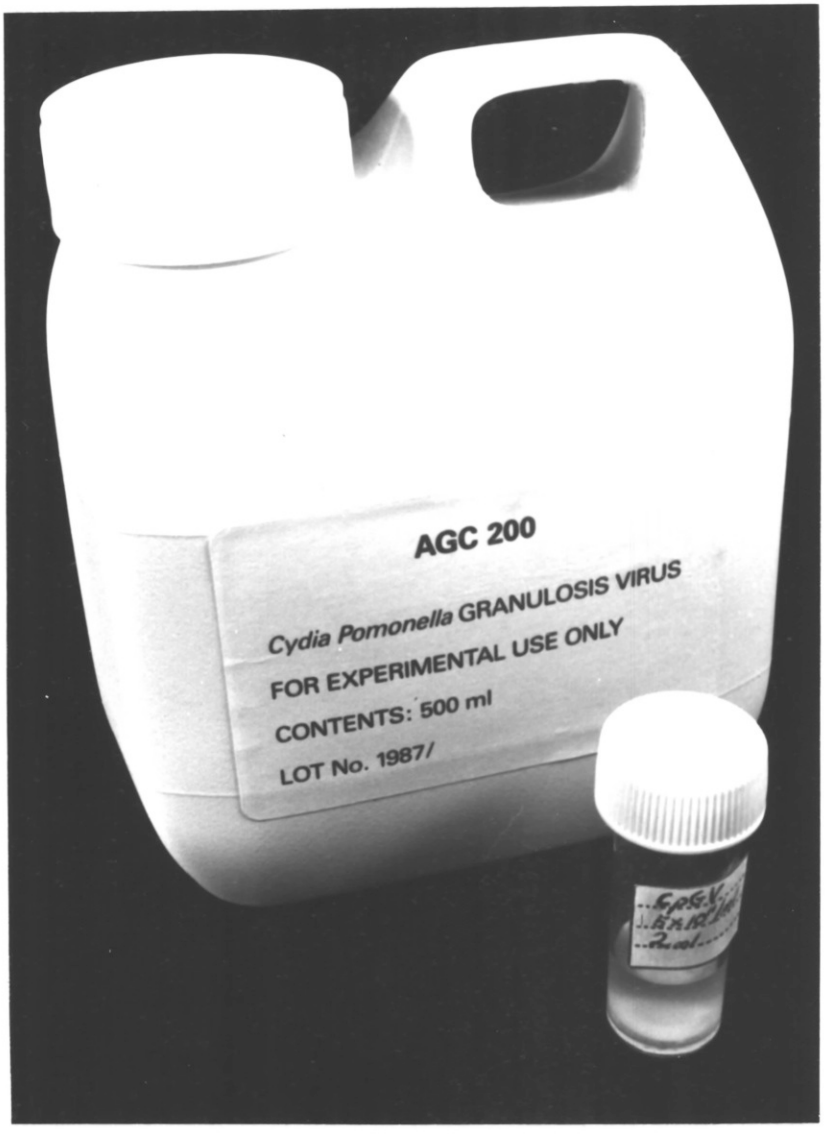


Plate 2.1 *Cydia pomonella* granulosis virus : Experimental product

product was bioassayed by incorporation, at known concentration, with insect diet, and the mortality response was compared with a standard sample of purified material (see section iv., below, for full details). The maximum virus yield was 3 x 10^{10} granules per larva (unpurified). Seven batches were produced for experimental trials at IHR, on commercial orchards and for trials in France, Spain and Italy (results not included here).

For the 1987 trials on commercial orchards (Chapter 8), an impure formulation of CpGV was supplied to growers in quantities sufficient to treat 0.5 ha with a single application: 500ml of CpGV at $5x10^9$ granules/l in opaque white polypropylene containers (plate 2.1). The final material was diluted from a stock preparation of known infectivity to give a standard CpGV concentration containing 40% glycerol (w/w) to improve storage life (Chapter 3, section III(c)). Growers were instructed to refrigerate the packs prior to use.

ii) Laboratory studies:

The storage life of impure CpGV homogenates was evaluated using two impure preparations, produced as follows:

Method I (standard): as above.

Method II (Diet only): Two "artificial" virus homogenates were made by homogenizing 100ml clean insect diet with 48ml purified CpGV ($1x10^9$ granules/ml) to give a final CpGV concentration of $1.92x10^8$ granules/ml. The homogenates were produced from diet containing or omitting ascorbic acid (a normal constituent of diet) to test its effect on virus storage: the homogenates were artificially produced using added CpGV to guarantee an equivalent virus titre in each sample.

iii) Production of dry CpGV formulations for laboratory and field trials:

Three methods of drying were used to prepare powders from each of the impure homogenate preparations described above:

I Freeze dried: 8-10ml of homogenate was freeze-dried for 24 hours in sterile plastic universal bottles using an Edward's Modulyo freeze-drier. The final material was milled in a pestle and mortar.

II Solvent dried: 100ml homogenate was shaken with 400ml acetone for two minutes and poured over a Whatman no. 1 filter paper in a Buchner funnel fitted to a vacuum pump. The collected material was washed through once with 25ml acetone before crushing with a pestle and mortar. The material was resuspended in 100ml acetone and the process repeated, washing twice with 25-30ml acetone. The material was left open to the air for three hours for the remaining solvent to evaporate, before milling with a pestle and mortar.

III Air dried: 10-15g homogenate was spread evenly in 9cm petri dishes and left for 24 hours in a fume cupboard with maximum air draught. The material was milled as before.

Materials were either stored in sealed plastic universal bottles, in vacuum packed 1ml vials (Edward's freeze drier vials), or in sealed universal bottles containing a muslin sachet of freshly oven-dried silica gel (2g per sachet).

iv. Bioassay of impure homogenates.

Impure homogenates were bioassayed by diet incorporation (section II(c)iii) using the following dilutions of starting material:

Homogenate: $9x10^{-9}$, $3x10^{-8}$, $9x10^{-8}$, $3x10^{-7}$, $9x10^{-7}$, $3x10^{-6}$, $9x10^{-6}$ ml homogenate/ml diet.

Dry powders: $3x10^{-9}$, $9x10^{-9}$, $3x10^{-8}$,$9x10^{-8}$, $3x10^{-7}$, $9x10^{-7}$, $3x10^{-6}$ g dry powder/ml.

For each bioassay, dilutions giving zero or 100% mortality were excluded from the analysis. Two bioassay series were set up for each sample and two standard curves of purified CpGV ($3x10^{2}$ - $3x10^{4}$ granules/ml diet) with virus-free controls were prepared for the interpolation of the virus activity in the unknown samples (LC_{50}s for unknown samples were recorded in terms of the *concentration* of starting material giving 50% mortality):

eg. for homogenates, CpGV concentration of unknown (granules/ml)

= LC_{50} standard (granules/ml)/LC_{50} unknown (ml/ml)

v) Assessment of bacterial contamination in impure CpGV

Bacterial contamination of impure CpGV preparations was evaluated by a standard plate count of bacterial colonies. Initial 1:10 dilutions of each sample were prepared in sterile peptone water (0.1g bacteriological peptone, 5g NaCl, 1000 ml sterile water) and sonicated for 15 seconds in order to disrupt any aggregated particles. Samples were diluted aseptically to give a dilution series of $1x10^{-3}$ to $1x10^{-8}$. 0.1 and 1.0 ml samples of each were thoroughly mixed with about 15ml molten nutrient agar (45-50°C) freshly poured into sterile petri dishes. Colony forming units (cfu) were counted for two replicates for each dilution after 24 and 48 hours at 37°C. Nutrient agar and peptone water controls were included with each test.

III. Quantification of CpGV by ELISA

The potential of enzyme-linked immuno-sorbent assay (ELISA) for determining CpGV titre in impure preparations was investigated using standard procedures described by Clark and Adams (1977).

i. Preparation of γ-globulin conjugate and ELISA plates:

γ-globulin was purified from antiserum from CpGV infected rabbits, and conjugated with alkaline phosphatase (Type VII, Sigma Chemicals) according to the method of Clark and Adams (1977). Polystyrene ELISA plates (InteMed) were coated by incubation at 37°C with 200 µl per well of purified γ-globulin (4µg/ml) in coating buffer (0.05M Na_2CO_3 at pH 9.6) for five hours in an air-tight container. Plates were then washed three times in PBS-Tween.

ii. Measurement of virus titre:

200µl of each test sample was added to each well, with six replicates for each sample. The outer rows of the ELISA plates were avoided because

of the tendency of irregularities in the plastic (Clark and Adams, 1977). The plates were incubated overnight at 6°C, washed three times in PBS-Tween, before adding 200µl of γ-globulin/enzyme conjugate to each well. The plates were again incubated for five hours at 37°C and washed as before. 200 µl of enzyme substrate (0.1% disodium ρ-nitrophenyl phosphate in 10% diethanolamine-HCl, pH 9.8) was added to each well and the resultant reaction stopped after 30 minutes by adding 50 µl 3M NaOH to each well. The virus concentration in each well was assessed photometrically at 405 nm in a Uniskan ELISA reader.

IV. Determination of genetic variation by agarose-gel electrophoresis.

CpGV produced for the three field seasons of the study was analysed each year by agarose-gel electrophoresis, following the methods of Maniatis et al (1982). The granulin coat of purified CpGV was dissolved in 0.1M Na_2CO_3 followed by the disruption of the lipid coat in 1% SDS. DNA was isolated by phenol/chloroform extraction (Maniatis et al, 1982) and dialysed in 10mM Tris 1mM EDTA (pH 8.0) for 36 hours. The purified DNA was degraded with EcoRI restriction enzyme (Sigma, UK) for 1.5 hours at 37°C. 1.2 µl buffered EcoRI (1µl X10 buffer + 3 units EcoRI) was used per 10µl DNA sample. The degraded DNA was run on a 0.9% agarose gel for 16 hours at 22V in 40mM tris acetate 1mM EDTA, staining with the fluorescent dye ethidium bromide. The method served as a reasonable check that the virus used was the same for each field trial.

V. Maintenance of continuous supply of apple foliage for laboratory tests

Fresh leaf material for bioassays was obtained from young apple seedlings (cv. Worcester Pearmain) which were maintained throughout the year at a minimum temperature of 10°C under glass with a 16 hour artificial photoperiod. Pest control in the glasshouses was by regular fumigation (ca. every two months) with dichlorvos, thus reducing the risk of residues which could affect the experiments.

VI. Laboratory bioassays on leaves

A new laboratory assay system was designed to study the speed of uptake of CpGV from leaf surfaces in the laboratory, and the effect of spray additives on virus ingestion. 15 mm diameter discs were cut from

fresh apple leaves (Worcester Pearmain seedlings) and 17 discs were placed lower surface uppermost on the inside of a 9 cm Petri dish lid, evenly smeared with sticky-trap glue (Tangletrap, The Tangle-Foot Co., Michigan, USA). Tangletrap was used to discourage neonate codling moth larvae from leaving the leaf discs (Mayas, 1969). The leaf discs were then sprayed with virus suspensions at different concentrations, with or without additives, using a Shandon "Jet-Pak" chromatography sprayer (Shandon Scientific, London, UK). Each lid was first covered with an "attenuator" made from a 9 x 1.5 cm metal ring supporting a single layer of nylon net (hole size 0.625 x 0.25 mm). The sprayer was held 45 cm from the plates and moved sideways at an even pace taking 2 seconds to spray three plates placed side by side. The "attenuator" reduced the droplet density and removed large droplets (see Chapter 6, table 6.7). A number of spray treatments containing CpGV at 1 x 10^7/ml (unless otherwise stated) and different formulation additives were applied. Agral (ICI Plant Protection Division) was included in each treatment as a wetting agent at 0.02% (v/v).

Once the leaf discs had dried, two neonate larvae were transferred to each disc using a fine-haired paint brush. The discs were transferred to a dark 26°C incubator. Larvae were left to walk around the leaf discs for set "exposure times" before transferring to clean diet: in later tests exposure times were standardised as: 3.5, 7.5, 15, 30, and 60 minutes. Larvae were transferred individually to each well of a 96-well microtitre plate (Alpha Laboratories, Eastleigh, Hampshire) half filled (ca. 150 µl) with heat-sterilised diet (20 minutes at 1 atmosphere pressure). Virus-induced mortality was recorded after rearing at 26°C for 5 days in darkness. 32 larvae were used for each exposure time for each treatment and non-virus deaths or missing larvae were subtracted from the total. The exposure-time mortality response was analysed by MLP and the treatments compared in terms of the slope, Log Exposure time for 50% mortality (LET_{50}) and the estimated fiducial limits. For each test, two controls were set up: (1) diet only - 32 larvae on sterile diet (2) leaf control - 32 larvae exposed to unsprayed leaf discs for one hour prior to transferring to clean diet.

VII. Laboratory assessment of CpGV and chemical compatibility

A number of pesticides used in conventional chemical orchard pest and disease control were tested for their compatibility with CpGV. The

infectivity of CpGV with or without mixing with these pesticides was compared by bioassay using neonate larvae of *C. pomonella*. Chemicals were mixed with CpGV at concentrations five times the maximum manufacturers' recommended dose for use in the field. The samples were incubated in the dark for three days at 27°C. A series of dilutions of CpGV with constant chemical concentration were made so that (after incubation) by diluting in water, and then into artificial diet, the concentration of chemical could be drastically reduced by 10,000 fold and CpGV would be present in artificial diet at $9x10^{2}$ - $3x10^{4}$ granules/ml. Using the method described above (section II(c)iii), the treated CpGV was bioassayed and compared to pure CpGV incubated in water for an identical period. Any loss in infectivity was shown by an increased LC_{50} calculated from MLP analysis.

VIII. Field trials

a) Plots

Field trials were carried out at the AFRC Institute of Horticultural Research, East Malling; Eden Farm, West Malling, and on three commercial orchards. Experimental Use permits for CpGV and the insect growth regulator fenoxycarb were obtained from the Ministry of Agriculture Fisheries and Food, Pesticide Registration and Surveillance Department. Permit numbers were, for 1986 - 01009/85, and for 1987 - COP 860084/A.

i. Orchard WF103, East Malling.

Trials to assess the effects of different virus doses (Chapter 4, section II(a)i), spray frequencies Chapter 4, II(a)ii), spray formulations (Chapter 6, II(a)i and II(b)v) and the impact on orchard ecology (Chapter 7, II(a), (b) and (c)), were carried out over three seasons on a one ha plot of Laxton's Fortune and Cox apples. The orchard consisted of large trees, approximately 3 m in height, square-planted 7.25 m apart. Two rows of Laxton's Fortune were interspaced with a single row of Cox, these being incorporated as guard rows. Plots consisted of four trees with four replicates in four blocks to account for variation in yield or codling moth infestation level across the site (Figs. 2.1-2.3).

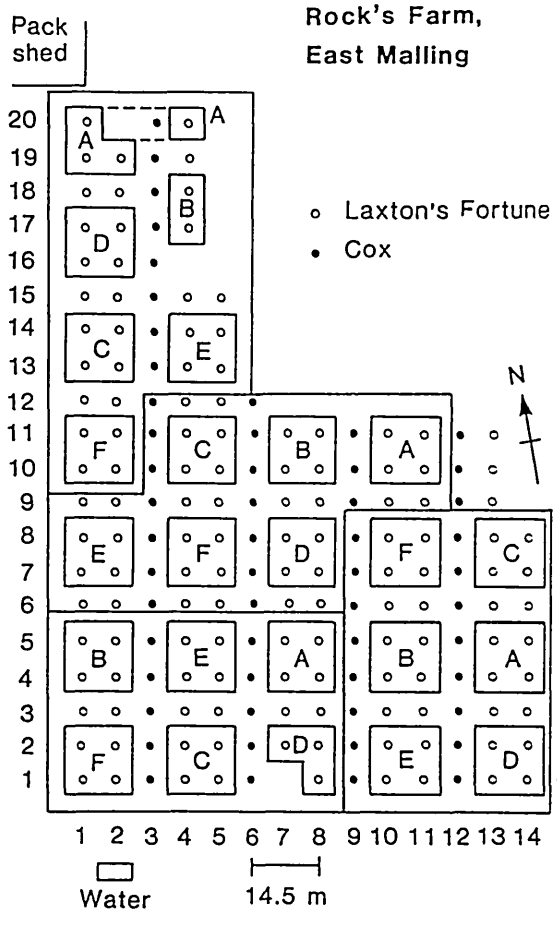


Fig. 2.1 Field layout: Plot WF 103, 1985

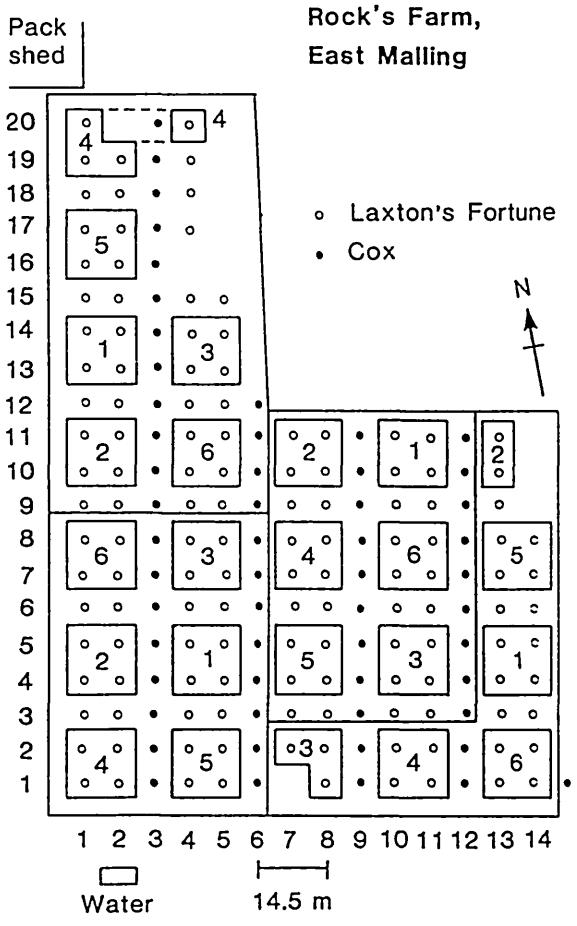


Fig. 2.2 Field Layout: Plot WF 103, 1986

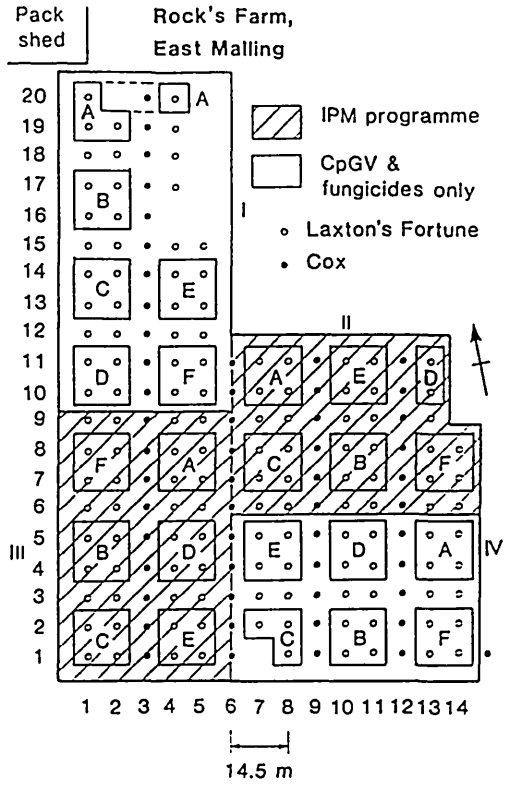


Fig. 2.3 Field Layout: Plot WF 103, 1987

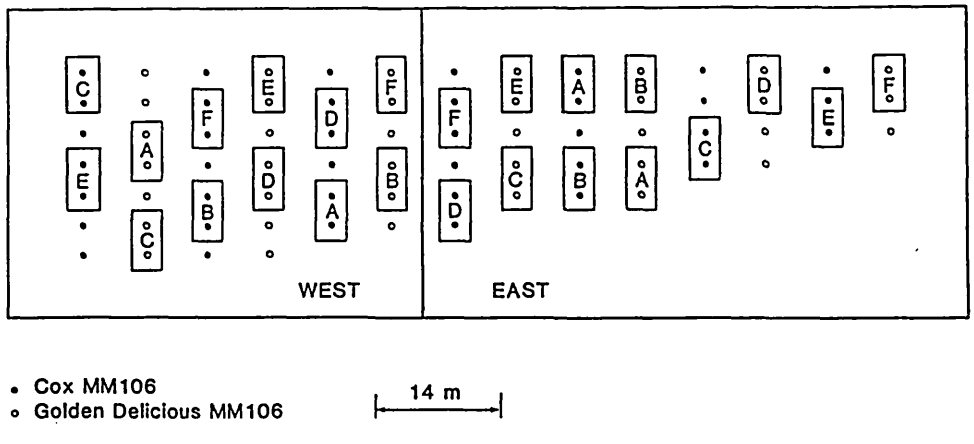


Fig. 2.4 Field Layout: Plot HG 115, 1987

ii. Orchards DR 119, HG115 and LF 113, East Malling.

Other experimental trials were performed on smaller sites using 1.5m trees. The 1986 fungicide compatibility trial (Chapter 4, II(a)iii) was carried out on Discovery (M26 rootstock) 4.6 x 2.3 m planting (orchard LF113); the 1987 spray volume trial (Chapter 4, II(a)iv) on Bramley (M26 rootstock), 4.5 x 6 m planting (DR119); and the 1987 formulation trial (Chapter 6, 2(c)i) on Cox and Golden Delicious (MM106 rootstock) 3.5 x 7.0 m planting (HG115). In each trial, plots consisted of two trees, with four replicates. LF113 was completely randomized, DR119 and HG115 designed as randomized blocks with four blocks. Apple variety was controlled for, by blocking, on site HG115 (Fig. 2.4).

Selected trees on DR 119 were used for an additional trial to evaluate the uptake of CpGV by codling moth damage from fruit and foliage (Chapter 5, II(b) - see section VIII(c) below, for details).

iii. WE 149, East Malling.

A trial testing the persistence of CpGV was carried out on 1.5 m Cox and Worcester trees on M9 rootstock, 5 x 2.5 m planting. The trial was designed as a randomized block with "plots" consisting of single branches, two branches per tree, blocked for apple variety. Three trees were used in each block (three Cox blocks, one Worcester).

iv. Eden Farm West Malling

A one hectare orchard of Tydeman's Early Worcester at Eden Farm, had not been chemically-sprayed for at least three years and was pruned from a derelict state prior to the trial. The orchard was to be used for the testing of the insect growth regulator Fenoxycarb. Since fenoxycarb is recommended to control the spring generation of leafroller larvae, treated areas must be sufficiently well shielded to prevent immigration of adults from unsprayed areas - a minimum separation of 20 m is recommended between treated and untreated areas (Dr R. Maag Ltd, Technical Information). In order to minimize immigration from untreated plots, experimental plots should be as large as possible and sampling avoided from the plot edges. With limited orchard space, increased plot size would inevitably sacrifice replication. However, for these trials a system was

planned where a "matrix" design allowed adequate replication and minimized immigration in a small orchard. Treatments were superimposed, matrix-fashion on a blanket application of fenoxycarb (Fig. 2.5). The experimental area was thus sprayed almost entirely with fenoxycarb but four small plots were left untreated. The design is best visualized as a "negative image" of a conventional field plan where treated plots would normally be superimposed on a large untreated area. Plots comprised four 3 m trees and were a minimum of 25 m apart. The four trees in each untreated plot were guarded from spray drift by leaving the inside edges of surrounding trees unsprayed. Four 'treated' plots were designated from within the blanket fenoxycarb area. The likelihood of moths migrating from the small untreated plots to the treated plots would be small due to the distance of and the dilution through 360°. The experimental area was "guarded" from neighbouring orchards by spraying a wide band around the trial orchard (Fig. 2.5).

v. Congelow Farm, Yalding, 1986; commercial organic farm.

In 1986, a small unreplicated trial was undertaken in a 1.5 ha orchard of apple variety Edward VII. A 0.3 ha square plot comprising 36, 3-4 m trees was chosen for CpGV treatment. The plot was bordered on three sides by untreated trees. All trees were sprayed with dispersible sulphur to control scab (*Venturia inaequalis*) and powdery mildew (*Podosphaera leucotricha*) but no additional insecticides were applied (further details, see Chapter 8, section II(b)i).

vi. Congelow Farm, 1987.

In 1987, a larger scale, fully replicated trial was undertaken at Congelow Farm. A 2 ha orchard of Laxton's Fortune was divided into eight plots, four treated and four untreated (fig 2.6).

vii. Folly Fruit Farm, Eastergate, West Sussex

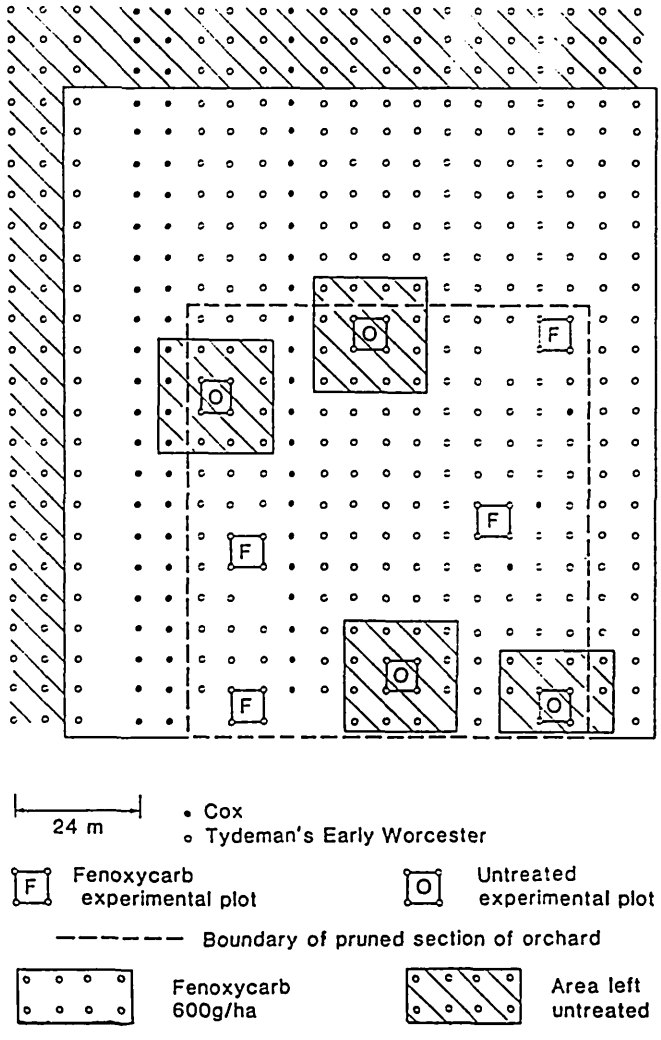


Fig. 2.5 Field Layout: Eden Farm, West Malling, 1986

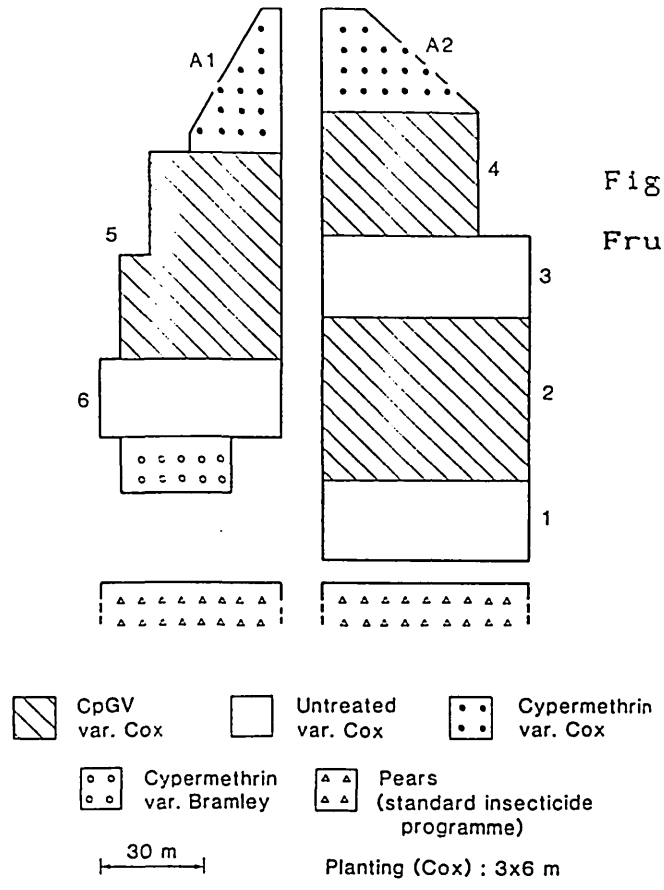


Fig. 2.6 Field Layout: Folly Fruit Farm, Eastergate, 1987

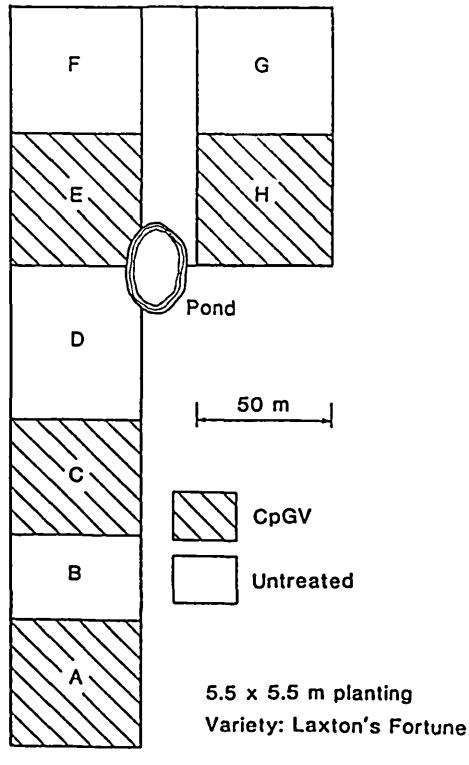


Fig. 2.7 Field Layout: Congelow Farm, Yalding, 1987

The trial site was a 1.5 ha orchard of Cox with Laxton's Fortune, Tydeman's Early Worcester, and James Grieve pollinators. Untreated plots were agreed by the farm owner, although these were only half the size of the treated plots. The orchard was divided into eight plots: three CpGV treated (0.8 ha), three unsprayed (0.4 ha) and two additional plots (0.3 ha) sprayed under the standard chemical programme used on the farm, serving as a useful comparison for the success of CpGV. The trial plan (fig. 2.7) was blocked - east and west - to account for variation in infestation; a derelict orchard on the eastern border was believed to be a source of codling moth adults.

viii. Little Barton Farm, Canterbury.

A 2 ha site, 'Pigeon Field' of variety Cox was divided into two equally sized plots.

b) Artificial release of adults

At two small sites (DR 119 and LF113), it was feared that pest infestation would be low owing to chemical pesticide control programmes in earlier years. To supplement the natural population, codling moth adults were artificially released. on orchard LF 113, in 1986 (Chapter 4, II(a)iii), 300 "wild" pupae were distributed in small nylon gauze bags pinned to each tree trunk. These pupae had been collected the previous year from corrugated traps on the trunks of unsprayed trees and stored outdoors in a bird-proof cage. Additionally, 200 laboratory-reared adults were released at the beginning of July. The 500 released pupae and moths were spread over 96 trees. In 1987, 786 laboratory-reared adults were released within the trial area of DR 119 over a week at the beginning of July (Chapter 4, II(a)iv).

c) Artificial release of eggs and neonate larvae

In a trial investigating the uptake of CpGV by larvae from sprayed foliage and fruit (Chapter 5, II(b)), the use of highly labour-intensive treatments required maximum pest damage in a very restricted trial area. Five 1.5 m Bramley apple trees on M26 rootstock, were chosen on part of site DR 119 in an area of low natural codling moth infestation. Twenty

branches from the five trees were selected, each with a good fruit set (16-29 apples per branch). The fruit was first inspected on the trees and any damaged apples were discarded from the trial branches. Each branch was covered in a loose fitting bag measuring 1.5 m in length and 0.7 m in width, made from fine nylon netting (hole size: 0.9 x 0.33 mm) (plate 2.2). The bags were tightly secured to the base of each branch with wire. To each bagged branch 110 mature eggs and 90 freshly hatched *C. pomonella* larvae were introduced. The bags were designed both to reduce predation and keep the introduced larvae on the treated branches.

d) Spray application

In the first trial of CpGV dose and formulation at WF103, sprays were applied at 1000 l/ha (5 l/tree) using a petrol driven Minicomet sprayer with hand held lance and single cone nozzle. However in the two subsequent seasons, in order to achieve a spray output more akin to common UK farm practice, CpGV was applied at a rate of 250 l/ha using a Solo Minor mistblower (plate 2.3). The Solo Minor was also used at Eden Farm and at Congelow Farm in 1986.

On the smaller field sites, large-output machinery was impractical because of the difficulty in localising the spray on the small plots. For these trials medium volume 500 l/ha was achieved using a CP15 Knapsack sprayer. An ultra-low-volume (ULV) output of 25 l/ha was achieved using a Turbair Scamp, generator-driven controlled droplet applicator. Where only single branches were sprayed,, treatments were applied using a Knapsack compression sprayer fitted with a single cone-nozzle. 0.5 l was applied to each treated branch, equivalent to 1000 l/ha.

Large-plot application of *Bacillus thuringiensis* (Dipel 2X) and fenoxycarb (Insegar) was achieved using a tractor-drawn Berthoud Mistblower delivering 250 l/ha (plate 2.4). Trials on commercial orchards were conducted - under supervision - using the grower's own machinery and workers. Tractor drawn mistblowers were used in each case.



Plate 2.2 Exclusion bags to prevent predation and escape of codling moth larvae



Plate 2.3 Spray application with Solo Minor mist blower



Plate 2.4 Spray application with Berthoud mist blower

e) Spray timing

The decision to spray, and the timing of the first spray application each season was predicted using pheromone trap records. In 1985 and 1986 Pherocon 1C traps (Zoecon, USA) were used with Oecos delta traps (Oecos, Hitchin, UK) used in 1987. One trap was placed in each site from the end of May, with the baits replenished every six weeks. Spray timing was based on a system devised by Glen and Brain (1982) who recommended that the first insecticide spray should be applied 140 day-degrees above 10°C after the start of a 'reference week' in which more than 5 adult male moths were caught in a pheromone trap. A second spray should take place 100 day degrees later. The first spray would thus correspond to the beginning of egg hatch, determined by adult flight and the minimum development temperature for codling moth of 10°C. Day-degrees were calculated on an approximate basis (Glen and Brain, 1982).

$$\text{Accumulated day-degrees} = \left\{\left(\frac{\text{daily maximum} + \text{daily minimum}}{2}\right) - 10\right\} \text{°C}$$

The spray dates and day-degrees have been given for the main trials reported here where the sprays were aimed against a natural population. Where laboratory reared adults were released, later sprays were timed to coincide with expected oviposition, assuming egg hatch to be 140 day-degrees after adult release.

f) Assessment of fruit damage

In each trial - excepting trials on commercial sites, when samples only were taken - all windfall and harvested apples were carefully examined for superficial and severe damage by codling moth larvae. Damaged fruit were cut open to assess the severity of damage and to distinguish between damage caused by codling moth larvae and other caterpillar pests. Apart from minor superficial stings, damage at the calyx or stalk could be seen by the presence of larval frass. Despite the large numbers of apples involved in each experiment - ranging from 3,500 to 79,000 (*sic*) - it was considered worthwhile to examine all the fruit since this gave greater precision in distinguishing between treatment effects.

Lepidopterous pest damage was distinguished as follows:

i. **Codling moth.** Such damage was distinguishable from that caused by other lepidopterous larvae: superficial "stings" - caused by first instars - were small, well healed injuries often marked by a reddening of the apple skin. Tunnelling was characterised by the presence of wet, brown frass, the tunnels themselves oozing at the surface and invariably leading straight towards the apple core. Codling moth damage was classified according to its severity using a system taken from Glen (1981)

Category I: damage by early first instar larvae where the apple skin has healed leaving at most a red "sting".

Category II: small pits of browned tissue with no exterior frass.

Category III: (Plate 2.5 AB) larger messy holes with visible frass and a tunnel in the apple but not reaching more than half way to the core. Larva may be present.

Category IV: (Plate 2.6) interior and exterior frass, tunnel reaching more than half way to the core with the seeds often eaten. Larva may be present. Frass generally light brown and very wet.

ii. **Early caterpillar damage.** (Plate 2.7 A, B, E, F) Species such as winter moth *Operophtera brumata*, clouded drab, *Orthosia incerta* and March moth *Alsophila aescularia* cause damage at the fruitlet stage, which persists to harvest; typically this is extensive corky scarring often misshaping the fruit at the site of damage.

iii. ***Blastobasis decolorella.*** (Plate 2.5 AA) *B. decolorella* larvae damage maturing fruit from July onwards and the distinctive purple-brown larvae most commonly feed around the base of the stalk leaving dry black frass. Any tunnels are smooth walled and fairly dry.

iv. **Fruitlet mining tortrix, *Pammene rhediella.*** (Plates 2.8 and 2.9 Q)) *P. rhediella* cause damage at the fruitlet stage, though damaged apples could remain on the trees until harvest: tunnels of less than 2 mm diameter follow (unlike codling moth) a meandering path - never to the core - and often resurfacing in a U-bend. The tunnels differed markedly

from those caused by codling larvae since the walls were dry and often lined with a white powdery substance.

v. **Superficial tortrix moth damage.** (Plate 2.9 R) Such damage is attributable to a number of leaf-roller species including *Archips podana*, *Adoxophyes orana* and *Syndemis musculana*. Apple damage is mostly restricted to irregular 'skinning' of the fruit and, in *A. podana*, some surface pitting. Very often damage is accompanied by webbing and commonly a larva is found inside a web 'tent' between two touching fruits or a fruit and an adjacent leaf. *A. podana* tends to cause small irregular patches of fruit skinning, whereas larger continuous areas are attributable to *A. orana*. Superficial damage is common in maturing fruits in July and August.

g) Codling moth trap bands

The overwintering population of diapaused codling moth larvae was assessed using corrugated trap bands (Falcon *et al* 1968). Bands consisted of a double layer of 10cm wide corrugated cardboard protected from bird predation by tough nylon gauze. Traps were set at the beginning of August and the number of larvae which had crawled into the traps to overwinter were recorded after harvest.

IX. Monitoring other pests and beneficial arthropods

a) **Earwigs.** An estimate of the relative numbers of earwigs, *Forficula auricularia*, in each plot was gained by counting individuals in 15 cm corrugated card traps wrapped around the trunks of trees.

b) **Mites.** Twenty leaves from each plot were passed through a leaf brushing machine (Leedom Engineering, California, USA). Mites were collected on a glass disc coated with washing-up liquid, identified and counted.

c) **Woolly aphid.** Woolly aphid, *Eriosoma lanigerum*, numbers were estimated by counting the number of colonies - clearly visible owing to their white "woolly secretions" - around the trunk and branches of each tree.



Plate 2.6 Apple damage by codling moth, Category IV



Plate 2.5 Apple damage by:
(AA) *Blastobasis decolorella*
(AB) Codling moth, Category III

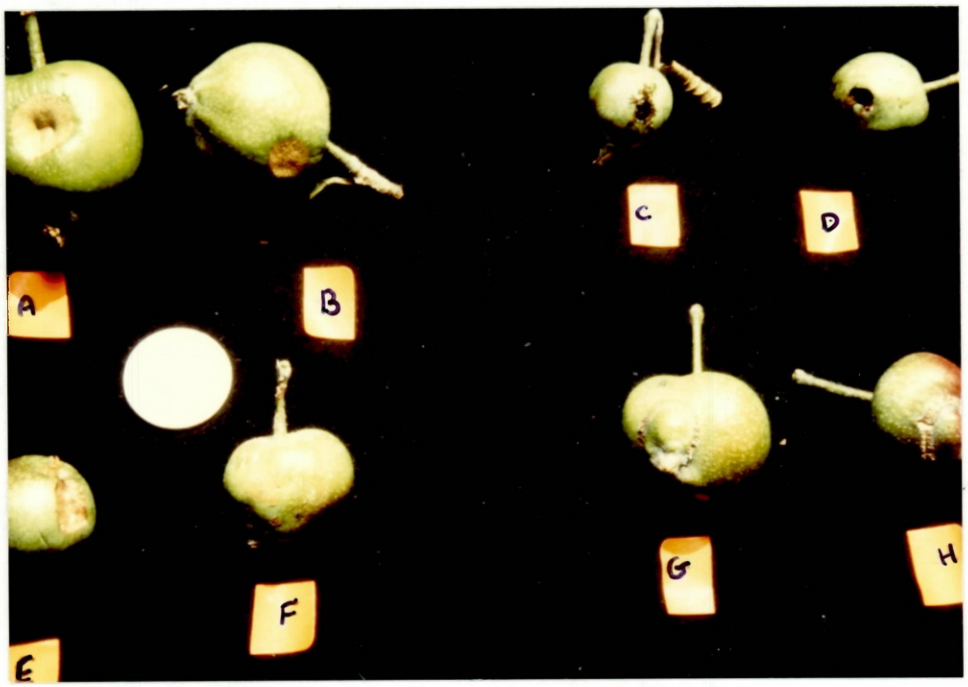


Plate 2.7 Damage to apple fruitlets:
(A,B,E,F) Early caterpillar damage
(C,D,G,H) Sawfly damage



Plate 2.8 Damage by friutlet mining tortrix (*Pammene rhediella*)

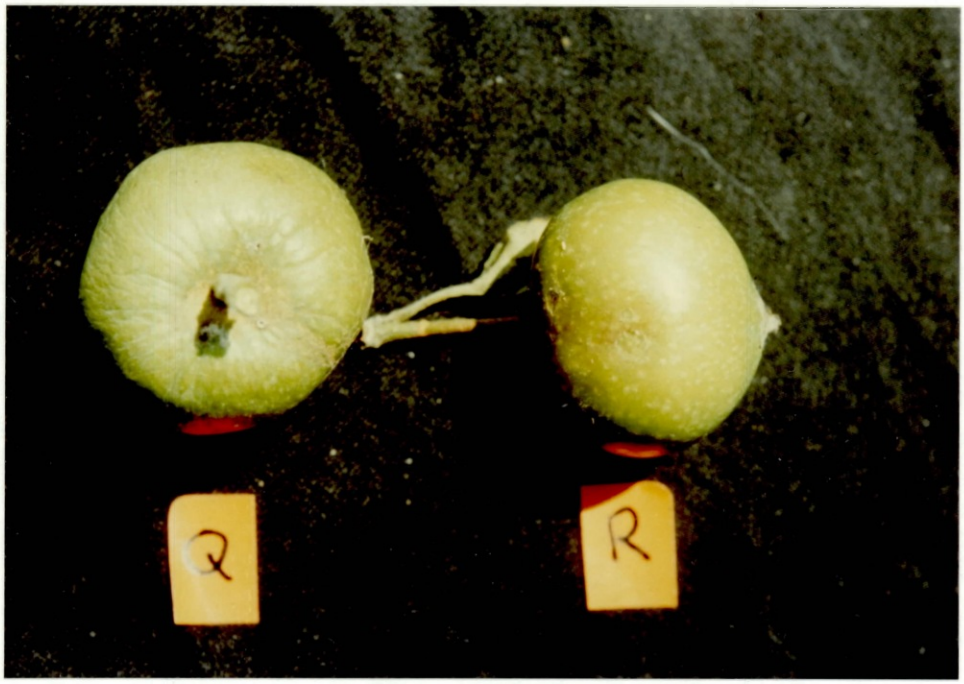


Plate 2.9 Apple damage by:
(Q) Friutlet mining tortrix
(R) Superficial tortrix moth damage



Plate 2.10 Bait pan for trapping adult tortrix moths

d) **Tree beating.** Various arthropod species were sampled by beating branches over a net funnel with collecting vial: two beats per branch, ten branches per plot or replicate. Samples were deep frozen for later identification.

e) **Suction sampling.** In the 1985 small plot trial, beating was avoided to prevent premature fruit fall. As an alternative, a petrol-driven 'Univac' suction sampler (Burkard Manufacturing Co. Ltd) was operated at maximum power for four minutes in each plot.

f) **Moth monitoring by bait-pans.** Bait-pans, following a design by David Glen (pers. comm.) were suspended at 2 m height within the tree canopy (Plate 2.10). Pans consisted of an 18x8 cm green plastic seed pan (B.E.F. Products Ltd., Billericay), half-filled with 10% molasses and dried active yeast in water, and covered by a second, larger pan (22x8 cm) forming a lid which was raised 30 mm above the smaller pan using wire spacers. Several moth species - predominantly leaf-rollers (Tortricidae) were attracted into the pans and could be identified after rinsing in water. Bait-pans rather than pheromone traps were chosen to attract moths from only a short range (David Glen, pers. comm.).

g) **Leafrollers.** 200 leaf clusters per replicate were examined for presence of leafroller larvae (Tortricidae), aphids and predators.

X. Analysis of spray output, penetration, deposition and persistence of CpGV

a) Virus deposition and persistence.

Spray deposition and the field-persistence of CpGV on foliage were measured by bioassaying leaf washings. Ten leaves were collected at random from each sprayed plot. From each leaf, a single 15 mm diameter disc was cut using a size 10 corkborer. The ten discs were shaken in 10 ml water in a conical flask for 2 hours at 20°C using a flask shaker at 250 rpm. Leaf washings were stored at -20°C prior to bioassaying.

0.1 and 1.0 ml of each sample were added to 9.9 and 9 ml molten sterile diet (50°C), dispensed into microtitre plates, and bioassayed against a known CpGV dilution series (section II(c)iii). Virus induced mortalities of neonate larvae were compared to the known-standard curve and the virus concentration in each sample estimated by interpolation. The two dilutions generally gave mortalities within the sensitive range of the bioassay, 3×10^{2} - 9×10^{3} granules/ml diet, but high concentrations (i.e just after high dose spraying) required a further dilution with 0.01 ml sample in 10 ml final mixture. Alternatively, very low concentrations (several days after spraying) required greater concentration by adding 2.5 ml sample to 7.5 ml diet. Four replicate samples were taken from the four plots in each experiment and a mean virus concentration was calculated for each treatment. Treatments were compared by analysis of variance.

A similar method was used to test laboratory sprayed leaf discs. Instead of using four replicates, an accurate estimate of CpGV concentration in a single leaf-washing sample of 15 leaf discs was made from a dilution series. Each sample was incorporated with diet at 2.5, 1.0, 0.1 and 0.01 ml/10 ml final diet and the resulting mortalities analysed by MLP analysis and compared to a standard curve.

b) Droplet size analysis

An assessment of both the droplet spectrum and the droplet penetration (below) within the orchard canopy was made for the Solo Minor mistblower used in the Laxton's Fortune orchard trials at IHR East Malling. Droplet sizes were measured from imprints on magnesium oxide slides placed in the spray path (Matthews, 1979). For each slide 100 droplets were measured using a microscope fitted with a drawing tube and recorded on a graphics board linked to an Epson HX-20 microcomputer. Droplet sizes were analysed using a specific programme which included a correction factor for the spread of droplets on the magnesium oxide (programme supplied by J.G. Allen, IHR East Malling). Five slides were placed in each of several positions within the tree canopy.

Droplets produced by the Shandon laboratory sprayer were measured on water sensitive paper (Ciba Geigy) in place of leaf discs. The droplet sizes were readily analysed with an image analyser (Digithurst Ltd,

Microsight 2) examining 220 droplets on three strips for each treatment. Droplet density was measured by counting fluorescent-marker (Uvitex) labelled droplets impacting on non-fluorescing graph paper. The number of droplets in each of twelve 0.5 x 0.5 cm squares (four for each of three replicate sprayings) were counted.

c) Spray penetration and drift

Spray penetration through the canopy, and the level of drift, to neighbouring plots was measured for the Solo Minor mistblower by incorporating a fluorescent tracer, Uvitex (40% w/v emulsifiable concentrate, ICI) at 0.75 l concentrate per 30 l. The spray was applied at 250 l/ha (as in field trials; Chapter 4, section II(a)ii and Chapter 6, section II(b)v). 20 leaves from each position in the canopy were collected and examined under ultra-violet light. Spray coverage for each leaf, both upper and lower surfaces, was estimated visually in percent categories: 0, 1, 2, 5, 10, 25, 50, 75, 90, 95, 98, 99 and 100%. A similar method was used by Furness & Pinczawski (1985) who used fluorescent Tinopal CBS (Ciba Geigy) and visual assessment categories of 0, 25, 50, 75 and 100%. They were unable to compare different spray machines by counting droplets due to large differences in mean droplet sizes and used "visual" coverage as a better comparison.

XI. Foliar disease

Sooty moulds (*Cladosporium* spp.) resulted from the application of certain spray additives. Their incidence and effect on leaf senescence was checked. 10 leaves from each replicate were collected and studied using a binocular dissecting microscope. Incidence of sooty mould was measured by counting the number of mould colonies growing on each leaf (the moulds nearly always appeared in spots or colonies). The effect on leaf senescence was measured by visually estimating the total area of leaf browning on each leaf and categorising browning in one of the following categories: 0, 1, 2, 5, 10, 25, 50, 75, 90, 95, 98, 99 and 100%. These categories were chosen since they were readily distinguished, visually, from each other.

XII. Statistical Analysis

Fruit-damage data was analysed in terms of the total numbers of damaged fruit, either by an analysis of variance on square-root transformed data or by analysis of deviance using a generalised linear model with a binomial error and logit link function. This latter method allowed suitable weighting for plots with a heavier yield which would thus give a more accurate estimate of the damage in each treatment. Means and standard errors were predicted from the model. The analysis was sub-divided into harvested, windfalls and total fruit, and also into deep damage (Categories III and IV, see below) and total damage (Categories I-IV). Within each analysis orthogonal comparisons of certain treatments or groups of treatments were performed allowing a more detailed analysis of multi-treatment trials.

Chapter 3

Virus mass Production, Quality control and long term storage

I. INTRODUCTION.

Lack of efficient inexpensive methods of mass production is one of the major restrictions for the wider use of insect viruses (Huber 1988). At present, CpGV can only be realistically produced by *in vivo* culture, which is, by nature, time consuming and laborious: optimizing production, quantification and quality control is thus a key area in the development of CpGV as a commercial agent. Major cost savings can be made by simplifying processing steps. Purification, for example, can be omitted entirely for commercial production (Huber, 1988). Similar savings should be possible by streamlining the quantification of the final product prior to dispatch.

In order to facilitate both the commercialisation and registration of CpGV, a final product should fulfil a number of criteria:

1. It should be effective as a control agent

2. Cost of production should be minimized

3. The final product should be standardized; consistent in virus activity and genetic makeup.

4. The material should be convenient to use and retain viability when stored under appropriate conditions.

5. Microbial contamination should be within acceptable limits and should not significantly increase with storage.

Use of CpGV in orchards, whether for commercial production or for experimental trials requires an effective production system. A programme of experiments was designed to produce an efficient rearing system for

small scale mass-production with potential for expanding into pilot-scale commercial production. These experiments included both insect rearing and virus infection.

Impure CpGV preparations using homogenized infected insects and diet - both wet and dry formulations - formed the basis for a study of quality control and storage, aiming to fulfil criteria 2 to 5. Successful preparations were later tested in field and laboratory trials to evaluate their potential as control agents (Chapter 6, sections III(b)iv & (c)). Genetic variation in CpGV produced over the three-year study was monitored by agarose-gel electrophoresis of purified viral DNA treated with the restriction endonuclease EcoRI.

Purified suspensions of CpGV can be easily quantified by counting virus capsules in a counting chamber using dark-ground illumination under a microscope (Burges and Thomson, 1971; Glen and Payne, 1984) or by an analysis of virus protein using the Folin test (Lowry *et al* 1951). However, neither of these methods is suitable for the analysis of an impure homogenate owing to the presence of insect and diet material. Impure preparations could, hitherto, only be quantified by bioassay (Payne, 1981). However, although bioassays are essential as the only means by which the infectivity of CpGV - impure or purified - can be tested, its routine use for every production batch would be both extremely time consuming and require large numbers of larvae (ca. 200 per test). The enzyme-linked immunosorbent assay (ELISA) has proved an effective and sensitive method for detecting the dissolved granule protein of granulosis viruses (Crook and Payne, 1980), and in this chapter, the potential of a double-antibody-sandwich (DAS) ELISA as a rapid method of quantification of CpGV in impure homogenates was evaluated and a method suitable for batch-processing of a final product was developed. DAS-ELISA was chosen as it is more able to discriminate between closely related granulosis viruses and least affected by impurities than direct- or indirect-ELISA techniques (Crook and Payne, 1980)

II. MATERIALS AND METHODS

a) Mass-rearing trials with English codling moth stock culture.

From attempts to mass-produce CpGV for field trials it was soon apparent that rearing larvae in open trays was impractical owing to the incidence of premature CpGV infection seemingly inherent in the insect culture despite efforts to prevent contamination (including the location of an insect rearing unit in a separate building from the virus work, restrictions on staff access to the unit and regular disinfection of work surfaces). Death of one larva from virus in an open tray leads to the rapid spread of virus to others in the same tray. Mass-rearing trials with the existing codling moth stock were restricted to individually reared larvae. A number of experiments were set up to evaluate factors influencing rearing conditions. Larvae were always reared on diet containing a juvenile hormone analogue, methoprene (Altosid 5E, Zoecon Corp.), at 2ppm which has the effect of increasing the final weight of mature larvae (Glen and Payne, 1984).

i. Development rate of larvae.

Knowledge of the development rate of codling moth larvae is important for determining when, for example, they reach final instar and thus are ready for infection with CpGV. Fifty freshly-hatched larvae were set up individually in repliplates with 2-3 mm diet, and kept at 27°C in darkness. Larvae were examined daily and the development rate was assessed by determining the larval development stage indicated by measurement of head capsule width (Weitzner and Whalon, 1987). Head capsule width was distinct for each instar:

I = 0.3-0.4mm; II = 0.45-0.6; III = 0.7-0.85; IV = 0.95-1.25; V = 1.4-1.9

ii. Effect of larval density.

For the production of virus for field trials, larvae were reared in compartmentalised 'repliplates' (see Chapter 2, section II(a) for full details). The influence of crowding on the yield of larvae was assessed by rearing different numbers of larvae, from one to five, in each compartment.

Larvae were reared on 2-3 mm depth diet at 27°C in darkness and the number surviving to maturity was recorded after 17 days.

iii. Comparison of rearing containers.

Larvae reared for the maintenance of the insect culture were housed in individual 'polypots' (Chapter 2, section I). Although this method was very reliable, and helped prevent the spread of airborne infection, use of polypots was time consuming and required considerable space. It was also possible to rear larvae individually but in compartmentalised trays, or 'repliplates' which were much easier to handle (with only one lid for 25 compartments) and the individual compartments were much smaller than polypots (Chapter 2, section II(a)). In order to test whether the use of repliplates was as effective at producing a high yield of healthy larvae as the use of polypots, and what other factors affected yield, a number of comparative tests were set up with different container and diet conditions. Productivity was measured in terms of the number and size of mature larvae resulting in each rearing system. Larvae were always reared at 27°C in darkness, although the time to 'harvest' varied from 17 to 21 days in different trials.

Trial iii.1: The effect of rearing larvae in 'polypots' or 'repliplates'.

The suitability of repliplates with shallow (2-3 mm) diet as rearing containers was compared with the more capacious polypots (as used in the maintenance of the stock culture) containing diet cubes (1.5 cm^3).

Trial iii.2: The influence of the physical nature of the diet surface and the effect of larval numbers on establishment and development.

In this test, each repliplate compartment contained a thin layer (2-3 mm), of diet. Diet layers were either produced by pouring molten diet directly in the trays - producing a smooth, shiny, surface - or by the slightly more lengthy process of cutting sheets from a block of solid diet and pushing the sheets into the compartments; this latter system producing a rough, granular surface. One or two larvae were added to each repliplate

cell. Four repliplates were set up for each treatment. As a control, fifty larvae were also reared individually in polypots.

Trial iii.3: Effect of ascorbic acid in diet on establishment and development

Ascorbic acid is a normal constituent of codling moth diet. However, unlike the other ingredients it must be added after the molten diet has cooled to below 70°C to avoid degradation. Removing this step would further streamline the process of diet preparation. Furthermore, there was some initial concern about the effect of ascorbic acid on the shelf-life of CpGV in impure homogenates containing diet. In order to test whether ascorbic acid was an essential component of diet for larvae intended only for virus production, repliplates were set up containing either normal diet (with ascorbic acid at 0.5% w/w) or diet without ascorbic acid. Five trays with one larva per cell were set up for each treatment.

Trial iii.4: Effect of surface moisture on larval establishment and growth.

After preparation of rearing trays, the surface of the diet is noticeably wet. The effect of this surface moisture on larval establishment and development was tested by preparing six repliplates for each treatment with one larva per cell. The cut diet surface was either dried with tissue or left moist prior to introducing larvae.

b) **Mass-rearing trials with a stock culture obtained from France**

Although the use of repliplates for the individual rearing of codling moth larvae proved adequate for the production of CpGV for small-scale field trials, this process would probably be too labour intensive for commercial production. Attempts to rear larvae *en masse* in open trays were unsuccessful with the existing codling moth stock at IHR Littlehampton, and this lack of success could perhaps have been due to the stock itself. To test this possibility, a different codling moth stock was imported from France (I.N.R.A., Station de Zoologie et d'Apidologie, 84140 Montfavet) and reared in the same conditions as the English stock. Some basic comparisons were set up to study English and French larvae in terms of establishment and size. All tests were performed in controlled environment cabinets, at 25°C and an 18 hour photoperiod.

i. Comparison of French and English stock larvae in open tray rearing

The establishment and development of French and English neonate larvae were compared in open trays, with 100 larvae added to 17x11x5 cm deep trays containing 8-10 mm diet. French larvae were also set up on thin layer (2-4 mm) diet, and thick (8-10 mm) diet with one hundred 6 mm diameter holes bored into the diet in each tray. Holes were included as a potential way of preventing larval interference and cannibalism by encouraging larvae to remain in small diet 'cells'.

ii. Size of English and French larvae

English and French larvae were compared by rearing individually in polypots containing 1.5 cm^3 blocks of diet. The number establishing, and their final weights were recorded after 19 days at 25°C.

c) **Infection with CpGV: optimal virus yield**

The final biological stage in the mass production of CpGV is the infection of mature larvae and subsequent harvest once infection is complete. This experiment was designed to evaluate the earliest harvest date which would give maximum virus yield; prolonging the time between larval infection and harvest would increase the chances of unwanted microbial contamination as larval tissues break down and saprophytic microbial growth occurs.

Seventy-five codling moth larvae were reared individually in repliplates (Chapter 2, section II(a)). After 17 days, larvae were infected with purified CpGV (60 µl at 10^7 granules/ml per repliplate cell). After 1, 2, 3, 5, and 8 days, seven larvae were removed from the repliplates and washed twice in sterile water to remove any superficial CpGV. The seven larvae were weighed and, with the addition of 5 ml sterile water, were ground in a glass tissue grinder. The resultant homogenates were stored at -20°C pending bioassay by the diet incorporation method described in Chapter 2 (section II(c)iii). From the MLP analysis of the bioassays, estimates of the virus yield per larva were made for each of the five tested dates.

d) Storage

Whether or not various CpGV preparations had an adequate shelf-life was determined by the titre of active virus and level of unwanted microbial contamination after storage. Liquid-homogenates, dry powder preparations, and purified CpGV were stored at a range of constant temperatures in darkness, and in a range of packaging. The effects of pH, the presence or absence of individual diet ingredients, and the addition of glycerol (40% v/v) to CpGV were also tested. Above-normal storage temperatures were used in some tests in order to accelerate deactivation of CpGV: loss of activity is three times quicker at 37°C than at 26°C (Huber, 1988).

Purified CpGV and impure homogenates were prepared as described in Chapter 2 (section II(d)). Impure preparations were either composed of infected insects and diet (tests i-iii), or from an artificially "infected" sterile diet homogenate to which purified virus had been added (test v). Dry powders were prepared from each of these products by air-, freeze-, or solvent-drying (Chapter 2, section II(d)iii). Materials were stored at a range of constant temperatures, in sealed bottles - with or without silica-gel drying agent - or in vacuum-packed vials. Virus titre was determined at intervals by bioassay, in comparison with a standard purified CpGV control (previously stored at -20°C). Bacterial contamination was determined by standard plate counts (Chapter 2, section II(d)v). In preparation for field trials, on research station or commercial orchards (including some in France, Spain and Italy; data not presented), impure CpGV homogenates were produced in seven batches (I-VII). Changes in microbial contamination during storage were monitored for Batch II only (a batch with a higher than acceptable bacterial count, rejected for use in field trials). Details of storage experiments are given below.

i. Effects of drying and the addition of glycerol on the shelf-life of an impure CpGV homogenate.

Treatments:

A. Impure CpGV homogenate
B. Impure CpGV homogenate + 40% glycerol (w/v)
C. Impure CpGV homogenate - Air-dried

D. Impure CpGV homogenate - Solvent-dried

E. Impure CpGV homogenate - Freeze-dried

F. Purified CpGV

G. Standard purified CpGV control

Samples were stored in sealed bottles at 4°, 20° and 37°C.

ii. Shelf-life of impure and dry preparations at -20°C.

Treatments:

A. Impure CpGV homogenate

B. Impure CpGV homogenate - freeze-dried

C. Impure CpGV homogenate - solvent dried

D. Standard purified CpGV control

Samples were stored in sealed bottles at -20°C

In this test, the material used (production batch VI) was prepared for the laboratory and field assessment of impure and dry powder formulations (Chapter 6,section III(c)).

iii. Effects of pH, drying and packaging on the inactivation of impure CpGV in accelerated storage tests.

Treatments:

A. Impure CpGV homogenate - (normal pH = 4.8)

B. Impure CpGV homogenate - pH adjusted to 7.0 by pH 11 phosphate buffer

C. Impure CpGV homogenate - freeze-dried

D. Impure CpGV homogenate - freeze-dried + silica gel packs

E. Impure CpGV homogenate - freeze-dried, vacuum packed

F. Impure CpGV homogenate - solvent-dried + silica gel packs

G. Impure CpGV homogenate - air-dried + silica gel packs

H. Purified CpGV

I. Standard purified CpGV control

Samples were stored at 37°C.

iv. Effect of diet ingredients on the inactivation of purified CpGV in storage.

Treatments:

A. Pure CpGV
B. Pure CpGV + Ascorbic acid 5% (ten times concentration in diet)
C. Pure CpGV + Ascorbic acid 0.5% (normal concentration in diet)
D. Pure CpGV + 0.25% (w/w) benzoic acid (normal concentration in diet)
E. Pure CpGV + 0.2% (w/w) methyl parahydroxybenzoate (normal concentration in diet)
F. Standard purified CpGV control

Samples were stored at 4°, 27° and 37°C.

v. Effect of ascorbic acid on the inactivation of a CpGV homogenate

Absence of ascorbic acid would affect the development of larvae and consequently the composition of an impure homogenate produced from infected insects and diet. For this reason, it was impossible to produce two batches of impure CpGV from homogenized infected insects and diet, identical in all but the presence or absence of ascorbic acid. In this test an artificially "infected" homogenate was prepared by adding known quantities of CpGV to diet with or without ascorbic acid (Chapter 2, section, II(d)ii).

Treatments:

A. CpGV/diet homogenate (CpGV added to diet homogenate at 1.92×10^8 granules/ml)
B. CpGV/diet homogenate - Air-dried
C. CpGV/diet homogenate - Solvent-dried
D. CpGV/diet homogenate - Freeze-dried
E. CpGV/diet homogenate - No ascorbic acid in diet
F. CpGV/diet homogenate - Air-dried - No ascorbic acid
G. CpGV/diet homogenate - Solvent-dried - No ascorbic acid
H. CpGV/diet homogenate - Freeze-dried - No ascorbic acid

I. Purified CpGV

J. Standard purified CpGV control

Samples were stored at -20°, 4°, 15°, 27° and 37°C.

e) **Uniformity of virus strain.**

In order to standardise a final product, it was important that the genetic composition of the virus should be checked regularly. Analysis by agarose-gel electrophoresis of viral DNA treated with the restriction endonuclease EcoRI (Chapter 2, section IV) was used to test CpGV produced in the three years of field studies.

f) **Protein analysis by Folin test**

Purified CpGV used in field trials was checked by a capsule count followed by a bioassay to test for infectivity. In addition, the different batches of purified CpGV used in the three field seasons reported here were further checked for uniformity by the Folin test for protein concentration (Chapter 2, section II(c)i).

g) **Development of an ELISA for quantification of impure CpGV.**

Samples of impure CpGV homogenates were prepared from infected insects and diet (Chapter 2, section II(d)i). The final product was tested for virus activity by bioassay against a pure standard sample of virus. A similar, but virus-free homogenate was prepared using uninfected, healthy fifth instar larvae. Homogenates with **known** CpGV concentrations (between 10^6 and 10^9 granules/ml) were then prepared by adding purified CpGV to dilutions of the virus-free homogenate to give a series of concentrations.

Each experiment involved the dilution of the preparations in 0.1M Na_2CO_3 which had the effect of dissolving the virus capsule protein, which has been found to increase the sensitivity of granulosis virus assays more than ten-fold (Crook and Payne, 1980). Preparations were subsequently further diluted in PBS-Tween (phosphate buffered saline + 0.05% Tween 20)

to prevent prolonged exposure to high pH. Three methods of sample preparation were compared:

i. After addition of purified CpGV (to give final virus concentrations of 10^6, $2x10^6$, $5x10^6$, 10^7, $2x10^7$, $5x10^7$, 10^8, $2x10^8$, $5x10^8$ and 10^9 granules/ml), the impure samples were diluted ten-fold in 0.1M Na_2CO_3 and allowed to stand for 35 minutes to allow capsule dissolution. The samples were further diluted three, ten and one-hundred fold in PBS-Tween before loading onto the ELISA plates (Chapter 2, section III). A pure CpGV standard was also prepared to give ten final virus concentrations from 10^6-10^9 granules/ml (as above). The pure standards were first diluted ten-fold in 0.1M Na_2CO_3 and then a further 100-fold in PBS-Tween. Na_2CO_3-PBS-Tween and virus-free homogenate controls were tested in the same way.

ii. A high level of background absorbence by impurities in the homogenates greatly reduced the sensitivity of method (i) (see results; section III(f)). In an attempt to reduce or eliminate this interference, in the second test samples were prepared as in method (i) (with ten- and one hundred-fold dilutions in PBS, and virus dilutions of $2x10^6$-$2x10^8$ granules/ml), but prior to loading, the samples were centrifuged at 1000 rpm for one minute (Sorvall RC5B centrifuge) in 1.6 ml aliquots. Centrifugation precipitated insoluble material leaving a clear supernatant for loading onto ELISA plates. Batches of impure CpGV in which the virus concentration was not known were treated in the same way.

iii. The ELISA was chosen as a straightforward method of quantifying virus titre. In this third method, the potential to store the standard samples prior to use was tested, since, if successful, this would allow its use as a pre-prepared kit. Samples were prepared as in method (ii), but assays were carried out before and after storing sample supernatants at -20°C for fifteen days.

III. RESULTS

a) Insect Mass Rearing

The development rate of codling moth larvae at 27°C is shown in Figure 3.1. Final instar moults commenced after 13 days with 57% reaching fifth instar after 16 days. By interpolation, the median point for development to fifth instar was about 15 days at this temperature. An attempt to rear larvae at 37°C was unsuccessful with all larvae dying within six days, none developing past the first instar. The effect on larval survival of crowding larvae reared in repliplates was investigated by varying the number placed in each cell. In three trials, regardless of the number starting in each cell, the number of larvae reaching maturity rarely exceeded one per cell and there was no advantage in increasing the number per compartment (table 3.1).

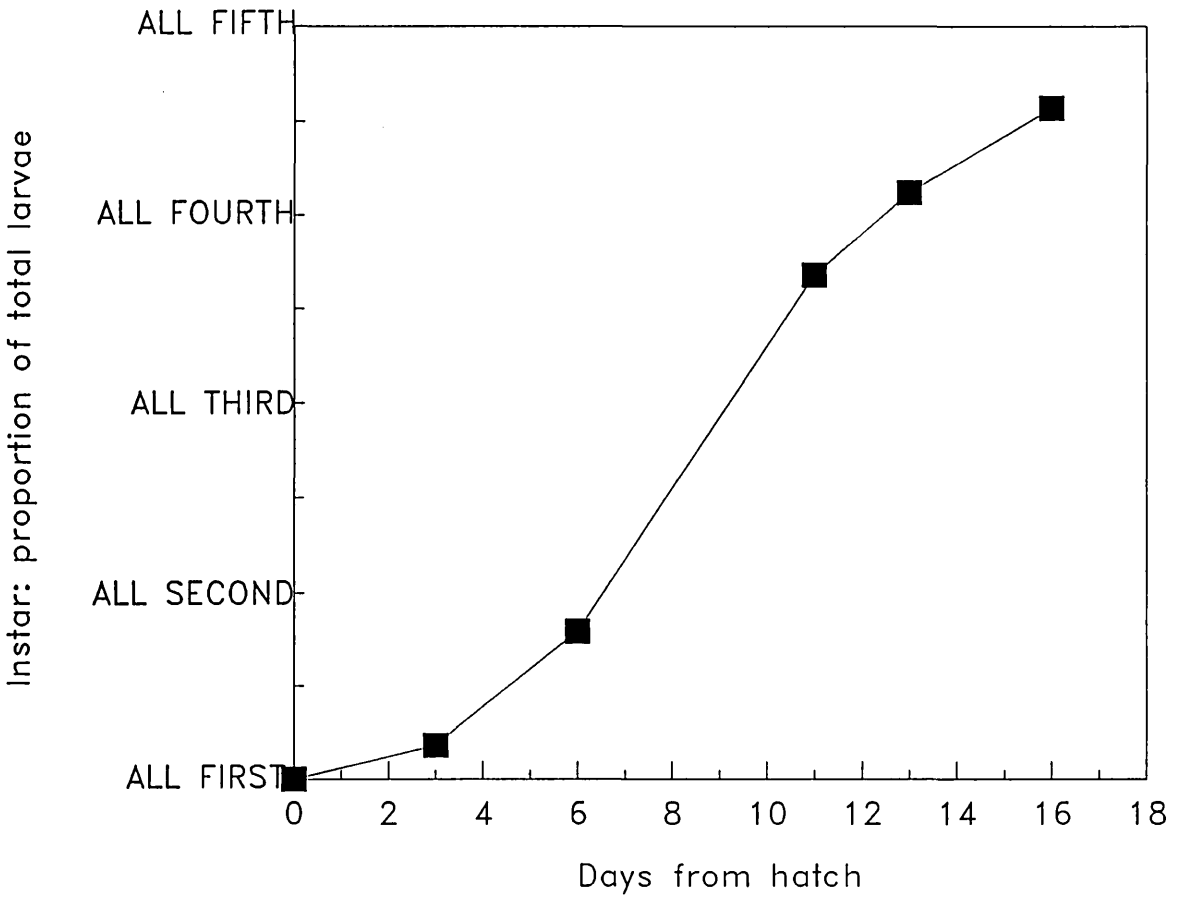
Table 3.1 Effect of crowding on compartmentalised rearing

Initial No. larvae/cell	Mean Final No.	S.E	Max No. per cell
1	0.90	0.10	1
2	0.80	0.17	2
3	0.67	0.13	1
4	0.53	0.13	1
5	0.90	0.15	2

The results of comparative rearing trials using both English and French stock codling moth larvae are given in tables 3.2 and 3.3. Survival of larvae reared individually - either in repliplates or polypots - was better than when reared in twos or threes, with up to 88% survival in polypots using cut diet. The final number emerging from repliplates was also little affected by the number initially placed in each cell, and this was consistent with the observations from the crowding experiment, above.

The diet surface was also important in larval development: although the level of establishment was not significantly different, the overall rate of development and weight at 'harvest' was lower in repliplates with a smooth diet surface - resulting from pouring molten diet directly into

Fig. 3.1 Development rate of codling moth larvae at 27° C



trays - than on diet with a rough surface produced by cutting sheets from a diet block. After 19 days, only 45% of larvae reared on poured diet had reached fifth instar, compared to almost 98% on the cut diet. The presence of surface moisture on cut diet did not affect establishment. Lack of ascorbic acid in the diet drastically reduced the development rate and weight at harvest, though establishment was unaffected.

Table 3.2 EXPERIMENTS ON ENGLISH LARVAE.

TRIAL	CONT-AINER	N°/POT OR CELL	DIET	THICKNESS (mm)	NUMBER Start	NUMBER Finish	SIG♦	%	WEIGHT (mg) MEAN	WEIGHT (mg) SE	WEIGHT (mg) MAX	WEIGHT (mg) MIN	SIG(t)	N° DAYS REARED
1	POLY	2	C	15	50	24		48	76,5	3,37	94	45		17
	REP	2	C	2-3	50	23		46	72,8	3,00	103	45	n.s	17
2	POLY	1	C	15	50	44		88	83,20	3,23	102	56		19
	REP	1	C	2-3	100	78		78	57,25	2,73	81	40	***	19
	REP	2	C	2-3	200	82	n.s	41	65,14	3,72	98	31	(cut	19
	REP	1	P	2-3	100	70		70	26,73	2,89	52	17	vs,	19
	REP	2	P	2-3	200	62		31	39,90	2,33	56	26	poured)	19
3	REP	1	C	2-3	125	71		56,8	88,2	2,20	122	47		21
	REP	1	-AS	2-3	125	79	n.s	63,2	21,4	3,67	79	6	***	21
4	REP	1	DRY	2-3	150	113		75,3	54,9	1,68	92	32		17
	REP	1	WET	2-3	150	111	n.s	74	53,8	2,20	87	22	n.s	17

♦Data compared by analysis of variance.

Level of significance: *** = P<0,001 n.s = not significant.
Rearing containers and diet: POLY = polypots; REP = repliplates; C = cut diet; P = poured diet; -AS = cut diet without ascorbic acid; DRY/WET = cut diet with or without surface moisture (see Chapter 2, sections I & II(a) , for full details).

Although some success was recorded using an open tray mass-rearing system with the English stock, many attempts to rear larvae in this way for virus production were dogged by very poor, and often zero survival of larvae to fifth instar. A certain amount of loss could perhaps be expected by larval aggression or perhaps cannibalism (consistent with the crowding trials) but by far the biggest problem was the spread of virus infection

in spite of rigorous attempts to preserve hygiene. There was insufficient time to test fully the French stock in extensive trials, but the encouraging results obtained in limited tests warranted the inclusion of these results. In a comparison of the existing English codling moth stock with eggs imported from a French laboratory culture, larval establishment was far superior using French rather than English larvae in open trays. Establishment was better than in any previous attempts using English larvae, and the final weight at harvest was also improved (98 mg mean weight in French larvae, and 61 mg in English - both reared individually for this comparison, on diet containing methoprene - with respective maximum weights of 137 mg compared to 93 mg). Cutting holes in the diet had no real effect on larval establishment or harvest weight, though it was interesting to observe that most of the larvae did occupy these 6 mm diameter holes, webbing the entrance to the holes after several days occupancy. Larvae could be reared adequately on thick (8-10 mm) or thin (2-4 mm) diet.

Finally, in an unreplicated test, an egg sheet was attached to the lid of a tray containing diet; 72 French larvae were reared in a single tray from about 200 eggs. The final weight of larvae was high, averaging 92 mg. The French stock was obtained at the end of the research project. However, results such as this suggest that, by using a virus-free healthy stock, large savings could be made in the cost of mass rearing.

French larvae responded equally to infection with CpGV:

LC_{50} neonate English larvae = 1524 granules/ml diet (1043-2180);
dose-mortality slope = 1.89

LC_{50} neonate French larvae = 1520 granules/ml diet (1075-2147);
dose-mortality slope = 1.85

Table 3.3 A comparison of English and French codling moth stock for mass-rearing

TRIAL	STOCK	CONT-AINER	N°PER TRAY/POT	DIET	THICKNESS (mm)	% Survival	WEIGHT (mg) MEAN	WEIGHT (mg) MAX	N° DAYS REARED
i	ENG	TRAY	100	C	8-10	7,0*	54	90	19
	FRE	TRAY	100	C	8-10	48,0	94	137	19
	FRE	TRAY+holes	100	C	8-10	60,0	96	121	19
	FRE	TRAY	100	C	2-4	60,0	91	131	19
ii	FRE	POLY	1	C	15	76,0	98	137	19
	ENG	POLY	1	C	15	72,0	61	93	19

* Many virus deaths.

Rearing containers and diet: TRAY = 17x11x5 cm plastic tray; holes = 100 6mm diameter holes bored through diet in each tray; POLY = polypots; C = cut diet (see Chapter 2, section I and II(a), for full details).
Larval stock: FRE = French larvae; ENG = English larvae

b) Optimal harvest of infected larvae (English stock).

Table 3.4 gives the yield of virus at different times after infection. Virus yield was excellent, with over $3x10^{10}$ granules produced from each infected larva. Maximum virus yield was achieved only five days after infection, at 27°C (before the larvae had died). A low level of infection after only two days may have resulted from some premature infection due to contamination or virus present in the English stock used. Plate 3.1 shows larvae (on artificial diet) at various stages of disease after infection with CpGV, culminating in disintegration of the cadaver.



Plate 3.1 Stages of disease in codling moth following infection with CpGV

Table 3.4 Optinal harvest of infected larvae

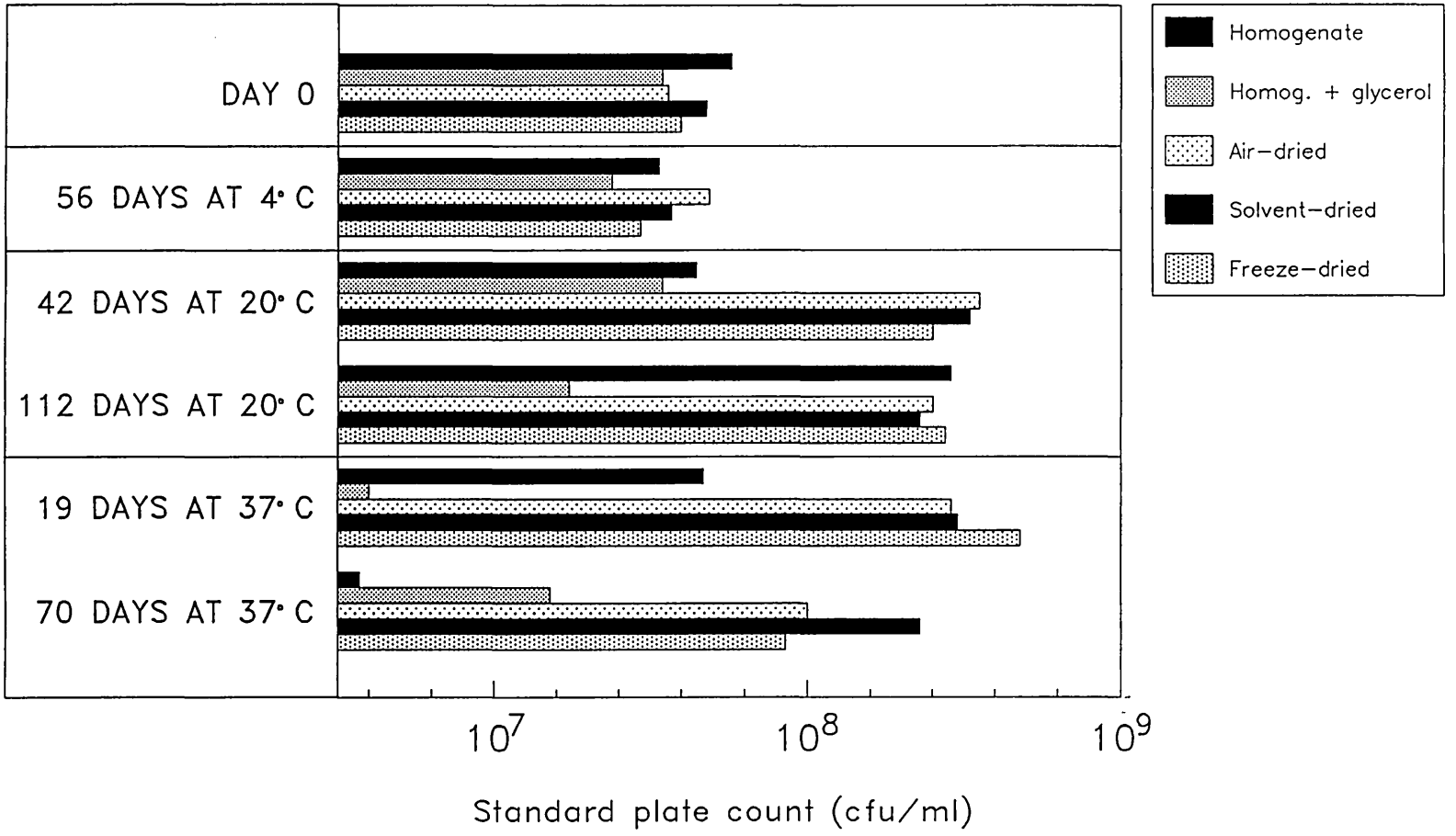
Days after infection	Weight of seven larvae (g)	Live/ dead?	CpGV per larva	Fiducial limits
1	0,52 g	All live	0	-
2	0,55 g	All live	$9,62x10^{9}$	$0,62-1,66x10^{9}$
3	0,55 g	All live	$1,48x10^{10}$	$0,92-2,43x10^{10}$
5	0,45 g	All live	$3,74x10^{10}$	$2,30-6,14x10^{10}$
8	0,42 g	3 dead, 4 live	$3,30x10^{10}$	$2,38-4,74x10^{10}$

c) Storage of CpGV

The effect of storage temperature, formulation, processing and packaging on the long term-shelf life of CpGV was investigated in terms of virus activity and the level of bacterial contamination. Bioassays to determine virus activity were analysed by MLP, and the activity remaining in the test material - with confidence limits - was thus derived. The data for activity and contamination have been summarised in Figures 3.2-3.8 highlighting the effects of different treatments. Virus activity of each sample after storage has been compared with its measured activity at day 0.

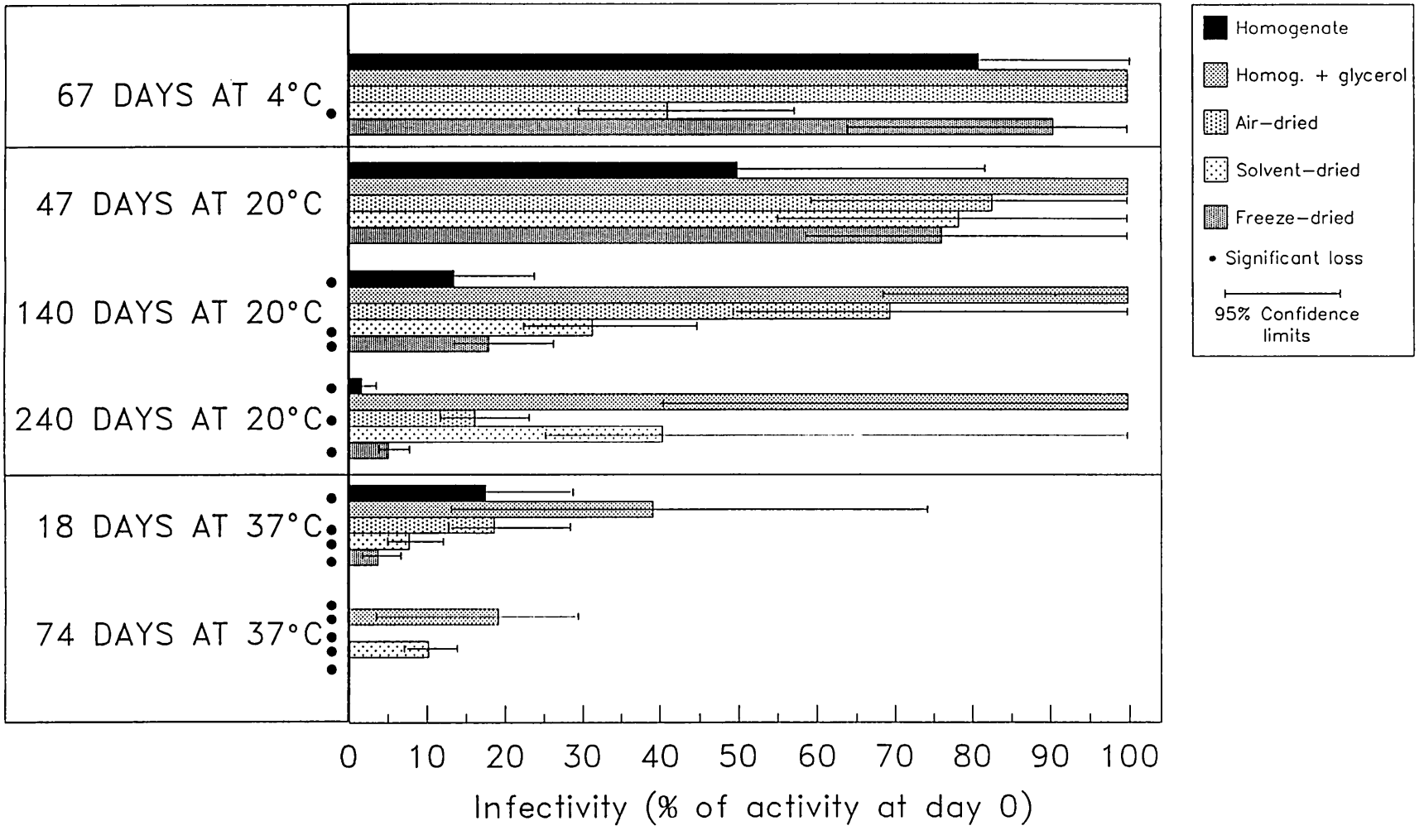
Bacterial contamination was monitored for an impure CpGV homogenate (production batch II) over several weeks at 4°, 20° and 37°C (Fig. 3.2). Bacterial counts were initially similar for all treatments - the processes of drying had little effect, presumably due to spore survival - and did not change significantly after 8 weeks storage at 4°C. However, at 20° and 37°C, bacterial counts in the dry powders increased by up to ten fold. The addition of glycerol maintained a low bacterial count in the impure homogenate at all temperatures whereas the count in the unformulated homogenate increased five-fold after four months at 20°C.

Fig. 3.2 Bacterial contamination of impure CpGV preparations



Note: dry preparations resuspended to original wet volume

Fig. 3.3 Long term storage of CpGV
1. effect of storage temperature (Batch II)



Note:Purified CpGV also retained 100% activity after 240 days at 20 C

Fig. 3.4 Long term storage of CpGV
2. Cold storage (−20° C)

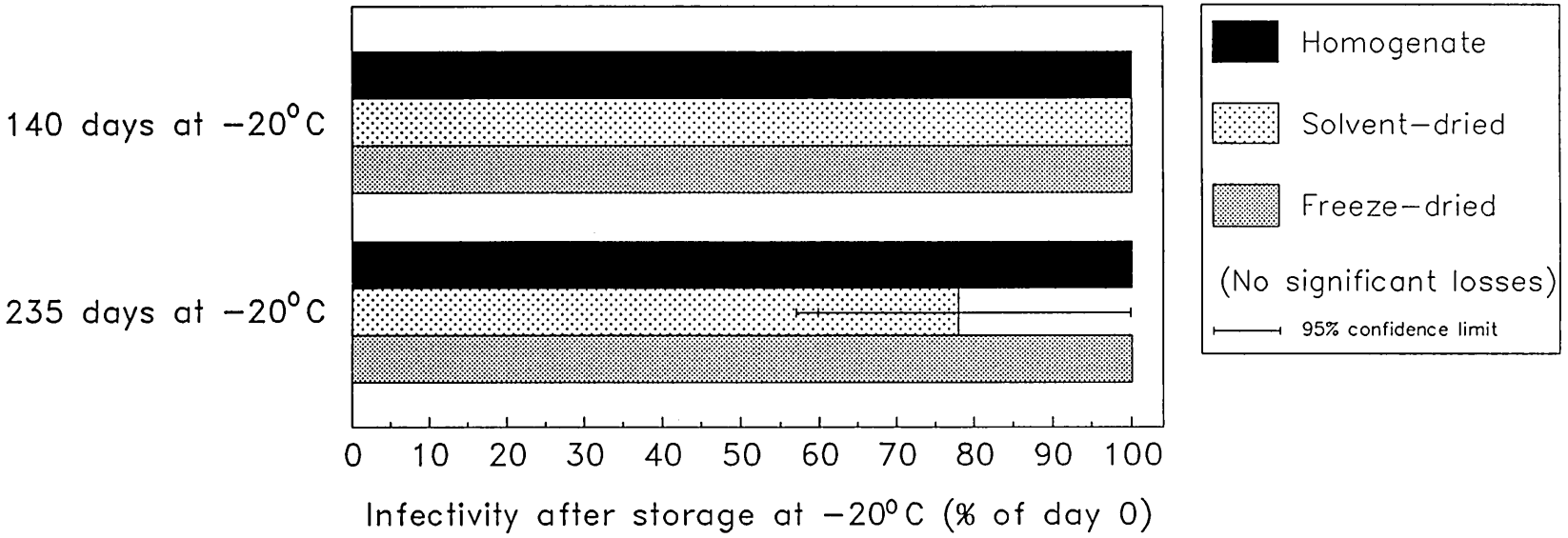


Fig. 3.5 Long term storage of CpGV
3. Effect of preparation and packaging

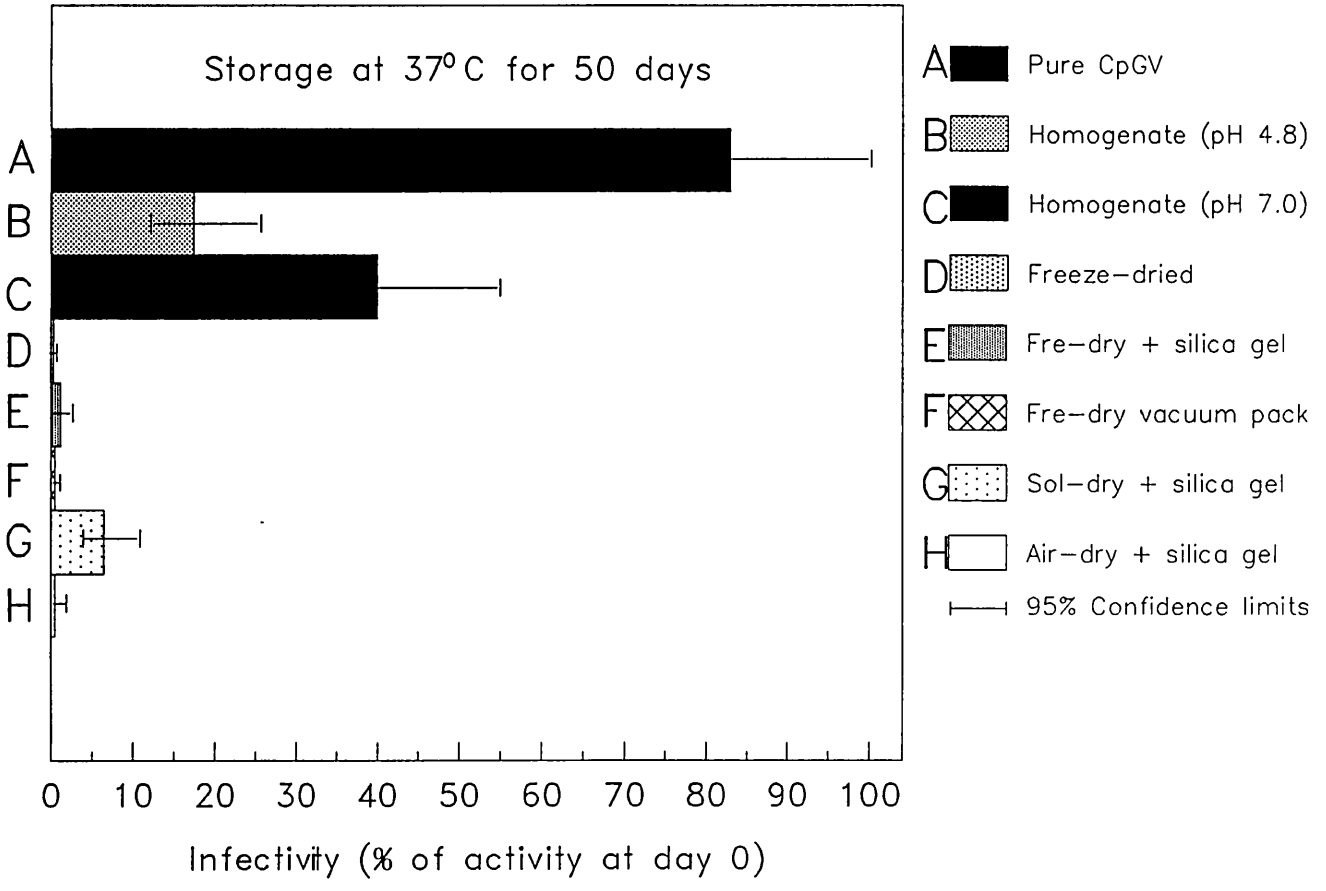


Fig. 3.6 Long term storage of CpGV

4. Effect of diet ingredients on pure virus

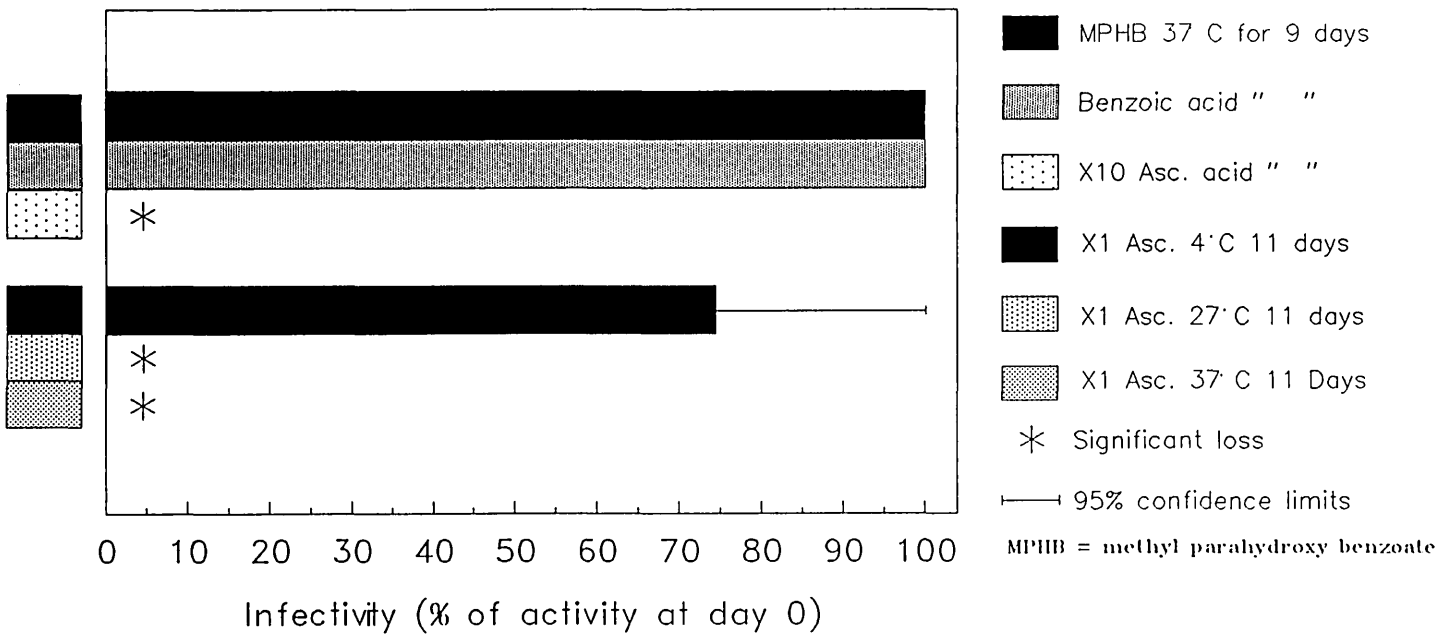


Fig. 3.7 Long term storage of CpGV
5. Effect of ascorbic acid on impure virus

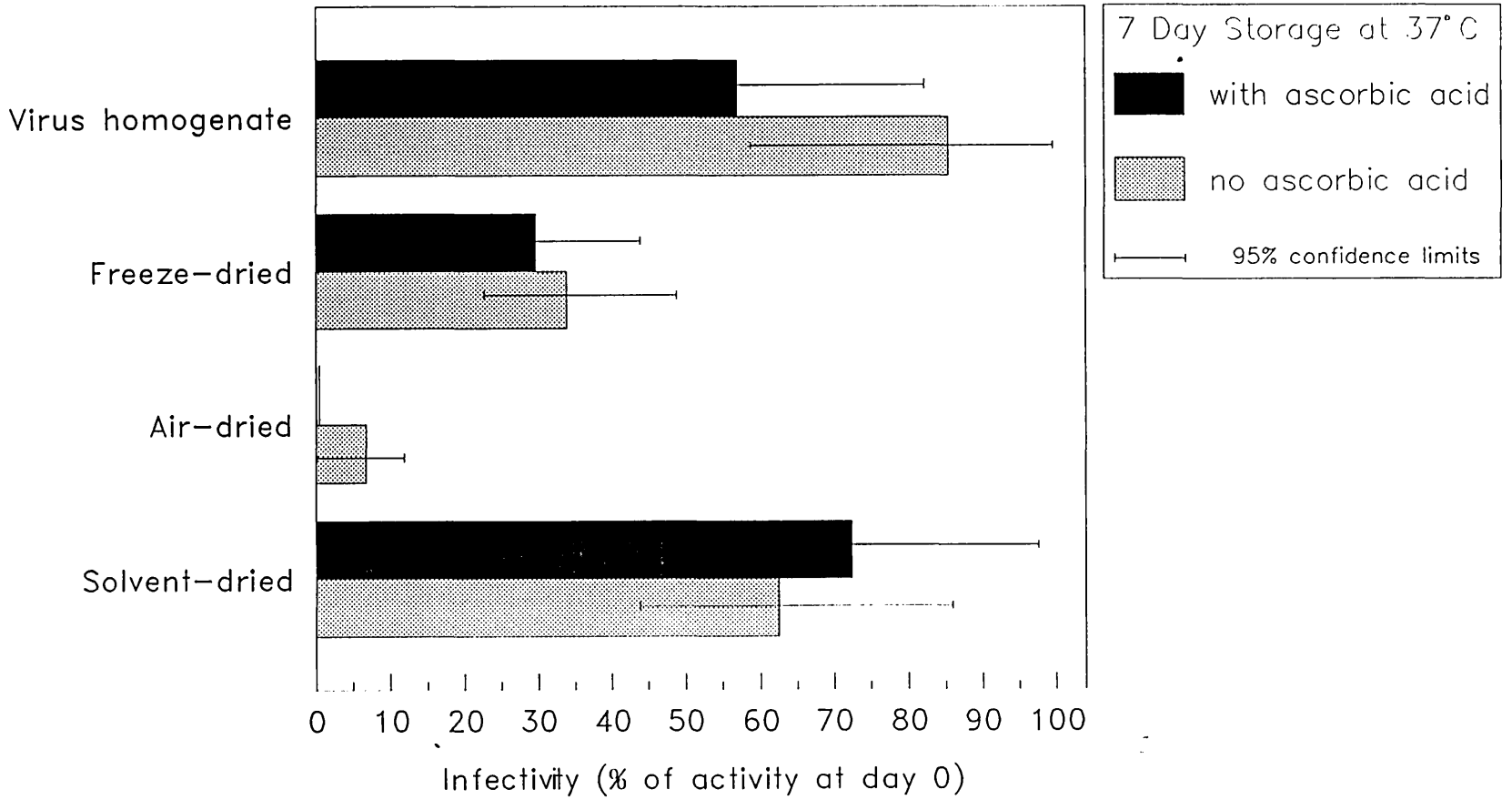
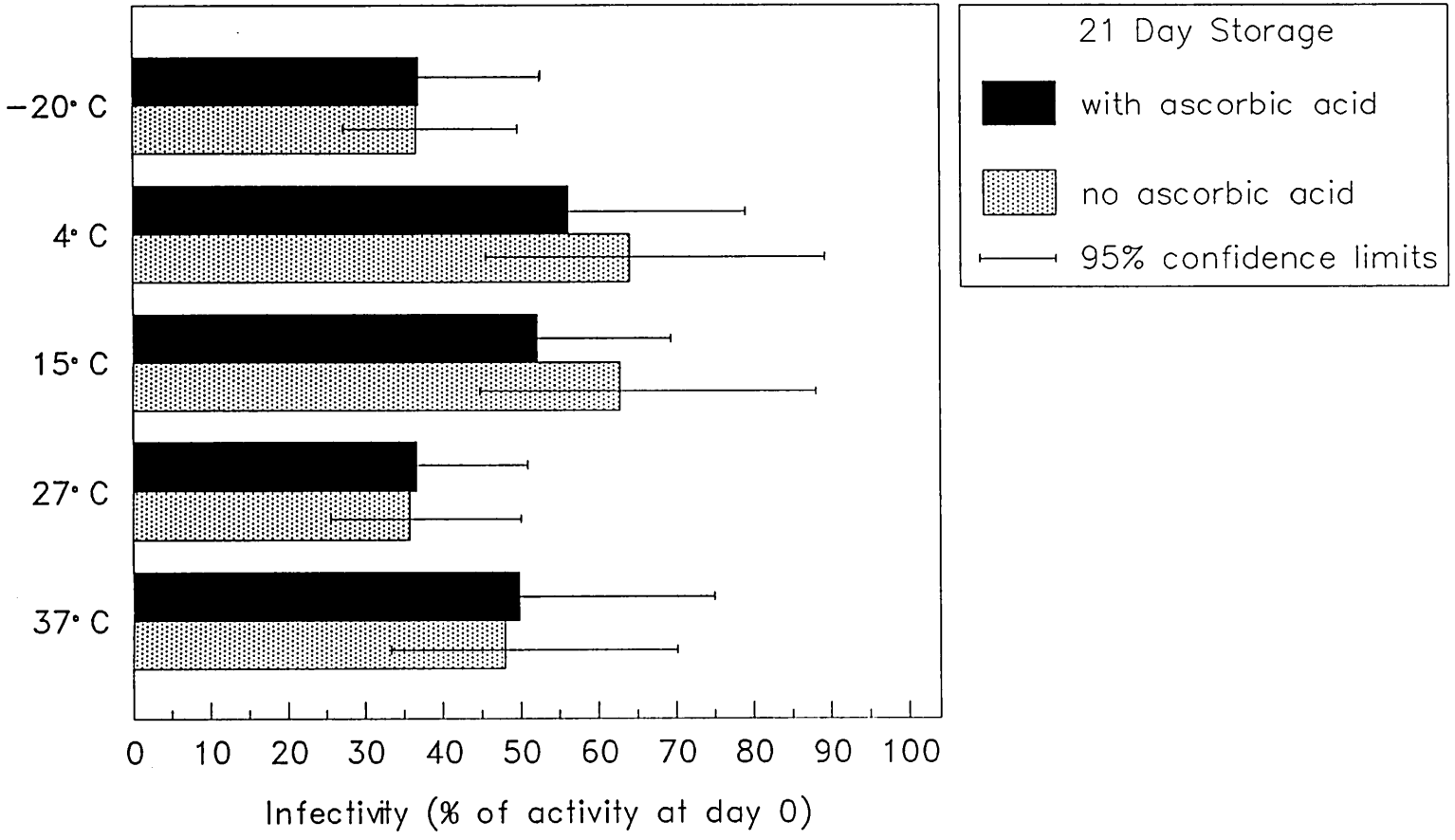


Fig. 3.8 Long term storage of CpGV
6. ascorbic acid at different temperatures:
effect on impure virus



Impure homogenates prepared for field trials were diluted to give a standard $5x10^{12}$ granules/litre (equivalent to one hectare dose). At this virus concentration standard plate counts (measured in colony forming units, cfu) for the batches used for field trials were as follows:

Batch I:	$3.6x10^6$ cfu/ml
Batch III:	$6.7x10^6$ cfu/ml
Batch IV:	$9.0x10^6$ cfu/ml
Batch V:	$3.4x10^6$ cfu/ml
Batch VI:	$5.8x10^6$ cfu/ml

CpGV itself proved to be tolerant to storage, even at comparatively high temperature: A sample of pure CpGV left for 50 days at 37°C retained 83% activity - the loss was not, in fact, statistically significant - (Fig. 3.5), and 100% activity when stored for 240 days at 20°C.

Impure homogenates and dry homogenate powders stored exceptionally well at -20°C, with no loss recorded after 235 days (Fig. 3.4). Above-freezing temperatures were however, a major influence on deactivation (Fig. 3.3). Little or no losses were observed in impure homogenates or powders after 67 days at 4°C. However, 74 days at 37°C reduced the activity of the same standard homogenate and air and freeze dried powders to levels below the detection limits of the bioassay. Storage at 20°C was better, with no significant losses occurring after 47 days, although the activity of the standard homogenate, and air- and freeze-dried powders fell to only 2-16% after 240 days. Solvent drying and the addition of glycerol to the homogenate caused significant improvements in storage at higher temperatures. Solvent dried powders were marginally better than freeze- and air-dried powders and the standard homogenate, retaining 40% activity after 240 days at 20°C. However, the most significant improvement was with the addition of glycerol; full activity was retained in the homogenate even after 240 days storage at 20°C, with 19% remaining after 74 days storage at 37°C. Adjusting the pH of the homogenate from 4.8 to 7.0 using a pH 11 phosphate buffer resulted in a slight improvement in storage life (Fig. 3.5): after 50 days at 37°C the sample adjusted to pH 7.0 retained 40.0% activity (confidence limits = 27-55%) compared with only 17.4% activity remaining in the pH 4.8 sample (confidence limits = 12-25%).

Neither vacuum packing nor the presence of silica gel in storage bottles had any major influence on shelf life (Fig. 3.5). A freeze-dried homogenate lost activity at a similar rate with or without these "improvements". Storage of air- and solvent-dried samples packed with silica gel was also poor with only 0.5 and 6.5% activity remaining after 50 days at 37°C

Neither of the diet ingredients benzoic acid nor methyl parahydroxybenzoate were detrimental to purified CpGV (Fig. 3.6). However, ascorbic acid at one or ten-times diet concentration caused almost complete loss of activity when stored at 27° or 37°C in less than two weeks. Ascorbic acid had, however, no effect on the storage life of the impure virus homogenate or dry powders, its effect presumably buffered by the presence of the other diet ingredients and insect debris (Figs. 3.7 and 3.8).

d) Uniformity of virus strain

EcoRI restriction enzyme analysis of CpGV DNA produced for the 1985, 1986 and 1987 field seasons suggested no major strain variation, with no change in the banding pattern observed following agarose-gel electrophoresis (plate 3.2).

e) Protein analysis of purified CpGV for field trials

Protein analysis by the Folin test gave consistent results for the three batches of purified CpGV used in field trials:

Batch A (sample 1) = 3.79 mg protein/10^{11} granules CpGV
(sample 2) = 3.88 mg protein/10^{11} granules CpGV
Batch B = 3.55 mg protein/10^{11} granules CpGV
Batch C = 4.87 mg protein/10^{11} granules CpGV

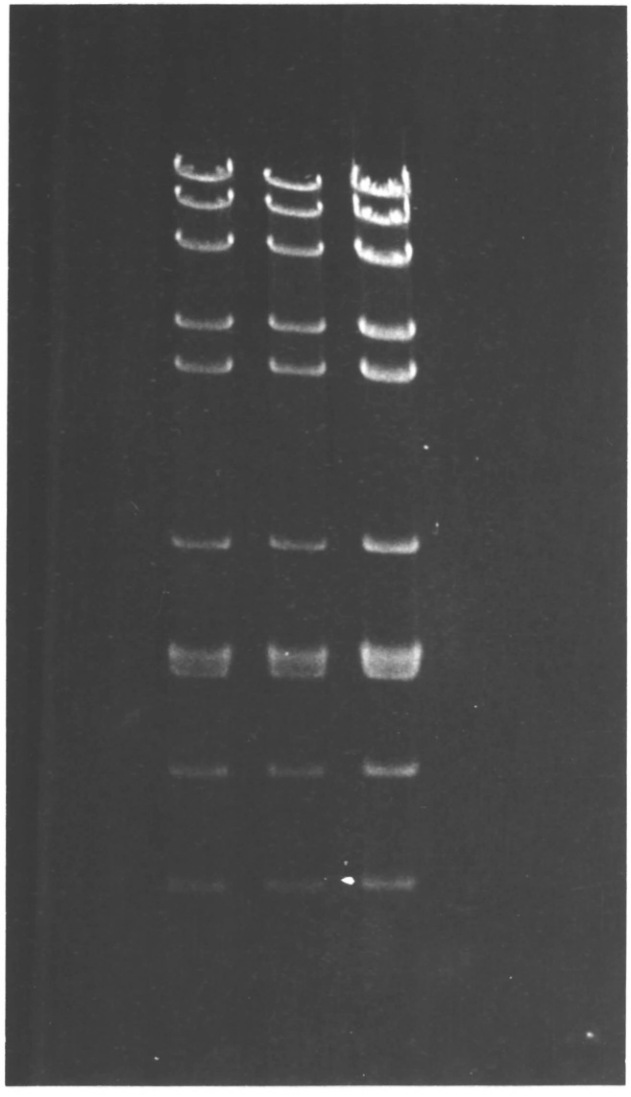


Plate 3.2 **Agarose-gel electrophoresis of CpGV DNA after EcoRI restriction enzyme degradation:**
From Left to Right : 1985, 1986, 1987 Production batches

Similar analysis by Glen and Payne (1984), gave figures of 2.5 ± 0.5 mg protein per 10^{11} granules, although the infectivities of their virus preparations (average LC_{50} = 2.4 ± 1.3 x 10^3 granules/ml diet; slope = 1.38 ± 0.34) were similar to those reported here (average LC_{50} based on 20 assays = 2.39 ± 0.23 x10^3 granules/ml diet; slope = 1.61 ± 0.06)

f) Development of an ELISA for quantification of impure CpGV

The results of ELISA tests on impure CpGV homogenates are expressed in Figures 3.9-3.11. In the first test where CpGV concentration series were assayed at three impure virus homogenate dilutions - 1:30, 1:100 and 1:1000 - and for pure CpGV, the response of the ELISA was clearly reduced with increasing homogenate concentration, presumably by non-specific interference from diet and insect impurities. The colour intensity increased between CpGV concentrations of $2x10^6$ - $1x10^9$ granules/ml (Plate 3.3).

Low speed centrifugation of the impure samples proved to be an effective method for removing non-specific interference. By dissolving the virus granules in alkali, the antigen protein stayed in solution whilst impurities were precipitated. All three curves, 1:100 and 1:1000 impure virus homogenate dilutions and pure CpGV, responded similarly and the ELISA was sensitive over the tested range of $2x10^6$ - $2x10^9$ granules/ml. The ODs of unknown samples diluted 1:100, 1:300 and 1:1000 were plotted against the mean response of the two homogenate standard curves. Three estimates of protein concentration were thus interpolated for each unknown sample giving an overall estimate of virus concentration for each impure sample. Agreement between these results (including a repeated experiment) and those obtained by two replicate bioassays were excellent (table 3.5).

Samples could be stored at -20°C, after centrifugation, for subsequent ELISA. The resultant assay was sensitive at CpGV concentrations above $5x10^6$ granules/ml (Fig. 3.11) and gave good estimates for the unknown samples (table 3.5).

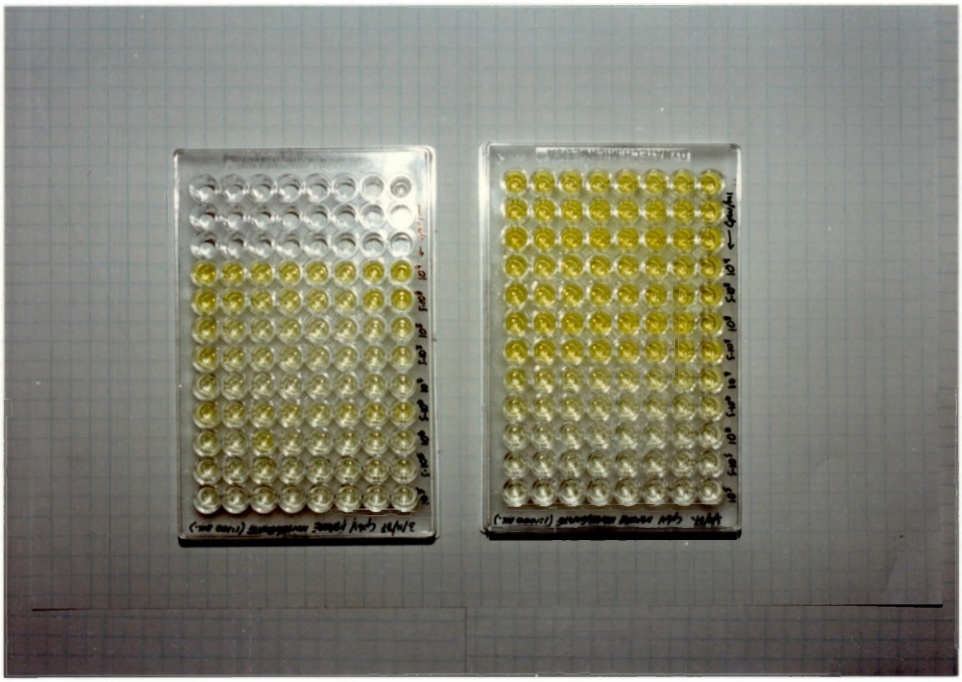


Plate 3.3 Quantification of CpGV in impure homogenate by ELISA

Fig. 3.9 An ELISA for impure CpGV:
a. Effect of homogenate dilution

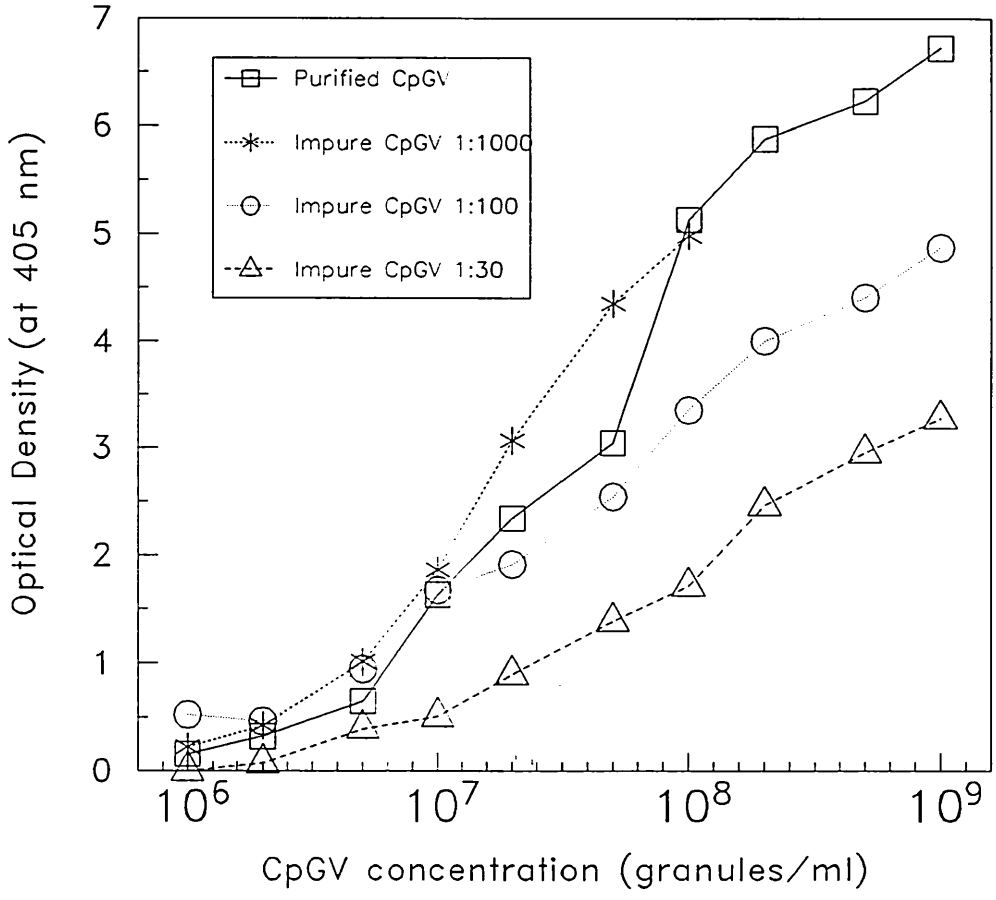


Fig. 3.10

ELISA: b. Removal of non-specific interference by low speed centrifugation

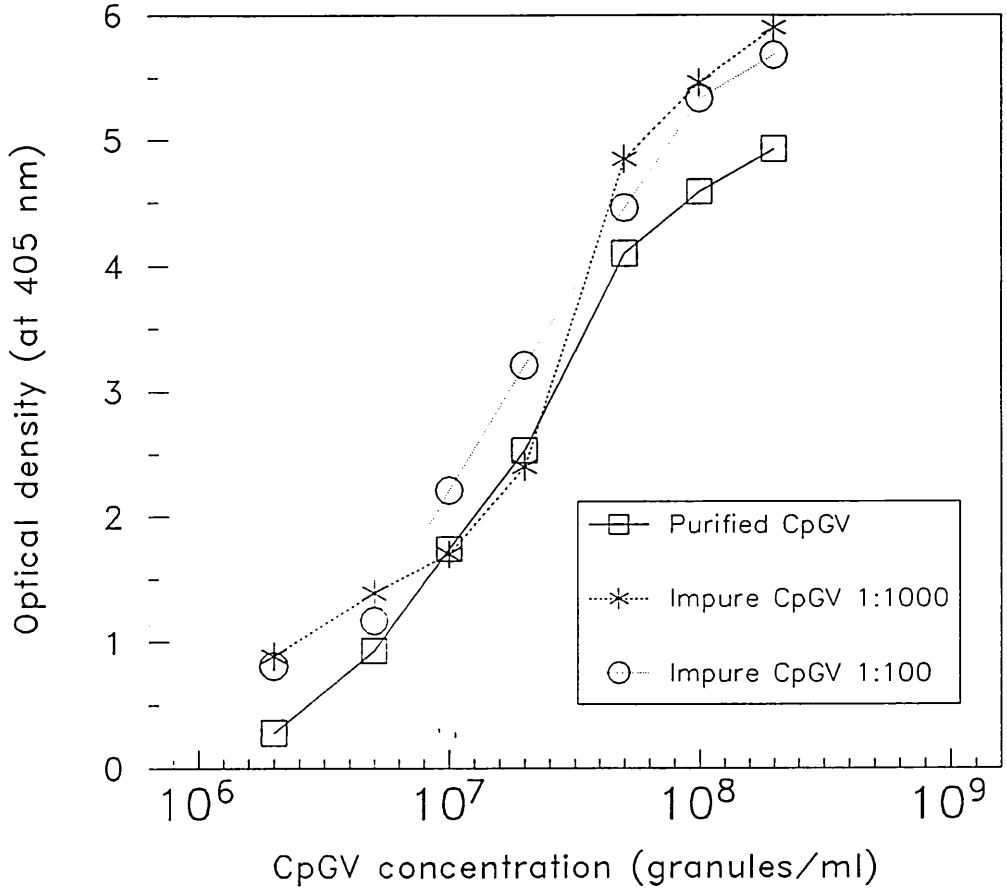


Fig. 3.11

ELISA: c. Low speed centrifugation followed by storage of samples at −20 C

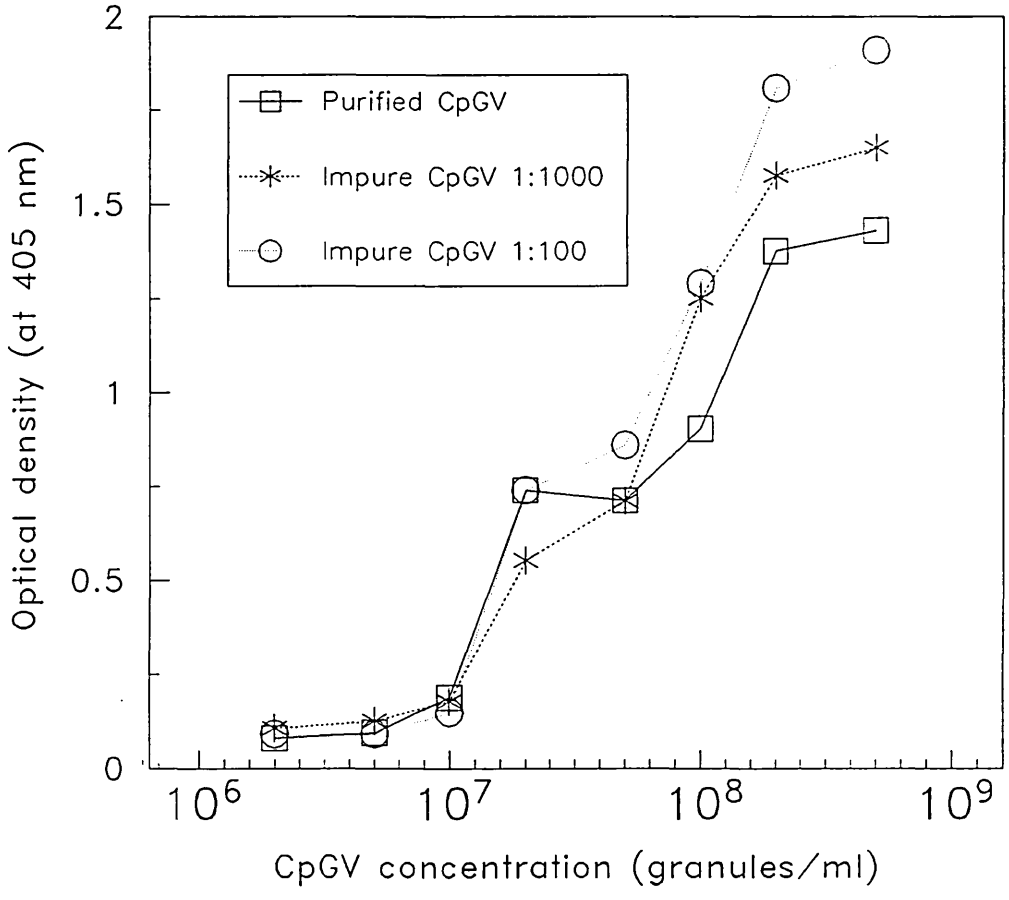


Table 3.5 Estimation of CpGV titre in unknown samples using ELISA and bioassay

SAMPLE	Estimates of CpGV concentration (granules/ml): ELISA (1)	ELISA (2)	ELISA (stored samples)	Bioassay (1) (Fd limits)	Bioassay (2) (Fd limits)
A	$7,19x10^{9}$	$6,75x10^{9}$	$8,32x10^{9}$	$4,02x10^{9}$ (2,52-6,37)	$4,61x10^{9}$ (2,48-9,08)
B	$1,32x10^{10}$	$1,56x10^{10}$	$1,85x10^{10}$	$1,47x10^{10}$ (1,01-2,20)	$1,67x10^{10}$ (1,15-2,56)
C*	$1,31x10^{10}$	-	-	$1,01x10^{10}$ (0,66-1,59)	

* Sample C was a commercial impure CpGV preparation, *DECYDE*, (MicroGeneSys, USA).

IV. DISCUSSION

A range of experiments has provided fundamental information about the production of CpGV. This data, combined with the development of an ELISA for use with impure CpGV preparations, has indicated that mass-production and quantification of CpGV could be achieved, and that the final product, with suitable formulation would have an adequate shelf-life for commercial use.

Larvae reared in small cells were susceptible to over-crowding, and emergence from a single cell rarely exceeded one individual irrespective of the initial number: cannibalism has been reported in the wild (Geier, 1963) and in laboratory rearing (Huber, 1986) and could explain this observation. For the production of CpGV for the experimental field trials, mass-production was achieved by individual rearing in compartmentalised trays. The codling moth culture appeared highly susceptible to unwanted granulosis virus infection and open-tray rearing proved virtually impossible. Compartmentalised rearing methods would, however, be far too costly for commercial production.

A new stock of codling moth larvae, imported from France, proved to be very different from the culture established at IHR, Littlehampton.

French larvae were larger - averaging 98 mg maximum weight compared to 61 mg for the English stock (Glen and Payne, 1984, recorded an average mass of 65 mg in the final instar for the same stock at IHR, also reared on methoprene-diet) - and were compatible with open-tray *en masse* rearing without incidence of unwanted virus infection. The possibility of a latent infection of CpGV existing in the English stock culture cannot be ruled out, and would explain the differences observed in rearing this and the French culture. Apparently "spontaneous" instances of GV infection was noted by Etzel and Falcon (1976) who also support the notion of a latent infection in their stocks. However, stressing their larvae (by various combinations of starvation, temperature and humidity changes) did not provoke expression of any latent virus. Open-tray rearing was possible with neonate French larvae, or directly from French egg-sheets placed within the rearing container. A thin layer of diet, only 2-4 mm thick (approximately 1 g per larva), was adequate to support 60 larvae to maturity in a tray measuring 17 x 11 cm. This contrasts Huber's (1986) finding that extra diet was needed to prevent cannibalism in open trays, with 2-6 g of diet required per larva. The nature of the diet surface proved to be important for larval establishment; larvae required a rough surface, though holes bored into the diet gave no advantage. Ascorbic acid was also essential for larval development.

Yield of CpGV was $3\text{-}4\times10^{10}$ granules/infected larva. This compares with a yield of 1.6×10^{10} granules/larva reported by Glen and Payne (1984); their figure is for purified virus and the lower yield probably results from losses in the purification process. Falcon *et al* (1968) achieved an average yield of 6.6×10^{9} granules/purified larva, though these were reared without the growth-inducing juvenile hormone analogue methoprene used in this, and Glen and Payne's study. Maximum virus yield could be realised only five days after infection, before death of the larvae. Early harvest reduces the chances of microbial contamination in dying larvae.

An impure CpGV homogenate proved to be highly effective in field trials (Chapter 6, section III(b)iv & (c), and Chapter 8, section III.ii, iii & iv). In storage tests the survival of virus in the unformulated preparation was however inferior to purified CpGV. Storage of purified CpGV was excellent even at 37°C. Cold storage, at -20°C provided one remedy, with no loss of activity recorded in any of the tested materials over 235 days. Drying the homogenate had little benefit in terms of

storage (virus activity or bacterial contamination). Solvent drying was superior to air-, freeze- and non-dried homogenates, but the room temperature shelf life was still inadequate. Although suitable spray-drying equipment was not available during this study, spray drying was reported to reduce bacterial contamination without damaging the virus (Huber 1986, 1988).

Incorporation of glycerol at 40% w/v gave a massive improvement in the storage life of the impure homogenate; bacterial contamination was maintained at a low level and no loss in virus activity has been recorded even after 240 days at 20°C. These findings were in keeping with those of Huber (1986) who found that glycerol incorporation at 50% also improved storage life; an impure CpGV dry powder resuspended in 50% glycerol had a half life of 200 days at 26°C (Huber, 1986). A high level of glycerol would increase the osmotic potential of the homogenate which might act to prevent bacterial growth (though not killing spores) which could be contributing to the hydrolysis of the virus granules. Altering the pH of the CpGV homogenate from pH 4.8 to 7.0 gave a slight improvement in shelf life; low pH could explain the loss of activity when pure CpGV was mixed with ascorbic acid - a principal component of the diet. Ascorbic acid was not detrimental to the impure homogenate, the effect presumably buffered by the presence of other homogenate components. Although the low pH of the impure preparation may contribute to its deactivation, it also reduces bacterial multiplication (Huber, 1986).

Impure homogenate batches produced for the 1987 field trials all contained less than $1x10^7$ bacterial colony forming units/ml. This compares very favourably with the average contamination level recorded in commercial *Lymantria dispar* NPV (Gypchek) of $6x10^{8}$ cfu/g (Podgwaite and Bruen, 1978). No detectable strain variation was observed in the CpGV used over the three field seasons; maintenance of a continuous genetic line may prove essential for registration purposes; major genetic changes could also affect infectivity.

DAS-ELISA proved to be a sensitive method for measuring virus granule protein in impure preparations of CpGV. A simple process of low speed centrifugation prior to sample loading eliminated the problem of high background readings due to non-specific reactions with diet

ingredients and larvae. The ELISA detected the presence of virus capsule protein which could be dissolved in alkali, thus staying in solution after centrifugation. The ELISA responded over a range of CpGV concentrations from $2x10^6$ - $2x10^8$ granules/ml which proved ideal for testing dilutions of unknown impure preparations. There was excellent agreement between the estimates for virus protein concentration by ELISA and the estimates of virus infectivity by bioassay. Large scale batch processing of impure CpGV for field trials, or for commercial production, requires an accurate, but rapid method of quantification. This method of DAS-ELISA combines accuracy and convenience. Pre-prepared standard virus dilutions could be stored at -20°C until use, and, according to Clark and Adams (1977), coated ELISA plates can also be stored for several weeks at -18°C. Presence of virus protein does not necessarily imply the presence of active virus and the only true measurement of activity of CpGV is by its infectivity; measured by time-consuming bioassay. However, in the preparation of fresh samples, there was excellent agreement between ELISA and bioassay and, on an industrial scale, quantification of all batches could be achieved by ELISA with a double-check by bioassay on randomly selected batches. The large scale commercial use of purified baculoviruses for insect pest control is impractical due to the cost of processing (Huber, 1988). This method of DAS-ELISA could prove highly useful as a rapid quantification tool for a whole range of impure baculovirus products.

In conclusion, despite inherent difficulties, mass-production and processing of CpGV should be feasible on a large scale. Provided that a healthy stock is maintained, larvae could be reared *en masse* in open trays from egg sheets as demonstrated by Guennelon *et al* (1982). The room temperature shelf-life of an impure homogenate was excellent when formulated with glycerol (cheaper alternative agents to increase the osmotic potential of the material could perhaps be found) and bacterial contamination was comparatively low. Virus yield can be maximised by the incorporation of the juvenile hormone analogue, methoprene, in the diet (Glen and Payne, 1984) and by optimizing the harvest date after infection. Quantification of each production batch could be achieved conveniently by DAS-ELISA, using the low-speed centrifugation technique described above.

Chapter 4

Optimum application strategy for use of CpGV to control codling moth in UK orchards.

I. INTRODUCTION

Since its isolation in Mexico in 1963 (Tanada, 1964) CpGV has been extensively field tested throughout apple growing regions of the world, and its potential as a viable control agent has been widely reported (e.g. Falcon *et al.*, 1968; Keller, 1973; Huber and Dickler, 1975; Jaques *et al.* 1977; Glen and Payne, 1984). Its commercial exploitation relies on achieving adequate control of fruit damage at economically viable application rates using conventional application methods.

This chapter reports research aimed at defining the optimum strategy for CpGV application, in terms of virus dose, frequency of application, spray volume and the potential of integration with other pest and disease control agents.

II. MATERIALS AND METHODS

a) Field trials

All field trials were carried out at the AFRC Institute of Horticultural Research (IHR), East Malling between 1985 and 1987. Field sites, numbered according to field plans at IHR East Malling, and plot layouts, are described in Chapter 2 (section VIII(a))

i. Effect of virus dose on codling moth damage (1985)

The effect of virus dose on codling moth damage was tested with CpGV at three levels in a comparative trial with deltamethrin as a chemical pesticide comparison. A fifth treatment was included in the trial to test the effect of formulation on CpGV at low dose; this will be discussed in

Chapter 6 (section III(a)i). A randomized block design was utilized with four blocks, and six treatments with four replicates on four-tree plots (Chapter 2, section VIII(a)i).

Trial site and apple variety: Plot WF103 (Fig. 2.1), Laxton's Fortune.

Treatments:

A. Unsprayed control
B. CpGV at 10^{12} granules/ha + 0.2% (w/v) skimmed milk powder
C. " " 10^{13} " " " " " " " "
D. " " 10^{14} " " " " " " " "
E. " " 10^{12} " " + 15% (v/v) cane molasses
F. Deltamethrin (2.5% a.i.) at 250 ml/ha

Spray dates: Two applications were made (27 June and 17 July) the second 140 day-degrees after the first spray, timed to coincide with the expected peak egg hatch. Figure 4.1 shows the pheromone trap records and the predicted egg-hatch based on the accrued heat sum after threshold trap recordings (see Chapter 2, section VIII(e) for full details).

High, medium and low doses of purified CpGV were formulated with skimmed milk powder - intended to improve virus persistence on foliage (Keller, 1973) - and 0.02% Agral (ICI Plant Protection Division UK) as a wetter. Two spray applications were made, separated by 20 days to coincide with expected egg hatch: June 27th (138.6 day-degrees) and July 17th (284 day degrees). Sprays were applied at 1000 l/ha (5 l/tree) using a Minicomet hydraulic sprayer (Chapter 2, section VIII(d)).

All apples, windfalls and harvest, were examined for codling moth damage; injured fruit were cut open and damage categorised into four groups according to severity (Chapter II, section VIII(f)i). Fruit-damage data was analysed using a generalised linear model with a binomial error and logit link function. This method allowed suitable weighting for plots with a heavier yield which would thus give a more accurate estimate of the damage in each treatment. Means and standard errors were predicted from the model. The analysis was sub-divided into harvested, windfalls and total fruit, and also into deep damage (Categories III and IV; see

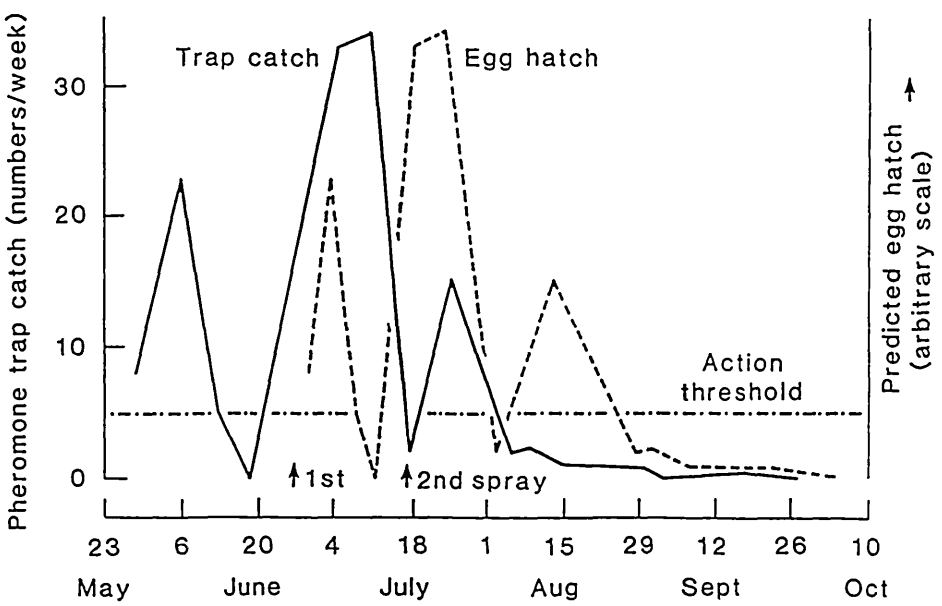


Fig. 4.1 Codling moth pheromone trap catches, 1985, East Malling (Plot WF 103).

Chapter 2, section VIII(f)i) and total damage (Categories I-IV). Within each analysis the following orthogonal comparisons were made:

CON vs ALL - unpsrayed control against all treatments
DELT vs VIR - deltamethrin against all CpGV treatments
LINEAR - low dose CpGV vs high dose
QUADRATIC - low dose + high dose vs 2x medium dose

The comparison "FORMULATION", analysing the effect of the two formulation additives at low CpGV dose, was tested in a separate analysis (see Chapter 6, section III(a)i) but is included in the table of results for this chapter.

Overwintering larvae in each plot were also recorded in corrugated trap-bands placed around each tree trunk (Chapter 2, section VIII(g)).

ii. Effect of spray frequency on virus efficacy (1986)

In 1985 an optimum CpGV spray dose was established: in the following year, a trial was designed to test the optimum strategy for using a given dose of CpGV; in a "standard" application of two high concentration treatments, or more frequent low virus concentration treatments. In this trial the same total quantity of CpGV for a season was divided into two, four or nine doses and the efficacy of each was assessed. Three spray regimes were tested using virus at two concentrations.

Spray application and formulation: In order to achieve a spray output more akin to common UK farm practice, CpGV was applied at a rate of 250 l/ha using a Solo Minor mistblower. All treatments were water-based suspensions of purified CpGV and included 10% (v/v) molasses to improve virus efficacy (Chapter 6, Section III(a)i.) and Agral (ICI) 0.02% as a wetter in the final formulation.

Trial site and apple variety: Plot WF103 (Fig. 2.2), Laxton's Fortune.

Treatments:

A Unsprayed control

B High total dose (1.125 x 10^{13} granules/ha total for season) divided into nine weekly doses of 5 x 10^{9} granules/l (1.25 x 10^{12} granules/ha)

C High total dose (as B) divided into four weekly doses of 1.125 x 10^{10} granules/l (2.81 x 10^{12} granules/ha)

D High total dose (as B) divided into two fortnightly doses of 2.25 x 10^{10} granules/l (5.62 x 10^{12} granules/ha)

E Low total dose (1.125 x 10^{12} granules/ha total for season) divided into nine weekly doses of 5 x 10^{8} granules/l (1.25 x 10^{11} granules/ha

F Low total dose (as E) divided into two fortnightly doses of 2.25 x 10^{9} granules/l (5.62 x 10^{11} granules/ha)

Spray dates (adverse weather conditions prevented spraying to the exact schedule of 7 or 14 day intervals):

Treatments D + F - June 30 and July 14 (146 and 247 day degrees)
Treatments B + E - June 27 (114 day degrees); July 6; 10; 19; 29; August 4; 11; 18 and 25
Treatment C - June 27; July 6; 10 and 19

The first weekly treatment was timed slightly before 140 day-degrees to ensure coverage of the egg hatch period. Treatments D and F were sprayed at 146 and 247 day-degrees, close to the recommendation of Glen & Brain (1982) for insecticide sprays.

All windfall apples were collected and assessed regularly for damage. Apples were harvested on 11 September when all fruit was again assessed, with damage classified into four categories of severity. The data were analysed as in trial i.

iii. Tank-mixing with fungicide; effect on efficacy of CpGV (1986)

More frequent application of CpGV would incur additional application costs. However, this would be alleviated if CpGV could be incorporated in fungicide sprays regularly applied to British orchards. In order to test this possibility, a trial was designed to test the compatibility of CpGV for tank-mixing with a popular fungicide suitable for use in an integrated pest management (IPM) strategy. Purified CpGV was applied at 500 l/ha at a standard dose, either sprayed on its own, or tank-mixed with the fungicide bupirimate. In both cases Agral (0.02% v/v) and molasses (10% v/v) were included in the final formulation. Treatments were applied 20 days apart. The trial site (Plot LF113) was artificially infested with codling moth pupae and adults (Chapter 2, section VIII(b)).

Trial site and apple variety: PLot LF113, Discovery

Treatments:

A. Unsprayed control
B. CpGV at 2.5 x 10^{12} granules/ha
C. CpGV at 2.5 x 10^{12} granules/ha + bupirimate at 2.2 l/1000 l

Fruit damage in windfalls and harvest apples was analysed by analysis of variance on angular transformed % data.

iv. Effect of spray volume on virus efficacy (1987)

In 1985 and 1986, CpGV was applied at 1000 and 250 l/ha respectively. In this trial the potential of ultra-low volume application was compared with a standard spray-volume. Medium (MV) and ultra-low volume (ULV) applications of CpGV were tested using identical rates of 5 x 10^{12} granules/ha applied three times at ten-day intervals. The trial site (Plot DR119) was artificially infested with codling moth adults (Chapter 2, section VIII(b)).

Trial site and apple variety: Plot DR119, Bramleys.

Treatments:

A. Unsprayed control
B. CpGV ($5x10^{12}$/ha) at 500 l/ha (MV) + 0.02% Agral (v/v)
C. CpGV ($5x10^{12}$/ha) at 25 l/ha (ULV) + 20% Actipron (v/v)

An ultra-low volume output of 25 l/ha was achieved using a generator-driven Turbair Scamp, controlled droplet applicator with 20% carrier oil (Actipron), whilst CpGV was sprayed at medium volume using a CP15 knapsack hydraulic sprayer (Chapter 2, section VIII(d)). Apples were harvested on September 1st; fruit damage was assessed as for trials i-iii. Analysis of the damage data was by analysis of deviance, fitting a binomial model to consider damage as a proportion of total fruit. The analysis was split into deep damage (Categories III + IV) and total damage (Categories I-IV) for the total harvest and windfalls.

v. Harvest dates and assessment of fruit damage.

All windfall apples were collected and examined weekly. The harvest dates depended on fruit variety: Discovery, 3rd week August; Laxton's Fortune 2nd week September; Bramley (premature harvest) 1st week September; Golden Delicious and Cox (premature harvest) 4th week September. Two trials were harvested early to fit the field programme. All harvested apples were assessed.

vi. Assessment of spray penetration and droplet spectrum.

The spray outputs from the Solo Minor mistblower, and the Turbair Scamp controlled droplet applicator were both assessed for droplet spectrum and spray penetration according to the methods given in Chapter 2 (section X(b) and (c)).

b) Laboratory assessment of CpGV and chemical compatibility

A number of pesticides used in conventional chemical orchard pest and disease control, and one organic top-dressing fertiliser, were tested for their compatibility with CpGV. Choice of chemicals was based on their

suitability for orchard IPM or use in organic orchards. The infectivity of CpGV, with or without mixing with these chemicals, was compared by bioassay, using neonate *C. pomonella* larvae, after a fixed storage time of three days at 27°C (Chapter 2, section VII). Chemicals were mixed with CpGV at concentrations five times the maximum manufacturers' recommended dose for use in the field.

II. RESULTS

a) Effect of virus dose on codling moth damage

The results of the 1985 trial on Laxton's Fortune are given in Table 4.1. Significant treatment effects were observed in each analysis ($P < 0.001$) and the orthogonal contrasts revealed consistent significant effects:

1. The unsprayed plots had significantly higher damage than all the treatments ($P < 0.001$).

2. Deltamethrin gave improved damage control compared to CpGV ($P < 0.001$).

3. Comparison of damage control obtained at three virus doses demonstrated improved control with increasing dose ("LINEAR" comparison, $P < 0.01$ for total deep-damage) though spraying at 10^{14} granules/ha did not give significantly improved control compared to 10^{13} granules/ha.

Control of fruit damage was better when deep-damage was considered as a separate category.

Similar results were recorded in trap-bands used to monitor overwintering larvae (table 4.2). Numbers recorded were low; nevertheless a significant treatment effect was observed compared to the unsprayed control plots ($P < 0.05$), though differences between the various treatments were not significant. The early harvest of Laxton's Fortune (September 9th - 10th) was the likely cause of low trap-band numbers with many larvae remaining in the fruit at harvest.

Table 4.1. Effect of CpGV dose and formulation on apple damage

Treatment	Total fruit examined	% deep damage Mean	S.E.	Orthogonal comparisons	Sig+	% total damage Mean	S.E.	Orthogonal comparisons	Sig+
1. Windfalls (deep damage, Categories III and IV only)++									
Control (unpsrayed)	1044	28,9	2,37	Con vs all	***				
CpGV 10^{12}/ha + 0,2% skimmed milk	675	9,52	1,70	Delt vs vir	**				
CpGV 10^{13}/ha + " " "	567	3,71	1,16	Linear	***				
CpGV 10^{14}/ha + " " "	547	2,69	1,02	Quadratic					
Deltamethrin	453	0,43	0,44	Formulation	**				
CpGV 10^{12}/ha + 15% molasses	992	3,70	0,89						
2. Harvested fruit (deep damage, III-IV and total damage, I-IV)									
Control (unpsrayed)	5070	12,41	1,11	Con vs all	***	15,92	1,24	Con vs all	***
CpGV 10^{12}/ha	6009	5,28	0,68	Delt vs vir	***	8,87	0,87	Delt vs vir	***
CpGV 10^{13}/ha	4060	3,09	0,60	Linear	*	5,86	0,85	Linear	**
CpGV 10^{14}/ha	4082	3,18	0,62	Quadratic		5,30	0,82	Quadratic	
Deltamethrin	4977	0,42	0,20	Formulation	**	1,15	0,34	Formulation	**
CpGV 10^{12}/ha + 15% molasses	4843	2,65	0,54			5,42	0,77		
3. Total fruit (deep damage only)									
Control (unpsrayed)	6114	15,13	1,22	Con vs vir	***				
CpGV 10^{12}/ha	6684	5,73	0,74	Delt vs vir	***				
CpGV 10^{13}/ha	4627	3,15	0,63	Linear	**				
CpGV 10^{14}/ha	4629	3,10	0,64	Quadratic					
Deltamethrin	5430	0,42	0,21	Formulation	**				
CpGV 10^{12}/ha + 15% molasses	5835	2,82	0,56						

+ Significance - *** = P < 0,001; ** = P < 0,01; * = P < 0,05

++ Only deep-damage was classified for windfalls in this trial

Orthogonal comparisons: Con vs all = control versus all other treatments;
Delt vs Vir = deltamethrin versus all virus treatments;
Linear = low versus high virus dose;
Quadratic = non-linear dose effect (low + high dose versus 2 x medium dose);
Formulation = skimmed milk versus molasses at low virus virus dose.

Table 4.2. Effect of CpGV spray dose on overwintering population.

Treatment	Mean	f_{mean}	Orthogonal comparisons	Sig.
	Mean number of larvae recorded in trap-bands.			
Control (unpsrayed)	20,6	4,54	Con vs All	*
CpGV 10^{12}/ha + 0,2% skimmed milk	6,35	2,52	Delt vs Vir	
CpGV 10^{13}/ha + " " "	1,74	1,32	Linear	
CpGV 10^{14}/ha + " " "	2,53	1,59	Quadratic	
Deltamethrin	0,16	0,40	Formulation	
CpGV 10^{12}/ha + 15% molasses	2,46	1,57		
S.E.D.*		0,76		

Level of significance; * = P < 0,05
* S.E.D. = standard error of the difference of means

b) Effect of spray frequency on virus efficacy

Table 4.3 gives the results, analysed as in the first trial. There was a clear effect of treatments when compared with the unsprayed control (P < 0.001) and in each analysis the high dose treatments (1.125 x 10^{13} granules/ha) proved more effective than the low dose (1.125 x 10^{12} granules/ha) (P < 0.05). The control of deep damage at harvest and total damage in windfalls was significantly affected by the choice of spray frequency (P < 0.05) at high virus dose. Further study of the results reveals that the strategy of only two applications a fortnight apart was consistently less effective than the four or nine weekly applications. This is borne out by the confidence limits calculated from standard errors. The four-applications strategy gave the best overall results for the total apples sampled. Varying spray frequency at low virus dose had no apparent effect (P > 0.05) on the level of control.

Table 4.3. Effect of spray frequency on control

Means and standard errors produced from binomial model

Total dose for season (per ha)	No. applications	Total fruit examined	% deep damage (categories III-IV)		% total damage (categories I-IV)	
			Mean	S.E.	Mean	S.E.
1. Windfalls						
Unsprayed control	-	3937	6,16	0,985	6,40	0,931
$1,125x10^{13}$	9)	3298	1,75	0,571	1,96	0,562
$1,125x10^{13}$	4) high	3512	0,716	0,367	0,899	0,382
$1,125x10^{13}$	2)	3218	2,76	0,743	3,23	0,747
$1,125x10^{12}$	9) low	2847	3,95	0,923	4,18	0,884
$1,125x10^{12}$	2)	2468	3,18	0,928	3,42	0,894
Analysis of deviance:						
Control vs treatments			***		***	
High dose vs low dose			*		*	
Low x9 vs low x2						
High x9 vs x4 vs x2			*+		*	
2. Harvest only						
Unsprayed control	-	6085	3,27	0,523	3,53	0,570
$1,125x10^{13}$	9)	6290	0,280	0,154	0,627	0,240
$1,125x10^{13}$	4) high	6263	0,395	0,177	0,642	0,238
$1,125x10^{13}$	2)	6460	1,15	0,315	1,40	0,366
$1,125x10^{12}$	9) low	5807	1,25	0,345	1,61	0,410
$1,125x10^{12}$	2)	5474	1,27	0,378	1,51	0,389
Analysis of deviance:						
Control vs treatments			***		***	
High dose vs low dose			*		*	
Low x9 vs low x2						
High x9 vs x4 vs x2			*			

/Continued...

Table 4.3. (Continued)

3. Total fruit (windfall and harvested)

Unsprayed control	-	10022	4,56	0,209	4,81	0,214
$1,125x10^{13}$	9)	9588	0,873	0,0959	1,17	0,111
$1,125x10^{13}$	4) high	9775	0,521	0,0727	0,746	0,0869
$1,125x10^{13}$	2)	9678	1,82	0,142	2,15	0,154
$1,125x10^{12}$	9) low	8654	2,32	0,167	2,62	0,177
$1,125x10^{12}$	2)	7942	1,95	0,157	2,18	0,166

Analysis of deviance:		
Control vs treatments	***	***
High dose vs low dose	*	*
Low x9 vs low x2		
High x9 vs x4 vs x2	*++	*+++

Significance levels: P < 0,001 = ***; P < 0,01 = **; P < 0,05 = *

Note: three points just under 5% level of significance:

* Mean Deviance Ratio = 3,31 ($F_{0.05}$ = 3,68)

** Mean Deviance Ratio = 3,45

*** Mean Deviance Ratio = 3,38

c) Effect of fungicide treatment on virus efficacy

The analysis of variance (Table 4.4) showed a significant variance ratio (F) for the treatments compared to the unsprayed control (P = 0.004): the incidence of apple damage was clearly lowered by CpGV application. There was no difference between the damage recorded for the CpGV and CpGV/bupirimate treatments where, in both cases, deep damage was reduced by about 80%.

Table 4.4 Effect of tank-mixed fungicide on efficacy of CpGV.

Treatment	Mean yield (apples/plot)	% deep damage (actual means)	Angular transformed means
A. Unsprayed	263.2	28.97	32.3
B. CpGV only	262.5	5.90	13.9
C. CpGV + bupirimate	350.5	6.02	14.1
S.E.D.			3.80

Analysis of variance:
F Probability of treatment effect = 0.004

d) Effect of spray volume on virus efficacy

Excellent control of deep-damage was achieved in both treatments at 25 l/ha and 500 l/ha ($P < 0.001$) (Table 4.5). There were no differences in the levels of control achieved with either of the two spray volumes used ($P > 0.05$).

The droplet spectrum from the Turbair was analysed by measuring droplets impacted on magnesium oxide slides. By using 20% Actipron (British Petroleum Ltd.) carrier oil in the formulation, an average droplet size of 63 µm (volume median diameter, VMD) was produced with VMD: Number Median Diameter (NMD) ratio of 1.51. The size of droplets was thus fairly uniform and within a range suitable for impaction on foliage (Matthews, 1979). An analysis of the spray output from the hydraulic sprayer used in this trial was not undertaken.

Table 4,5 Effect of spray volume on virus efficacy

Treatment	Windfalls			Harvest			Total apples		
	Total fruit examined	% deep damage	% total damage	Total fruit exam,	% deep damage	% total damage	Total fruit exam,	% deep damage	% total damage
Unsprayed control	725	4,41	9,93	2295	1,22	1,96	3020	1,99	3,87
500 l/ha (MV)	623	0,32	4,01	1886	0,05	0,42	2509	0,12	1,32
25 l/ha (ULV)	646	0,15	2,48	1699	0	0,12	2345	0,04	0,77
Analysis of deviance: 1, Contr, vs treatments		***	**		***	**		***	**
2, ULV vs MV		NS	NS		NS	NS		NS	NS

Significance levels; * = P < 0,05 ** = P < 0,01 *** = P < 0,001 NS = Not significant

e) Penetration of spray droplets and spray drift

An analysis of the droplet spectrum, and spray penetration into the orchard of the Solo Minor Mistblower was also undertaken (Tables 4.6 and 4.7). In the sprayed plots, leaf coverage was generally good with a mean average of 52% on upper leaf surfaces and 37% on lower leaf surfaces. There was some variation through the plot canopy but this was relatively small. Downwind drift was recorded but only noticeable on the windward edge of the neighbouring plot and even there, the foliar coverage was considerably smaller than in the treated plots and almost non-existent on the lower leaves.

The droplet sizes recorded in the spray canopy did not differ significantly with the position in the canopy, with volume median diameters (VMD) ranging from 90-143 μm, and number median diameters from 50-71 μm. The VMD:NMD ratios were larger on the outer canopy leaves (1.85-2.82) than the inner canopy, (1.28-1.70) presumably, the largest droplets having insufficient momentum to penetrate the canopy and the smallest droplets having insufficient momentum to impact on the leaf surfaces. The glass slide technique used may not represent a definitive measure of droplet

sizes produced under field conditions since small droplets would tend to be blown passed in an airstream; leaves however move, breaking the airflow, thus aiding impaction. The consequence would be to bias the results towards larger droplets.

Table 4.6 Droplet spectrum from Solo Mistblower

Position in 3 m apple canopy	NMD (µm)	SE	VMD (µm)	SE	VMD:NMD
Inner, low	71,15	7,86	120,21	31,44	1,70
Inner, middle	69,00	10,60	116,12	9,88	1,68
Inner, high	70,32	10,89	90,30	48,26	1,28
Outer, low	52,43	12,64	110,97	32,90	2,12
Outer, middle	50,68	12,50	142,91	43,01	2,82
Outer, high	56,44	10,84	104,29	31,10	1,85

Table 4.7 Solo Minor Mistblower: penetration of spray deposits into treated plots (visual assessment)

Spray output: 250 l/h (aqueous formulation + Uvitex at 40 g/l)
Direction of sprayer travel: North to South; east row sprayed first
Wind direction: Easterly (light)
4-tree plots: 3 m canopy cv. Laxton's Fortune

Row*	Edge	Height (m)	Upper leaf surface Mean % coverage (20 leaves)	SE	Lower leaf surface Mean % coverage (20 leaves)	SE
a) Plot						
1	Windward (E)	3 (top)	13,4	4,8	30,2	8,3
1	Leeward (W)	3 (top)	58,5	7,3	39,3	6,7
1	Windward	2	51,5	7,6	52,2	9,0
1	Leeward	2	67,7	6,5	55,9	7,6
2	Windward	2	70,5	5,5	50,1	8,0
2	Leeward	2	66,5	7,0	56,8	8,6
1+2	North edge	2	56,1	6,9	29,4	7,3
1+2	South edge	2	44,6	8,7	13,2	6,2
1+2	Centre of trees	3	38,1	7,3	26,6	6,2
1+2	Centre of trees	2	54,6	7,4	17,6	6,1
Mean			52,15		37,13	
b) Downwind drift						
3**	Windward	2	30,5	6,5	10,4	2,1
3**	Leeward	2	2,2	0,7	0,95	0,40
4	Windward	2	10,4	4,1	0,50	0,34
4	Leeward	2	1,9	0,6	0,15	0,08
5	Windward	2	6,4	1,5	0,05	0,05
7	Windward	2	0,1	0,07	0	-

* Numbered east to west
** Guard row

f) Compatibility of CpGV with chemical pesticides

A number of chemicals were tested for compatibility with CpGV. Untreated or treated CpGV samples were bioassayed for their infectivity after a fixed storage time; Table 4.8 shows the LC_{50}s and fiducial limits for the various treatments. Of all the chemicals tested, only wettable sulphur caused any noticeable loss in virus infectivity (three-fold in three days). Even with sulphur, it is unlikely that any important loss of infectivity would occur at the field concentration used and in the time in which a tank-mix might be standing. Of the chemicals tested, CpGV appears thus to be largely compatible for tank-mixing.

Table 4.8 Compatibility of CpGV with pesticides

Chemical	Type	Initial conc.	LC50 CpGV + chemical	Fiducial limits	LC50 CpGV only	Fiducial limits
Dithianon (52,5% w/w)	Fungicide	5,25%	$4,5x10^3$	2,91-8,75x10³	$4,0x10^3$	1,84-8,52x10³
Fenarinol (12% w/v)	"	0,825%	$2,83x10^3$	1,99-4,07x10³	$3,86x10^3$	2,72-5,57x10³
Triadinefon (25% v/v)	"	2,5%	$4,35x10^3$	2,49-9,93x10³	$4,90x10^3$	2,93x10³-1,03x10⁴
Bupirinate (27% w/w)	"	0,5%	$2,64x10^3$	1,72-4,51x10³	$2,44x10^3$	1,57-4,24x10³
Wettable sulphur (80% ai w/w)	"	10%	$7,68x10^3$	5,23x10³-1,15x10⁴	$2,54x10^3$	2,72-5,57x10³
Wettable sulphur (Repeat)	"	10%	$1,36x10^4$	7,96x10³-2,25x10⁴	$5,024x10^3$	2,69-7,86x10³
Cyhexatin (50% w/w)	Acaricide	0,625%	$4,68x10^3$	3,10-7,23x10³	$5,05x10^3$	3,46-7,49x10³
"Maxicrop"+	Organic fertiliser		$4,67x10^3$	2,39-7,39x10³	$5,02x10^3$	2,69-7,86x10³

+ "Maxicrop" seaweed fertiliser (Maxicrop International, Great Shalford, Cambs.)

IV. DISCUSSION

In each of the field trials conducted between 1985 and 1987 it was necessary only to spray against a first generation of *C. pomonella*. Summer temperatures are rarely high enough to promote a substantial second generation in the UK. The level of damage caused by codling moth depends on temperature conditions and the level of damage in the previous year (Gruys, 1982). Poor weather during the three seasons of the study may have been partly the cause of low infestation levels in 1986 and 1987. Total fruit damage by codling moth in untreated Laxton's Fortune plots (left unsprayed in the previous season) was 15% (deep-damage) but in the following season damage had fallen to 4.6% and in a trial in 1987 (Chapter 6, section III(b) iv), damage on the same site fell to just 1.6%. Repeated use of CpGV on the site - albeit to only half of the trees in the orchard - has probably added to a progressive reduction in the local codling moth population. Analysis of large numbers of fruit proved essential where treatment differences were being studied and the level of damage was low.

Artificial supplementation of codling moth infestations in two trials appear to have succeeded, with a very high, 29 %, damage in the 1986 fungicide compatibility trial. Release of adult codling moths to supplement natural populations was not a novel strategy; Richards (1984) released adult moths to coincide with the natural population.

In a trial to determine an optimum application dose of CpGV, virus sprayed at 10^{13} granules per hectare gave significantly improved control over a dose of 10^{12} granules/ha. Increasing the dose a further ten-fold gave no further improvement in control. Other workers have reported relationships between spray dose and damage reduction. Since spray volumes used in different trials may vary, it is important to convert reported figures to virus dose/ha rather than granules/litre. Glen and Payne (1984) demonstrated a relationship between dose and damage reduction, from 10^{8} to 10^{11} granules/l or 6 x 10^{10} to 6 x 10^{13} granules/ha. They quoted a slope of 0.86 for log dose (granules/l) against probit % reduction in deep damage and a slope of 0.5 for total deep and shallow damage. Standard errors quoted by Glen and Payne do not, however, indicate a significant improvement in control beyond 2 x 10^{10}/l (1.2 x 10^{13}/ha) consistent with the findings reported here. Huber and Dickler

(1978) observed a gradual reduction in total damage from 16 to 60% by increasing the dose from 10^{9}-10^{11}/litre (about 10^{12}-10^{14}/ha at high volume). They quote a slope for total damage of 0.6; the effect of this shallow slope accounting for the necessity to increase dose massively to effect small improvements in control.

In Glen and Payne's study the lowest applied dose of 10^{8} granules/l (6×10^{10}/ha) did not significantly reduce the total damage; only the control of deep damage was improved. Total damage was not reduced with doses below 6×10^{8}/l (3.6×10^{11}/ha), the doses being insufficient to prevent larval death prior to shallow feeding. Control of total damage in these trials was also poorer with, in trial i., 67% control of total damage compared to 80% control of deep damage only; some feeding prior to death from infection was the most likely cause. In Canadian trials, Jaques *et al.* (1977, 1981) found that the level of superficial damage in CpGV-treated plots was actually higher than in untreated plots. Without increasing the speed of kill of CpGV there will always be a finite level of superficial damage caused before lethal infection is reached.

In these trials CpGV efficacy compared poorly with deltamethrin; CpGV gave only 80% control of deep fruit damage compared to 97% with deltamethrin. Two factors may be responsible and suggest that the poorer control is not insurmountable. Firstly, application was by hand lance in this trial and distribution of spray may have been poor in some places such as the upper central branches. Deltamethrin has a contact action and larvae could receive a lethal dose merely by crawling over sprayed leaves. CpGV is stomach-acting and a larva must not only encounter a treated area of leaf or apple but also ingest virus from that point. Better coverage is, therefore, more important for CpGV than deltamethrin. Secondly, CpGV has a very limited lifetime on foliage (Keller, 1973; Brassel and Benz, 1979; Glen and Payne, 1984) and with a spray interval of 20 days many larvae may have emerged and entered fruit when very little virus remained on foliage. Deltamethrin by contrast is relatively persistent remaining active for more than 45 days (Charmillot *et al.*, 1984).

To combat the poor persistence of CpGV, either a higher initial dose of virus could be used or the interval between sprays could be increased. Increasing the dose would appear not to be very effective since control was not improved beyond a dose of 10^{13} granules/ha. The effect of

reducing the spray interval was tested by distributing a defined quantity of CpGV over two large fortnightly doses or several smaller weekly doses. The weekly application regime at 1.125×10^{13} granules/ha/season in four or nine sprays gave the best level of control. Extending the spraying period over four or nine weeks probably killed emerging larvae over a longer time span than with just two sprays, and the frequent weekly sprays were likely to have maintained an effective level of virus on the foliage for more of the time. The nine weekly applications may have killed late emerging larvae or larvae emerging from a partial second generation but overall control was not quite as good as with the four-spray scheme. One consequence of the nine-spray strategy is to reduce the dose applied at each spray; the advantage of a longer time span may have been countered by lower doses at the period of main egg hatch early in the season. Damage in windfall apples - caused by codling attack well before harvest - was lower in the four-spray scheme; the higher doses in the early part of the season may account for better control in these windfalls than with the nine-spray scheme.

Comparing application over nine weeks or two weeks at a lower dose of 1.125×10^{12}/ha/season gave no improvements but it is likely that, with nine sprays at only 1.25×10^{11}/ha, the extremely low concentration of CpGV on foliage may have offset the advantage of its frequent application.

Huber and Dickler (1977) found that eight, weekly, applications of CpGV at 1×10^{11} granules/l (*c.* 10^{14}/ha) gave no significant improvement over four applications at the same dose applied every two weeks (damage was reduced by 83 and 86% respectively). However, such a high dose probably overshadowed any benefit achieved by more frequent sprays. In more recent trials Dickler and Huber (1986 and 1988) contradict their earlier conclusions. More frequent low-dose applications were recommended since they were "as effective" as a higher dose applied less frequently. However, their treatments were not strictly comparable since different total virus doses were used: nine applications totalling 4.5×10^{13} granules/ha/season compared with four applications totalling 2×10^{14} granules/ha/season and, once again, the high doses used may not have allowed discernible differences.

Increasing the number of sprays used could increase the labour requirement for spraying. However, in the UK, many growers regularly apply

fungicides at weekly or fortnightly intervals, to control scab (*V. inaequalis*) and mildew (*P. leucotricha*). In field and laboratory experiments it was demonstrated that CpGV could be mixed with a range of fungicides without loss of activity, and only one agent, wettable sulphur, did cause a slight loss in virus infectivity when mixed at a very high concentration (five times the manufacturer's top recommended dose) and stored at 27°C for three days. However, tank mixing generally occurs just prior to spraying and, in a short time, it is unlikely that any important virus inactivation would occur. Fungicides with low predator toxicity, suitable for orchard IPM, were chosen for the tests. Wettable sulphur is less suitable for IPM owing to its toxicity to predators such as *Typhlodromus pyri* (Collyer and Kirby, 1954, 1958). Keller (1973) reported compatibility of CpGV with two fungicides folvet-dinocap and zineb-captan, different from those tested above, and less suitable for use with CpGV in an IPM programme. The fungicides dinocap, mancozeb, orthocid, fenarimol and bitertanol have also been shown to be compatible with CpGV (Fischer-Colbrie, 1978, Dickler and Huber, 1986, 1988).

In the trials reported here, CpGV was successfully applied at 500 and 1000 l/ha by hydraulic sprayers fitted with conventional cone nozzles, and by mistblower at 250 l/ha. 250 l/ha is a typical spray rate used by many UK growers in the application of fungicides and insecticides. In a comparative trial, a ULV rate of 25 l/ha was shown to be as effective as a medium volume rate of 500 l/ha. Spray output in the ULV system was controlled using a fan assisted spinning disc with 20% carrier oil in the formulation which acted as an anti-evaporant and regulated droplet sizes to 63 µm (VMD) with a VMD:NMD ratio of 1.51. For good coverage of foliage Matthews (1979) recommends mean droplet sizes of between 40 and 100 µm (VMD) and stresses the need for a narrow droplet spectrum at ultra low volume in order that the volume and dose can be optimized.

CpGV was shown to be flexible in its use; the dose per ha being more important than the spray volume, provided that coverage and suitable spray characteristics are achieved. CpGV has been commonly applied at high spray volumes often to run-off: Glen and Payne (1984) applied at 600 l/ha; Huber and Dickler (1976) at 750 l/ha; Huber and Wunderman (1978) at 1100 l/ha by mistblower; Glen and Clark (1985) to run-off. Richards (1984) compared two spray volumes, 600 l/ha and 50 l/ha, but found that the latter required ten times the virus dose to achieve the same level of control,

despite equivalent virus deposition. However, the ULV application was carried out with an aqueous formulation (no carrier oil) applied with spinning disc equipment (Richards, 1984). A second trial using an air blast system with fine orifice cone nozzles did contain an anti-evaporant at 10% v/v (Richards, 1984). Richards reports a resultant droplet size (VMD) of 154 μm in this latter trial. Poor control achieved with either of these ULV methods could be attributed to poor penetration of droplets produced by the spinning disc application (Richards, 1984) and inadequately sized droplets with wide droplet spectrum in the air blast system.

These trials further support the potential of CPGV as a biological control agent. It is now clear that, in a normal UK season, spray strategy can be optimized by applying a standard virus dose of approximately 2 x 10^{13} granules/ha/season over four or nine weekly sprays. Although pheromone trap monitoring is likely to be useful for deciding when and whether to spray, the frequent spray strategy will make timing less critical. CpGV can be tank mixed with fungicide and applied at a range of spray volumes from 25 l/ha using controlled droplet application techniques, and from 250 to 1000 l/ha using standard orchard spraying equipment.

CHAPTER 5

Uptake of granulosis virus from the surface of apples and leaves.

I. INTRODUCTION

The virus particle of CpGV is surrounded by a proteinaceous layer - "granulin" - which probably confers protection to the virus for its survival outside the insect host. The virus is only able to enter its host by ingestion, whereupon the granulin dissolves in the alkaline midgut allowing the virus particle to infect the gut cells and spread from there to other susceptible tissues, leading to death of the host.

CpGV can be sprayed in aqueous formulations and remain active on the plant surface prior to ingestion by its host. Newly hatched codling moth larvae acquire virus from the branches - fruit and/or leaves - but not from sprayed eggs (Glen and Clark, 1985). So far, little has been known about the precise site of virus uptake, though Keller (1973) postulates that since most eggs are laid on the leaves surrounding the fruit - 75% according to Subinprasert (1988) - larvae must first come into contact with twigs and leaves, on which they nibble, thus providing the first opportunity to ingest CpGV. In trials, 30-40% of released larvae were found to have made their way to the fruit within 48 hours (Subinprasert, 1988), with 90% of larvae spending at least 10 minutes on leaves prior to moving to fruit (Glen and Clark, 1984). Larvae released on fruit took from a few minutes to two hours before deciding on a point of entry; penetration into the fruit took 1-2½ hours (Geier, 1963). Once inside the fruit, there seems to be no further opportunity for larvae to ingest virus; the timing of CpGV sprays is thus critical and sprays must be targeted against the neonate larvae prior to fruit entry. In France, where warmer conditions allow two codling moth generations each year, CpGV was found to be less effective against the second generation and this was accredited not to different climatic conditions, but to the different feeding behaviour of the two generations: the first spending more time outside the apples since fruits were less developed early in the season (Burgerjon, 1986; Burgerjon and Sureau, 1988).

Experiments were undertaken to clarify the precise sites of virus ingestion and consider whether CpGV could be ingested passively or only during active feeding. The implications for codling moth control are discussed.

II. MATERIALS AND METHODS

a) Laboratory trials.

A new leaf bioassay system was devised to examine the rate of uptake of CpGV from sprayed leaf discs (Chapter 2, section VI). Leaf discs were sprayed with CpGV at known concentrations, using a Shandon laboratory mistblower sprayed through a net "attenuator" (to reduce the droplet density and remove large droplets). Larvae were allowed to feed on the discs for set time periods prior to rearing on virus-free diet. Virus induced mortality was recorded after five days at 27°C. The exposure-time/mortality response was recorded and analysed by maximum likelihood probit (MLP) analysis.

b) Field trial

A field trial was designed to test from which parts of the host plant neonate larvae ingest CpGV.

Trial plot and artificial infestation: Five 1.5 m Bramley apple trees on plot DR119 were divided into twenty single-branch "plots" which were artificially infested with mature codling moth eggs and neonate larvae and caged to prevent predation and escape (Chapter II, section VIII(c), plate 2.2).

Spray application and treatments: One hour prior to caging and artificial infestation, the following CpGV treatments were applied:

A. Unsprayed control
B. CpGV applied to leaves only
C. CpGV applied to whole branch
D. CpGV applied to fruit only
E. Unsprayed branches without exclusion bags or introduced *C. pomonella*



Plate 5.1 Restricted spraying of apple foliage with CpGV

CpGV was formulated in water at 4 x 10^{10} granules/l with 0.02% agral. Treatments B and C were sprayed at high volume, close to 'run-off', using a hand held compression sprayer with a single cone nozzle. Large polythene bags were used as temporary shields to prevent unwanted drift onto other branches. Spraying in treatment B was restricted to the leaves by wrapping each apple in thin plastic "clingfilm" until the spray had dried (Plate 5.1). The same spray formulation was used for treatment D but was applied by painting each apple liberally to achieve a 'high-volume' application close to 'run-off'; so that each fruit was thoroughly covered but not dripping onto the foliage below. Treatment E was included to test the effectiveness of the artificial pest release, by measuring the background level of pest damage. CpGV was applied only once, on 30 July 1987.

Analysis of fruit damage: On 26th August, fruit damage was assessed by examining each apple and scoring damage attributable to *C. pomonella* according to severity (Chapter 2, section VIII(f)).

III. RESULTS

a) Laboratory leaf bioassays

Neonate codling moth larvae exposed to CpGV treated leaf surfaces, showed a clear response of increased virus-induced mortality to exposure time on the leaf surfaces; Figure 5.1 shows this relationship. A maximum likelihood probit (MLP) analysis enabled a figure for the LET_{50} to be calculated for each curve, where ET is the exposure time in minutes (table 5.1). In each case the points were shown not to differ significantly from a straight line ($P > 0.05$). Increasing the concentration of CpGV sprayed on leaf discs decreased the LET_{50}, indicating, not surprisingly, that larvae ingested more virus in a given time when the virus dose was increased. CpGV at 10^6 granules/ml appeared to provide too low a dose to cause infection. Even after larvae were left for nine days before assessment for virus-death (as opposed to the usual five) mortality was still negligible and the slope of the exposure time-mortality response was not significantly different from zero. In experiments where leaves were sprayed at 1 x 10^7 or 5 x 10^7 granules/ml, virus ingestion appeared merely as a function of time, with significant levels of infection occurring after only

3.5 minutes exposure to sprayed leaves. Light microscope examination of the leaf discs revealed that larvae rarely made any excavations of the leaf surface before fifteen to thirty minutes (Table 5.2). Other than definite feeding holes - as recorded in table 5.2 - no superficial channelling or mining of the leaf surface could be seen even with a scanning electron microscope (PLate 5.2).

Virus deposition on the leaf discs after spraying at 1 x 10^7 granules/ml was calculated from the MLP analysis of leaf washings, and related to the area of the leaf disc:

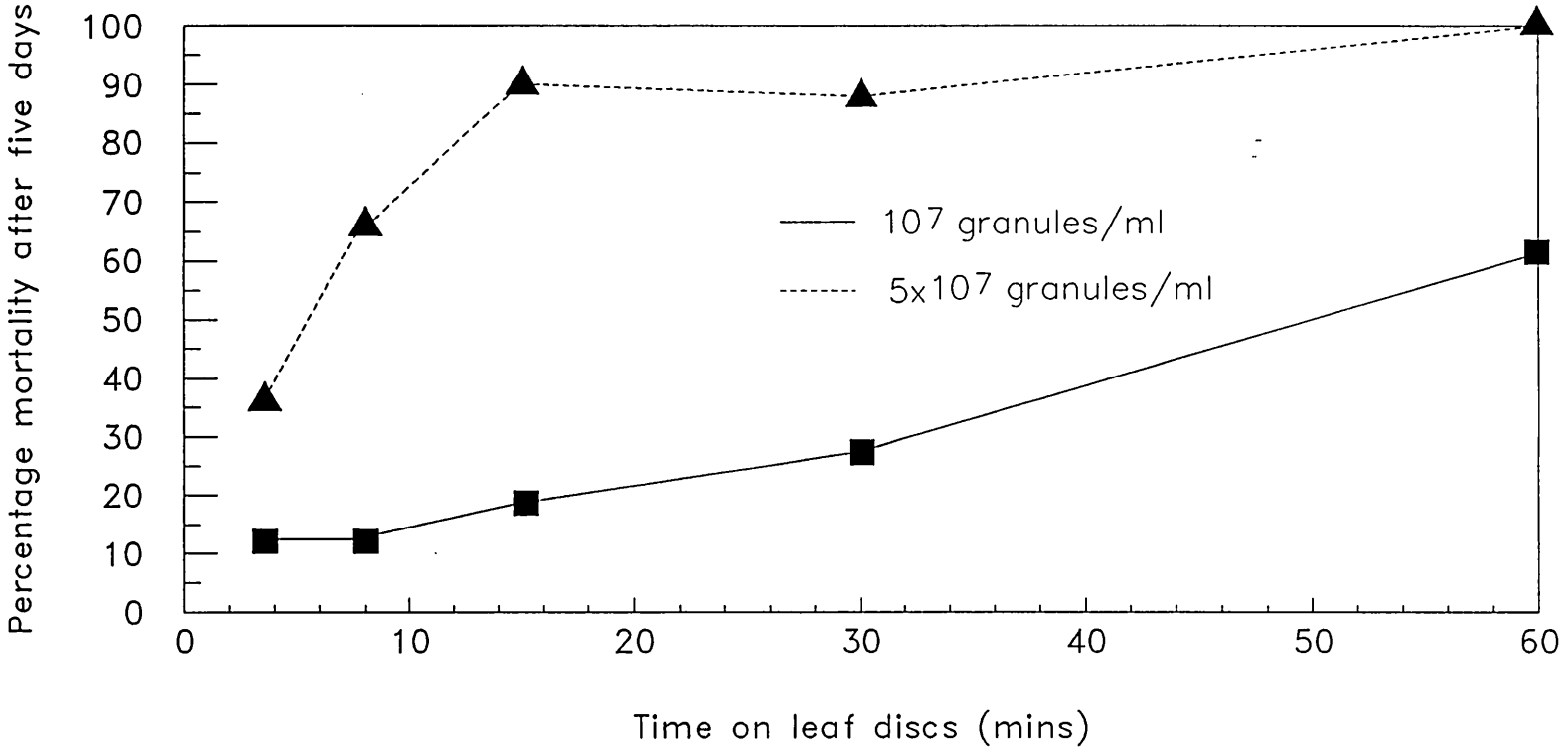
Concentration per disc = 7.64 x 10^3 granules/disc
(confidence limits = 1.37 x 10^3 - 1.23 x 10^4)

Concentration per cm^2 = 4.32 x 10^3 granules/cm^2
(confidence limits = 7.75 x 10^2 - 6.96 x 10^3)

With the "attenuator" fitted, droplet sizes produced from the Shandon laboratory sprayer averaged 76.4 µm (volume median diameter) with a volume median diameter : number median diameter ratio (VMD:NMD) of 3.8. The attenuator filtered out droplets larger than 150 µm (VMD without attenuator = 96.7 µm).

Fig. 5.1

Uptake of CpGV by larvae from sprayed leaf discs



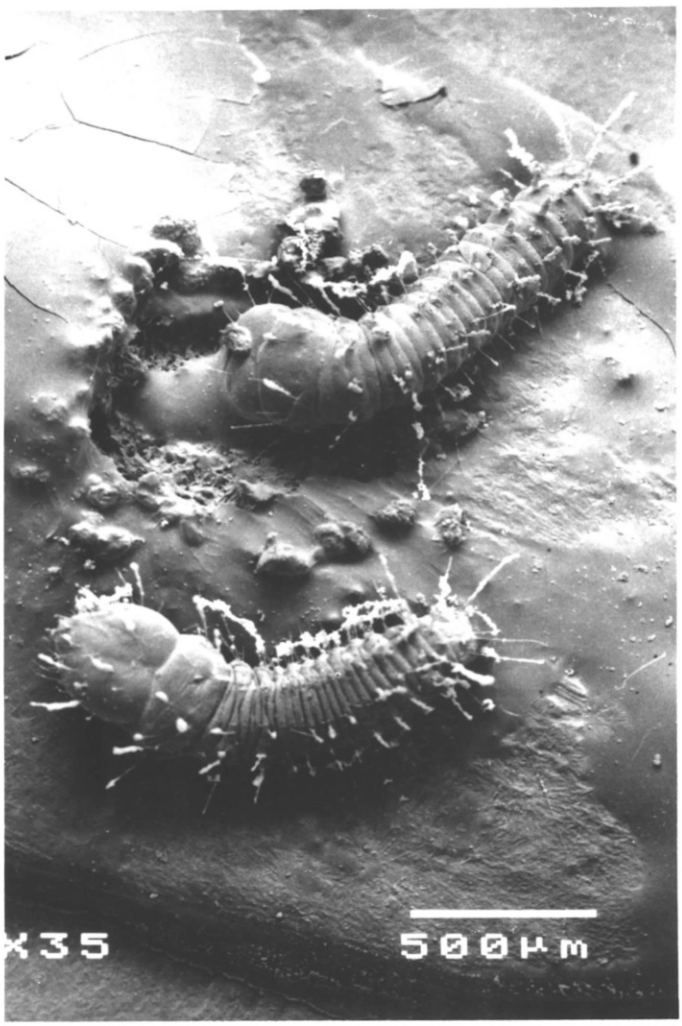


Plate 5.2 **Neonate codling moth larvae feeding on apple leaf disc (Scanning electron micrograph)**

Table 5.1 Effect of virus concentration on uptake of CpGV from leaf surfaces

Virus induced mortality recorded after 5 days at 27°C.

Treatment (per ml)	LET_{50} (mins)	Fiducial limits (mins)	Slope	Slope heterogeneity x2	Slope probability	Control mortality due to virus[a]
5×10^7	5,0	(2,9-6,8)	2,1	1,1 (2df)	>0,05	4%
5×10^7	4,4	(2,4-6,3)	1,8	4,1 (3df)	>0,05	0
1×10^7	57	(34-170)	1,2	4,2 (3df)	>0,05	0
1×10^7	103	(51-724)	1,1	0,5 (3DF)	>0,05	0
1×10^6 [b]	-	-	0	2,3 (4DF)	>0,05	0

[a] A separate control was used for each treatment

[b] The slope for 10^6/ml sprayed, was not significantly different from zero. Virus-induced mortality was negligible even after leaving larvae for nine days to succumb to any ingested virus.

Table 5.2 Active-feeding sites on experimental leaf discs: size and number of feeding holes excavated by larvae

Exposure time (mins)	No. larvae in test	No. holes	Mean area of holes (mm^2)	S.E. (mm^2)
3,5	101	1	0,0099	-
7,5	91	1	0,0109	-
15,0	93	3	0,0322	0,0630
30,0	53	8	0,1509	0,0167
60,0	60	19	0,3167	0,0344

b) Field trial: uptake of CpGV from apple plant surfaces

The method of artificial pest infestation and exclusion cages proved successful, with heavy fruit damage in the unsprayed branches (Plate 5.3). THe data for pest damage was computed by analysis of deviance. Since a defined number of codling larvae and eggs were introduced to each experimental plot it is reasonable to assume that the number of apples did not limit the pest infestation and consequent level of damage. The data

was thus analysed by a Poisson model which considered damage as a score and did not adjust for the total number of apples. (A second analysis adjusting for the number of apples in fact made little difference to the results.)

Table 5.3 shows the damage recorded and analysis of deviance for "deep" damage, where larvae have tunnelled into the apple flesh. The absence of any appreciable damage where larvae or eggs were not introduced, demonstrates that natural background pest infestation was negligible. Superficial damage in each treatment was immense and without relation to treatment. Up to ten superficial "stings" were found on a single apple. The high density of larvae in a confined area probably allowed a good deal of superficial feeding prior to larval death from virus or other causes. What is more interesting is the level of "deep" damage. Deep damage on the unsprayed branches was high with on average ten in each "plot" of 22 apples. On each of the treated branches, damage was reduced to less than 0.15 per branch (back transforming from a $\sqrt{}$ transformation analysis of deviance). The analysis of deviance (table 5.3) shows a highly significant control effect where the damage in unsprayed apples accounts for most of the observed deviance. There was a non-significant deviance for control x treatment; i.e. deviance accounted for between treatments.

CpGV application has achieved excellent suppression of "deep" damage, suggesting that larvae can ingest virus capsules from both the leaf and apple surfaces. Superficial damage was high but larvae causing such damage clearly died prior to excavating past the apple skin.



Plate 5.3 Heavy codling moth damage on apples following artificial infestation

Table 5.3 Apple damage at DR119: virus acquisition trial

Treatment	Mean "superficial" damage per plot (no. holes)	s	Mean "deep" damage per plot (no. holes)	s	Mean no. fruit
A Unsprayed	22,47	4,74	10,43	3,23	22,25
B Apples only	16,73	4,09	0,0625	0,25	17,5
C Leaves only	8,24	2,87	0,1225	0,35	18,75
D Whole branch	31,7	5,63	0,1225	0,35	20,25
S.E.D.*		0,804		0,408	
E (Open branch unsprayed)	0,25		0		

Analysis of deviance of "deep" damage

Fitted model	d.f.	Mean deviance ratio	Significance
Control	1	70,49	***
Control x treatment	2	0,18	

(Residual mean deviance = 1,241, 12 d.f.)

Significance level; *** = P < 0,001
* S.E.D. = Standard error of the difference of means

IV. DISCUSSION

In laboratory assays, CpGV was ingested directly from leaf surfaces, and the amount ingested appeared to depend simply on exposure time and the concentration of virus in the spray liquid. Ingestion of CpGV by young larvae did not require them to feed actively on the leaves and was presumably caused by larvae browsing on the leaf surfaces as they walked across the discs, perhaps in search of a suitable feeding site. In subsequent experiments (Chapter 6, section III(b)iii), CpGV was shown to be actively ingested during this "browsing" since larvae surface-sterilized after exposure, contracted CpGV at a similar rate to unsterilized larvae:

CpGV was not merely sticking to their legs or mouthparts and ingested later. A median lethal dose of CpGV was ingested in as little time as 3.5 minutes, well before significant numbers of larvae were observed feeding on the leaf tissue. A linear log time/probit mortality relationship could be fitted. The slope of this response increased with virus concentration in the spray over the three doses tested; an expected phenomenon since at zero time for each concentration the mortality from virus infection will be zero, hence each probit line must emanate from the same point, but larvae will respond more quickly to a higher virus concentration on the leaf surfaces.

Keller (1973) exposed codling moth larvae to field-sprayed leaves for 10 minutes and 3 hours, recording 71.3 and 78.4% mortality respectively after rearing on clean diet. Keller attributed the virus infection to active feeding on the leaf tissue, and ascribed the small size of the increase in mortality between the two feeding times, as resulting from larvae feeding on the same spot. The poor discrimination in mortality between the two exposure times is, however, much more likely to have been due to the very high virus dose applied -10^8 granules/ml to run-off - where virus deposition on leaves would be so high that mortality was approaching a maximum even at 10 minutes. A spray dose of 10^8 granules/ml gave a leaf surface concentration of 2404 granules/mm^2 (Glen and Payne, 1984) - over 50-fold higher than the virus deposition on leaf discs sprayed in this study at 10^7/ml with the spray "attenuator" in place. In Keller's work, the larvae which did not become infected probably never came into contact with CpGV, where spray coverage is likely to have been much poorer on field-sprayed foliage than on laboratory sprayed discs. This would explain a mortality of only 78% after three hours on Keller's field-sprayed leaves (at 10^8 granules/ml), compared with 90 and 100% mortality after 60 minutes, where leaf discs were sprayed in the laboratory at 5 x 10^7 granules/ml.

The bioassay technique used here has adequately discriminated between virus doses, but its advantage over other bioassay techniques is in the ability to test different formulations of virus for their field-spray suitability. Bioassays based on the incorporation of virus in semi-artificial insect diet (Payne, 1981) are excellent for testing the activity of a virus preparation but do not discriminate between preparations which may or may not be readily ingestible from leaf

surfaces, as in field application. CpGV formulations suitable for commercial development may contain impurities - removal of the purification stage of virus processing is a considerable cost saver (Huber, 1988) - and additives such as wetting agents, stickers and ultraviolet protectants. Additives and impurities may repel ingestion by larvae. Various dry formulations of CpGV have been produced (Huber, 1988; see also Chapter 2, section II(d)iii) but the drying process may cause aggregation of virus granules. Unlike pests which feed on exposed foliage, codling moth larvae tunnel into fruit from whence they can no longer ingest biological or chemical insecticides; Glen and Clark (1984) showed that larvae can reach fruit in as little time as 10 minutes. This bioassay technique mimics the field situations where exposure to virus spray is strictly limited in time. The technique is able to discriminate between preparations containing the same concentration of active virus but differing in their formulation (Chapter 6, section III(b) and (c)). Differences due to virus aggregation, impurities, additives and even larval attractants can theoretically be discriminated by this technique.

In a field trial where spray deposition was restricted to certain parts of the host plant, virus ingestion by larvae was demonstrated from both leaves and fruit. CpGV was applied to apples only, leaves only, or both. Superficial fruit damage was high in all treatments, including the unsprayed control and probably resulted from larval feeding prior to death by virus infection. Control of "deep-damage" was excellent with all three virus treatments giving over 98% damage reduction compared to the untreated control. There is clearly no need for larvae to eat the surface of fruits in order to ingest a lethal dose of CpGV. The phenomenon of virus ingestion whilst browsing on foliage is consistent with observations by Burgerjon and Sureau (1988), who found greater efficacy of CpGV sprayed against the first pest generation, which they concluded spent more time on foliage in the absence of developed apples. Furthermore, a greater number of eggs were found laid on fruit later in the season, decreasing the fruit-searching time in which larvae could contract CpGV from leaf and fruit surfaces (Burgerjon and Sureau, 1988).

These experiments confirm that CpGV can be ingested from both leaves, and apple surfaces, and that the longer time larvae spend browsing on foliage, the greater the probability they have of dying from virus infection. If suitable feeding attractants or stimulants for codling moth

larvae could be isolated it may be possible to encourage greater foraging on leaves prior to fruit entry, and encourage more active browsing on treated foliage. Any new formulations of CpGV, whether containing potential attractants, or spray adjuvants could be tested for efficacy by the described bioassay technique. This possibility is discussed in the following chapter (6, section III(b)).

Chapter 6

Improving the formulation of CpGV

I. INTRODUCTION

One way by which the field control of codling moth might be maximised could be to apply CpGV at very high doses to overcome the problem of poor persistence. However, in view of the high cost of virus production - and the lack of any improvement in control achieved using a high virus dose compared to a 'standard' dose (Chapter 4, section III(a)) - a more acceptable solution would be to optimize the application strategy for its use by frequent application (Chapter 4, section III(b)) and to improve the formulation. This chapter discusses the use of formulation additives to improve virus persistence, and feeding attractants to enhance virus uptake by larvae.

Much work has been published on the use of formulation additives to improve persistence and a number of agents have been claimed to prolong activity in laboratory tests, although this has not always been reflected in field studies (Richards, 1984). In the current study a number of agents with reported virus persistence-enhancing activity were tested directly in orchard trials, their choice also determined by their cheapness. A number of potential feeding attractants were chosen for laboratory and field testing, including natural and artificial sweeteners, fruit extracts and α-farnesene, the naturally occurring component of apples with reported olfactory attraction properties in codling moth larvae (Sutherland, 1972; Sutherland and Hutchins, 1972; 1973; Wearing and Hutchins, 1973).

In addition to formulation improvements to enhance CpGV persistence and uptake, this study was also concerned with the search for low-cost, convenient formulations. The costly process of virus purification was omitted in the preparation of an impure homogenate which was tested in both laboratory and field trials. Dry powder preparations - initially designed to test for storage potential (Chapter 3, section II(d)) - were analysed in the same way.

II. MATERIALS AND METHODS

a) Field trials on virus formulation

A field-trials programme was designed to examine the effects of formulation additives to CpGV sprays, testing for increased virus persistence and improved control of codling moth fruit damage. The apparent success of molasses in reducing fruit damage in trial i. (below) was followed by a series of laboratory and field experiments to identify the mechanism for this improvement and isolate other potential formulation additives with similar activity. The use of impure and dry CpGV formulations were also tested in laboratory and field studies. All field trials were carried out at IHR East Malling and Eden Farm, West Malling (see Chapter 2, section VIII(a)), for full details of orchard sites).

i. A comparison of molasses and skimmed milk as spray additives

Plot WF103, Laxton's Fortune, 1985 (Fig. 2.1).

Skimmed milk has been claimed as a UV protectant for CpGV (Keller, 1973) and used in field trials to improve virus persistence (Glen *et al.*, 1981b; Glen and Payne, 1984; Fischer-Colbrie, 1978). Another low-cost agent, molasses, has also been claimed to confer resistance to UV to other baculoviruses or improve their persistence (Shapiro *et al.*, 1983; Jones and McKinley, 1986). Both these formulation additives were included in a trial to compare their relative advantages when added to CpGV sprays. The comparison formed part of a CpGV dose and formulation trial (Chapter 4, section II(a)i) where CpGV was tested at three dose rates with a standard chemical insecticide comparison. A randomized block design with four trees per plot and four replicates in four blocks was used (Fig. 2.1). Six treatments were tested. All CpGV treatments contained 0.02% Agral as a wetter. The effect of the two formulation additives on virus persistence and fruit damage reduction was tested. Leaf samples were taken immediately after spraying, and after one and seven days. The amount of active virus remaining was measured by bioassaying leaf washings taken from each sample (Chapter 2, section X(a)). All fruit, windfalls and harvested apples, were examined for codling moth damage, grading damage according to severity (Chapter 2, section VIII(f)). Additional effects on

other orchard arthropods (Chapter 7, section III(a)) and foliar disease (Chapter 2, section XI) were monitored throughout the season.

Treatments:

A. Unsprayed control
B. CpGV at 10^{12} granules/ha + 0.2% (w/v) skimmed milk powder
C. " " 10^{13} " " " " " " " "
D. " " 10^{14} " " " " " " " "
E. " " 10^{12} " " + 15% (v/v) cane molasses
F. Deltamethrin (2.5% a.i.) at 250 ml/ha

Spray dates: Two applications were made (27 June and 17 July) the second, 140 day-degrees after the first spray, timed to coincide with peak egg hatch (see Chapter II, section VIII(e)). Sprays were applied at 1000 l/ha using a Minicomet hydraulic sprayer.

ii. The influence of skimmed milk and other spray additives on virus persistence

Plot WE 149 Cox/Worcester Pearmain, 1985.

In addition to the main field trial in 1985, a second smaller trial was undertaken where the effects of various formulation additives on virus persistence were studied. Six CpGV treatments containing different spray additives were applied to single-branch "plots" of 1.5 m apple trees (M9 rootstock). Four blocks (3 Cox, 1 Worcester Pearmain) consisted each of 3 trees with two treated branches on each. A randomized block design with four replicates tested the following treatments, all containing purified CpGV at 1 x 10^{10} granules/l (10^{13}/ha) and 0.02% Agral and sprayed at 1000 l/ha by knapsack compression sprayer,

Treatments:

A. CpGV only
B. CpGV + 0.2% (w/v) skimmed milk powder
C. CpGV + 0.2% (dry weight/v) Indian ink
D. CpGV + 0.2% (v/v) S600 anti-transpirant (Synchemicals, London)
E. CpGV + 0.2% (v/v) Crodabuild 50, synthetic rubber solution (Croda Paints Ltd, Hull)
F. CpGV + 0.2% (v/v) Nufilm 17 (Muller Fertiliser, Baltimore, MD, USA)

The additives chosen were those already claimed to have some protectant properties, such as skimmed milk (Keller, 1973; Glen *et al.*, 1981b) and Indian ink (composed of shellac and lampblack) (Bull *et al.*, 1976; Shapiro *et al.*, 1983) or were chosen for their ability to form fine films; Nufilm 17; Crodabuild - a synthetic-rubber solution used in paints - and S600 anti-transpirant spray. At the concentrations used, and the volumes applied, it was estimated that each of these additives would cost less than £10 per ha to spray (1985 prices). Foliar persistence of virus was measured as before, from leaf samples taken immediately after spraying, and after one, three and nine days.

Spray date: 13 August

iii. Effect of molasses on foliar persistence of CpGV.

Eden Farm, West Malling, Tydeman's Early Worcester, 1986.

The results of Trial i. had indicated that addition of molasses gave enhanced control of codling moth damage. In an attempt to determine whether this improvement was attributable to increased virus persistence, a study was conducted on virus persistence in the presence of molasses. In this trial, a high concentration of CpGV was sprayed on apple foliage and the persistence followed over three weeks. Two treatments were applied with four replicates to single-branch plots, two branches per tree. Purified CpGV at 4 x 10^{11} granules/l was used with Agral at 0.02% and sprayed at 1000 l/ha using a knapsack compression sprayer. CpGV was applied at a higher concentration of CpGV than used in the comparison of

molasses and skimmed milk in trial i. so as to allow differences in foliar virus concentrations to be more easily detected by bioassay. Leaf samples were taken immediately after spraying, and after two, eight and twenty-three days. Foliar persistence of CpGV was measured by bioassaying leaf washings as before (Chapter 2, section X(a)).

A. CpGV only
B. CpGV + 10% (v/v) cane molasses

Spray date: 2 September.

b) Laboratory and field studies on the effect of molasses and other compounds as 'feeding stimulants'.

i. Initial laboratory tests: Effect of molasses on uptake of CpGV

In field trials, improved codling moth control was achieved with CpGV sprays incorporating molasses; yet no improvement in virus persistence was observed (section III(a)i and iii). To determine whether or not molasses enhanced the uptake of virus by larvae, a new leaf bioassay technique (Chapter 2, section VI) was used to study the effect of molasses as a formulation additive for CpGV. Leaf discs were sprayed (see Chapter 2, section VI, for full details) with CpGV at fixed concentrations, 32 larvae were then allowed to feed for pre-set "exposure times" before being removed and reared on virus-free artificial diet. Larval mortality due to virus was recorded after 5 days at 27°C. In each test two controls were set up: a leaf control where 32 larvae were allowed to feed for 60 minutes on unsprayed leaf discs prior to transferring to virus-free diet; and a diet control where 32 larvae were set up directly on diet.

The assay effectively measured the relative rates of virus ingestion, since virus uptake by larvae - leading to infection in the event of a lethal dose being contracted - was limited to the pre-set times in which larvae were allowed to feed on the sprayed leaf discs. Any feeding stimulant, inhibitor or disruptor would result in an altered time-mortality response slope. The $\log_{10}$ exposure time plotted against probit mortality gave a straight line from which the exposure time corresponding to the

medial response (ie 50% mortality) could be derived. The exposure time giving 50% mortality (LET_{50}) could be compared for different formulations.

In test (1), molasses was incorporated at 15% (as in Field Trial i) with CpGV at $1x10^7$ granules/ml and Agral at 0.02%. The LET_{50} and confidence limits of the two response slopes were compared. In test (2) larvae were allowed to feed for 3, 9 or 27 minutes on leaf discs sprayed with CpGV at three concentrations, with or without molasses: the results were analysed by analysis of variance. As a comparative treatment, molasses was also tested at 1% (v/v) with CpGV at just one concentration; the results not intended for inclusion in the main analysis of variance.

(1) Treatments:
- (a) CpGV at 10^7 granules/ml
- (b) + 15% (v/v) molasses
- (c) Diet + leaf control

"Exposure times": 2, 4, 8, 16, 32, 60, 300 (minutes)

(2) Treatments:
- (a) CpGV only - 2 x 10^6 granules/ml
- (b) " - 10^7 granules/ml
- (c) " - 5 x 10^7 granules/ml
- (d) + Molasses 15% (v/v) - 2 x 10^6 granules/ml
- (e) " " " - 1 x 10^7 granules/ml
- (f) " " " - 5 x 10^7 granules/ml
- (g) " 1% " - 1 x 10^7 granules/ml
- (h) Diet + leaf controls

Exposure times: 3, 9, 27 minutes

ii. Laboratory screening of potential formulation additives

The same assay was used to test a range of possible additives (which could have similar effects to molasses) in a rapid screening programme prior to field-testing. In addition to molasses, natural sugars, sorbitol, artificial sweeteners, and two constituents of apples, pectin and malic acid, were tested. Agral at 0.02% (v/v) was always included as a wetter.

(1) Treatments: (a) CpGV at $1x10^7$ granules/ml
(b) + 15% (v/v) molasses
(c) + 10% (w/v) sucrose
(d) + 10% (w/v) fructose
(e) Diet + leaf controls

(2) Treatments: (a) CpGV at $1x10^7$ granules/ml
(b) + 2% (w/v) "Natrena" contains saccharine
(c) + 10% (w/v) "Candarel" contains aspartame
(d) + 10% (w/v) Lactose
(e) + 99% (v/v) Pure apple juice (Sainsbury's pure apple juice; 11.5% dry weight)
(f) Diet and leaf controls

(3) Treatments: (a) CpGV at $1x10^7$ granules/ml
(b) + 2% (w/v) Pectin (saturated solution)
(c) + 10% (w/v)Sorbitol
(d) + 10% (w/v) Molasses
(e) + 10% (w/v) Malic acid
(f) Diet and leaf controls

Exposure times for all tests: 7, 14, 28 minutes

iii. Laboratory analysis of feeding stimulants as spray additives

A third series of leaf bioassays was designed to investigate further the apparent "feeding stimulant" effect of molasses and sorbitol (both of which gave improved codling control in field trials; sections III(a)i and (b)iv), sucrose, and apple extracts. Previous tests (above; sub-section i), showed this bioassay technique to be most effective at discriminating additives enhancing CpGV uptake, if larvae were allowed to feed for up to one hour on leaf discs sprayed with CpGV at $1x10^7$ granules/ml. The bioassay was thus standardized with larvae left on leaf discs for set "exposure times" of 3, 7.5, 15, 30 and 60 minutes, prior to rearing on virus-free diet for five days at 27°C. In each experiment a number of spray additives were compared with unformulated CpGV (or, more precisely, CpGV with the addition of just the wetting agent Agral). Each experiment included untreated diet and leaf controls. The "exposure time"/mortality

response of each additive was compared with the unformulated virus treatment in terms of the LET_{50} and confidence limits. The treatments tested are outlined below, though the full details of the additives evaluated in each separate test are given in the table of results.

Sorbitol, sucrose, skimmed milk (used in Field Trial 1.), and apple skin and apple flesh homogenates were added to CpGV at $1x10^7$ granules/ml. Sorbitol was tested at 10% (w/v), the rate used in Field Trial 1., whilst skimmed milk was tested at 1% (w/v) (as used in field trials by Glen and Payne, 1984). Sucrose was tested at a range of concentrations from 0.75% to 6% corresponding to levels of reducing sugar found in cane molasses and in the apple extracts used in these tests (see below).

Apple skin and flesh homogenates were prepared by homogenizing 1.5 g skin or 2.0 g flesh (cv. Bramley) in 12.5 ml water. The homogenate was prepared by finely chopping the apple tissue, followed by crushing in a pestle and mortar (skin only), and blending in a Wearing laboratory blender for 3 minutes. The homogenates were strained through muslin. Mean dry weights were: apple skin, 1.6%; and apple flesh 1.7%. An analysis of these apple extracts revealed a mean reducing sugar content of 0.75% in the skin extracts and 2.5% in the flesh extracts (glucose via neocuprine-copper reagent analysis; Bittner & McCleary, 1963). Cane molasses contained 60% sucrose.

It could be argued that codling larvae might transmit virus to the diet from the leaf surface on legs, prolegs and mouthparts and ingest it later. In one experiment, larvae were surface-sterilized by bathing them for 2-3 minutes in 0.5% Hyamine 1622 solution (BDH Ltd, Poole) after exposure to sprayed discs to eliminate the possibility of passive carryover of CpGV. Hyamine 1622 is a cationic surfactant suitable for surface sterilizing larvae (W.A.L. David; unpublished observation).

The effect of molasses in enhancing the efficacy of CpGV was also tested using an impure CpGV homogenate - as used in the 1987 field trial - since impure preparations would be most likely to form the basis of a commercial product.

Volatile and non-volatile extracts of apple skin, and α-farnesene, the naturally occurring component of apple skin (which also gave improved

codling moth control in field trial (b)v; below), were compared to unformulated CpGV and to molasses and sucrose as spray additives. Apple extracts were prepared by Lester Wadhams (IACR Rothamsted Experimental Station): the crude extract (designated LAM5) was obtained from apple skin by solvent extraction with ether. Each sample was the equivalent extract of 20 g apple skin/ml, diluted 1:4 in the final virus suspension.

LAM5/129C = total, crude apple skin extract in ether
LAM5/132B = volatile fraction of 129C
LAM5/132C = non-volatile residue of 129C

The crude and non-volatile fractions were made miscible with the virus suspension by adding Agral (1% v/v), and absolute alcohol (10% v/v), followed by sonication. 0.1% α-farnesene was formulated with 1% Agral (v/v). LAM5/129C, 132B and 0.01% farnesene contained (E-E)- α-farnesene at 0.025% (w/v).

iv. Effect of molasses on larval feeding.

The effect of molasses on the number and size of larval feeding holes on leaf discs was also tested by comparing discs sprayed with or without 10% (v/v) molasses. Thirty seven larvae were left on 17 leaf discs for 60 minutes at 27°C.

v. Field Trial - Comparison of potential larval "attractants" and efficacy of purified and impure virus formulations.

Plot WF103, Laxton's Fortune, 1987 (Fig. 2.3).

In laboratory tests I and II, a number of additives were shown to increase the rate of ingestion of CpGV from leaf surfaces by young codling moth larvae. Of these, sorbitol and molasses were chosen for testing under field conditions, the trial also serving to contradict or confirm the previous success demonstrated with molasses in Trial (a)i. Another chemical, α-farnesene, has been shown to act as a larval olfactory attractant (Sutherland, 1972; Sutherland and Hutchins, 1973; Wearing and Hutchins, 1973) and was thus considered as a potential formulation additive

to attract larvae to spray deposits. In the same trial, the efficacy of an impure CpGV preparation containing homogenised infected codling moth larvae, frass and diet (Chapter 2, section II(d)i) was compared with purified CpGV, as used in all previous trials.

The same orchard as in trial (a)i. was used with a similar layout of four blocks, four replicates and four-tree plots (Fig. 2.3). Six treatments were applied in four sprays, with all virus treatments applied at 10^{12} granules/ha at 250 l/ha using a Solo Minor mistblower. All treatments contained Agral at 0.02% except treatment F, where Agral was incorporated at 1% to enhance solubility of the additive. The efficacy of each treatment was measured, as before, by analysing all fruit, windfalls and harvested apples, for codling moth damage. The presence of unwanted foliar disease caused by the additives was also monitored.

A. Unsprayed control
B. Purified CpGV
C. Impure CpGV homogenate
D. Purified CpGV + 10% (v/v) cane molasses
E. " " + 10% (w/v) sorbitol
F. " " + 0.08% farnesene

Of the four blocks, two were treated with an experimental IPM programme comprising early season sprays of pirimicarb and diflubenzuron and summer application of *Bacillus thuringiensis*. These treatments, for a separate study (Chapter 7, section II(c)) were chosen to have minimum effect on codling moth control, but were nevertheless blocked in the experimental design (Fig. 2.3).

α-farnesene was supplied as $POCl_3$-farnesene (containing 40% of the E,E- stereoisomer of α-farnesene) by Lester Wadhams of the Institute of Arable Crops Research, Rothamsted.

Spray dates: Following the success of more frequent applications used in 1986 (Chapter 4, section III(b)), in 1987 four virus applications were made, spaced at one-week intervals - 1, 8, 16, 24 July, commencing at peak egg hatch.

c) Laboratory and field evaluation of impure and dry formulations of CpGV

i. Effect of impure and dry formulations of CpGV on control of codling moth.

Plot HG 115, East Malling, Cox and Golden Delicious, 1987 (Fig. 2.4).

In a trial to compare the efficacy of impure CpGV and two dry-powder derivatives with purified virus, five formulations were tested at a dose of 5 x 10^{12} granules/ha at 500 l/ha. Impure CpGV was prepared from homogenised infected insects and diet and dry powders were prepared from the same material (Chapter 2, section II(d)i & iii). Molasses was included in treatment E to test its compatibility with impure CpGV. Four replicates of two-tree plots were arranged in a randomized block design (blocking for orchard position and apple variety) as shown in Figure 2.4. CpGV was applied at 5x10^{12} granules/ha, at 500 l/ha using a knapsack compression sprayer. The efficacy of each treatment was assessed by examining all fruit, windfalls and harvested apples for codling moth damage and damage severity.

Treatments:

A. Pure CpGV
B. Impure CpGV homogenate
C. Solvent-dried homogenate
D. Freeze-dried homogenate
E. Impure homogenate + molasses 10% (v/v)
F. Unsprayed control

All formulations contained Agral at 0.02% (v/v). Formulations were tank-mixed by agitation: materials were **not** specially treated by sonication to break up aggregated virus. Prior to application, CpGV formulations were kept at -20°C to prevent loss of activity. Bioassays of the stored materials confirmed that the original activity had been maintained: each preparation showed no loss of activity even after storing for 235 days at -20°C.

Spray dates: Treatments were applied five times: July 2; 8; 16; 23; and August 10; the last treatment timed to cover a late peak of adult flight activity.

ii. Laboratory evaluation of impure and dry formulations of CpGV.

Pure, impure and dry formulations - as used in field trial 5 - were tested using the above leaf bioassay method. Formulations were applied at the same rate of active virus, tested by diet-incorporation bioassay (Chapter 2, section II(c)iii).

CpGV was applied at 5 x 10^7 capsules/ml, a higher rate than in previous leaf bioassays since virus was being sprayed without feeding stimulants. Formulations were either mixed directly by agitation or additionally sonicated to break up groups of aggregated particles. Formulations were applied with 0.02% Agral. Larvae were exposed to sprayed leaf discs for 3.5, 7.5, 15, 30, and 60 minutes.

III. RESULTS

a) Comparison of molasses and skimmed milk as spray additives

i. Effect of CpGV dose and formulation

In the 1985 field trial the effect of CpGV dose and the addition of molasses at low CpGV dose was studied. An insecticide was included for comparison. The fruit damage was analysed by analysis of deviance, fitting a binomial error and logit link function. The details of the analysis are given in Chapter 4 (section III(a)); the effect of formulation, tested at the lowest dose of CpGV, was analysed by a separate orthogonal contrast.

The full results are given in Chapter 4, Table 4.1: the effects of virus dose and the chemical insecticide comparison have already been discussed (Chapter 4, section III(a)). Fruit damage in the unsprayed control plots was high with 15% damage in the total fruit (windfalls and harvest combined). Incorporation of molasses at a virus dose of only 10^{12}

granules/ha gave as good control as the skimmed milk formulation at 10^{13} and 10^{14} granules/ha. The orthogonal comparison, "Formulation", shows that molasses was significantly better at reducing deep and total damage than the skimmed milk formulation at the same virus dose ($P < 0.01$). Using CpGV at low dose (10^{12} granules/ha) for the formulation comparison, allowed greater sensitivity: the addition of molasses improved damage reduction from 62.1% to 81.4% of total-fruit deep-damage. A higher virus dose might have overshadowed such an improvement since the proportional change would have been less.

Leaf samples were taken just after spraying and after certain time intervals. These were processed and an estimate of virus deposition and persistence calculated, and compared, for the two formulations; 15% molasses and 0.2% skimmed milk powder, both with virus sprayed at 10^{13} granules/ha. Estimates of virus activity made from the four replicates were examined by analysis of variance on a $\log_{10}$ transformation of the original data. From the estimated means, figures for the amount of virus per leaf surface area have been calculated (Table 6.1). There were no significant differences between treatments.

Table 6.1 Effect of skimmed milk and molasses on virus persistence

Days after spray	Treatment	Mean concentration log10 granules/ 0,25 ml sample	S.E.	F prob.	Foliar concentration granules/cm2 (upper and lower surfaces)
0	Skimmed-milk	2,723	0,1061		598
				>0,05	
	Molasses	2,799	0,0872		712
1	Skimmed-milk	2,490	0,1165		350
				>0,05	
	Molasses	2,748	0,0773		634
7	Skimmed-milk	2,519	0,0712		374
				>0,05	
	Molasses	2,330	0,0952		242

After spray application it soon became apparent that the molasses treatment was encouraging the growth of sooty moulds (*Cladosporium* spp.) on the foliage. Table 6.2 (a and b) shows the incidence of sooty moulds and its effect on leaf senescence for the molasses and control treatments. On three sampling dates there was a significant increase in the recorded incidence of sooty moulds with molasses (24 July $P < 0.01$; 16 August $P < 0.05$; 24 September $P < 0.001$). Sooty moulds were not noticeable on any of the other treatments. The effect on leaf browning was minimal with a rise from about 0.1% total area with the unsprayed leaves to around 0.2% with molasses; the effect was significant on two of the three sample dates (24 July $P < 0.05$; 26 September $P < 0.01$). Leaf browning was significantly correlated with sooty moulds, though the effect was small:

Correlation coefficient = 0.1154 (476 d.f.); P = 0.0116

Table 6.2 Effect of molasses on formulation of sooty moulds and leaf senescence

Treatment	24 July	16 August	26 September
a) Sooty moulds: mean no colonies/leaf			
Unsprayed	0,145	0,102	0,188
CpGV + molasses	19,0	18,9	7,01
F significance	**	*	***
b) Leaf senescence: mean % leaf area browning			
Unsprayed	0,084	0,128	0,083
CpGV + molasses	0,210	0,237	0,240
F significance	*		**

Significance levels: * = P < 0,05 ** = P < 0,01
*** = P < 0,001

ii. Effect of spray additives on foliar persistence of CpGV

Leaf samples collected from formulated CpGV sprayed branches were processed and statistically analysed as for the spray deposit measurements in Trial (a)i. All CpGV treatments were applied at 1000 l/ha with 10^{10} granules CpGV/ml (10^{13}/ha). Table 6.3 summarises the results. Standard errors have been calculated from the four replicate estimates interpolated from the standard curve. Overall, the mean treatment foliar concentration of virus decreased 36 fold over 9 days and nine fold over the first 3 days. None of the treatments gave any useful level of protection, there being a fairly rapid decline in all six treatments, though skimmed milk was the best protectant after one day, when a significant treatment effect was observed.

Table 6.3 Effect of spray additives on foliar persistence of CpGV

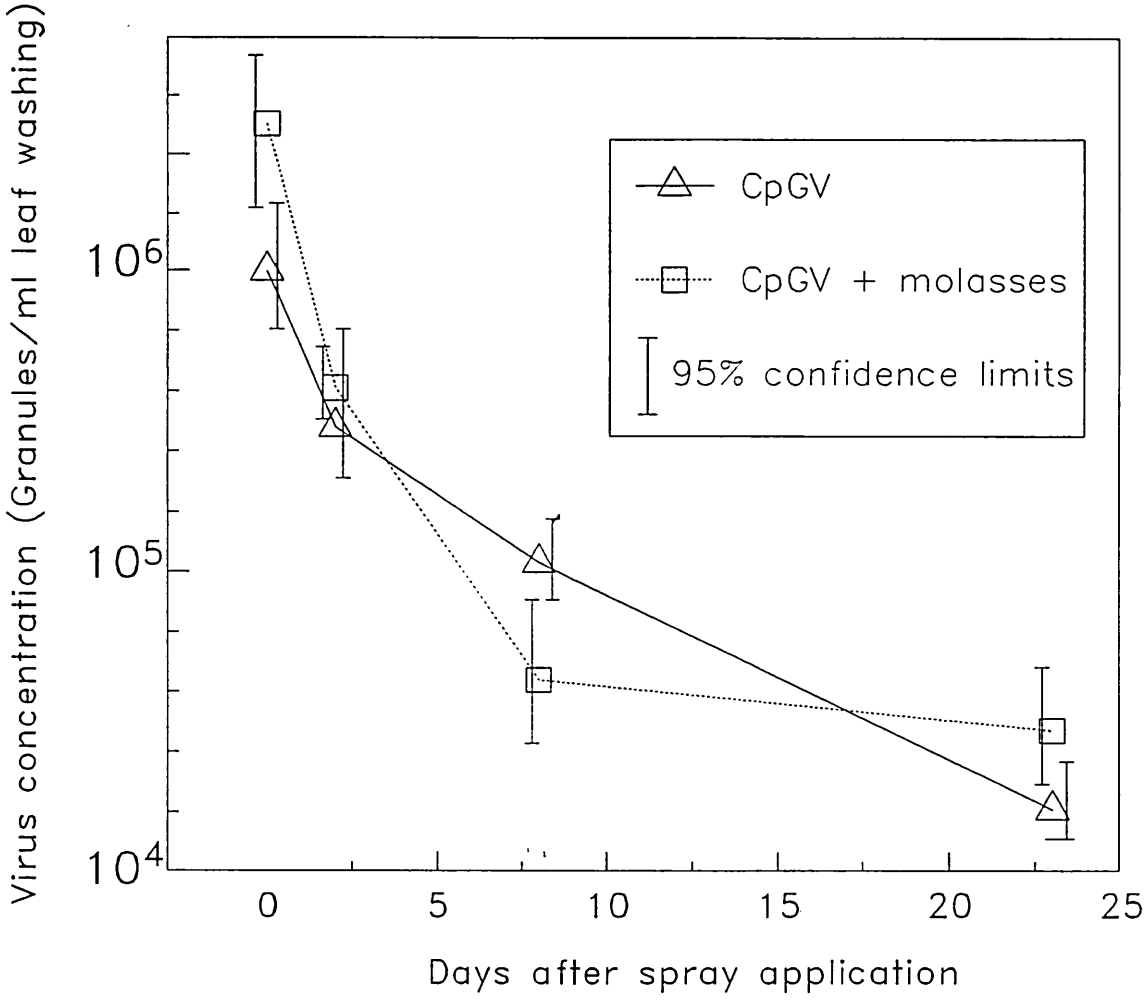
Days after application		Treatments						
		CpGV	Skimmed	Indian	S600	Nufilm	Croda-build	Mean
0	Mean concentration (LOG_{10} granules/0,1 ml)	3,475	3,3998	3,454	3,518	3,855	4,109	
	S.E.	0,148	0,204	0,142	0,147	0,180	0,221	
	Foliar concentration (granules/cm²)	8447	$2,82\times10^4$	8048	9326	$2,03\times10^4$	$3,64\times10^4$	$1,85\times10^4$
	F prob. Treatment effect: >0,05							
1	Mean concentration (LOG_{10} granules/0,1 ml)	2,629	3,137	2,343	2,295	2,784	2,852	
	S.E.	0,107	0,111	0,122	0,127	0,106	0,103	
	Foliar concentration (granules/cm²)	1204	3879	623	558	1721	2012	1666
	F prob. Treatment effect: <0,01							
3	Mean concentration (LOG_{10} granules/0,1 ml)	3,022	3,057	2,586	2,794	2,785	2,723	
	S.E.	0,121	0,101	0,149	0,123	0,136	0,131	
	Foliar concentration (granules/cm²)	2976	3226	1090	1760	1724	1495	2045
	F prob. Treatment effect: >0,05							
9	Mean concentration (LOG_{10} granules/0,1 ml)	2,314	2,466	2,100	2,328	2,039	2,167	
	S.E.	0,105	0,939	0,1172	0,105	0,123	0,115	
	Foliar concentration (granules/cm²)	583	827	356	602	309	416	515
	F prob. Treatment effect: >0,05							

iii. Effect of molasses on persistence of CpGV

In 1986 a trial was designed to test the effect of molasses on foliar persistence. Molasses had been shown to improve the efficacy of CpGV in controlling codling moth (Trial (a)i), and measurements of active foliar deposits from this trial, whilst not demonstrating any improvement in persistence, were perhaps not wholly conclusive since very low doses of CpGV were applied. In this trial a high dose of 4×10^{11} granules/l was applied making the assessment of persistence much easier, and more accurate. The activity of foliar deposits for the two treatments have been plotted in Figure 6.1, showing a rapid decline in activity, very similar for both treatments, with an initial half-life of around 1 day.

Fig. 6.1

Persistence of CpGV on foliage



b) Laboratory and field studies on the effect of molasses and other compounds as 'feeding stimulants'.

The new bioassay system designed to measure different rates of virus ingestion from leaf surfaces produced consistent exposure-time/mortality responses. Virus ingestion was a function of the time larvae spent on virus-sprayed leaves ("exposure time") and there was no threshold minimum time before virus was ingested (Fig. 6.2). Virus uptake is apparently not dependent on any active feeding on the leaf tissue (chapter 5, section III(a)). For each response curve a straight line relationship could be fitted by MLP analysis and a figure for the Log_{10} exposure time for 50% larval mortality (LET_{50}) derived. LET50s could be compared between treatments. Treatments affected both the LET_{50} and the slope of the response curve. The linearity of the response was statistically analysed in each experiment. With very few exceptions, the heterogeneity X^2 values were non-significant and the points did not differ significantly from a straight line.

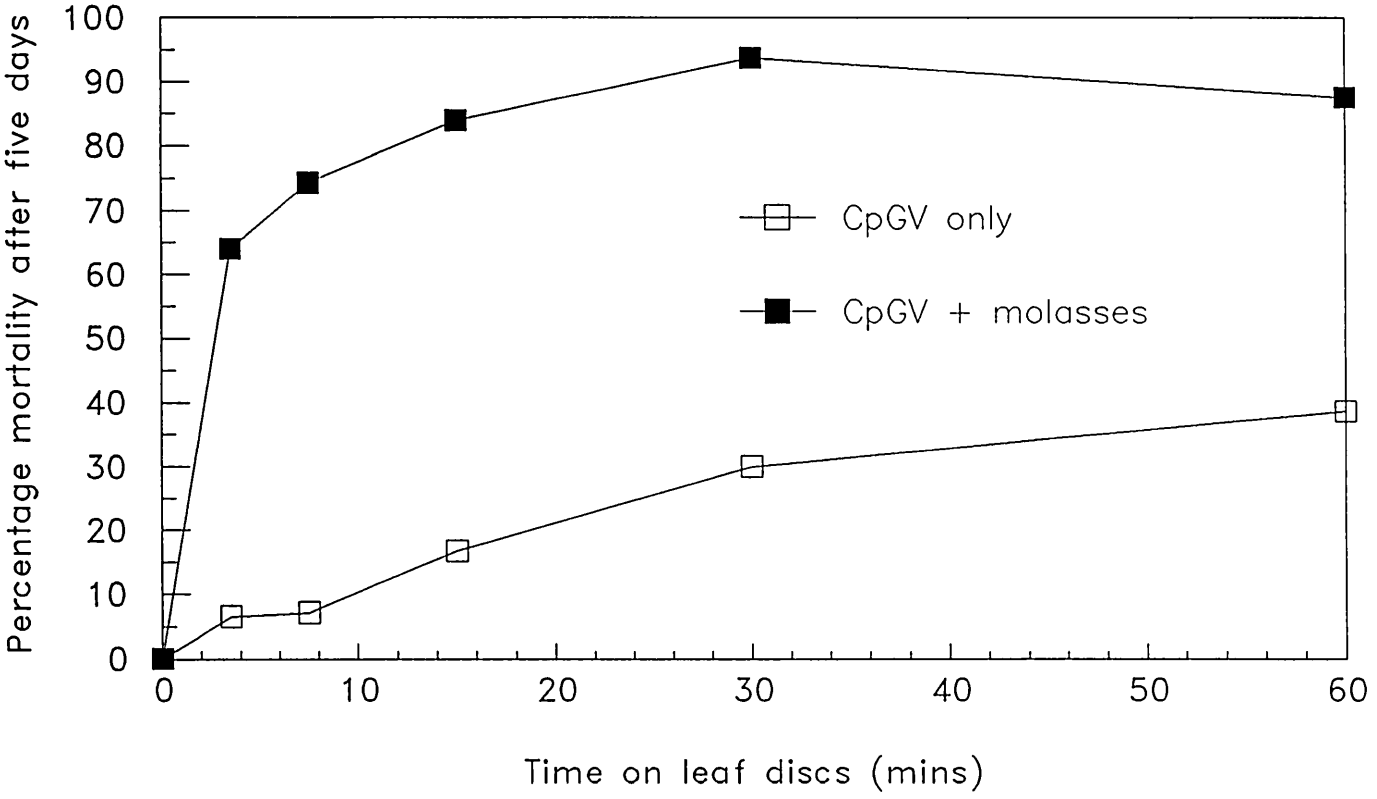
i. Laboratory evaluation of molasses as a feeding stimulant:

Table 6.4 gives the results of the initial tests. CpGV was sprayed with and without molasses and in both tests (1) and (2), the LET_{50}, with the addition of molasses was reduced considerably and the confidence limits of the two treatment LET_{50}s were distinct.

In a factorial design (test 2) larvae were allowed to feed for 3, 9 and 27 minutes on leaf discs sprayed with CpGV at three concentrations, with or without molasses. Increased CpGV concentration, exposure time and the addition of 15% (v/v) molasses produced highly significant increases in virus-induced mortality ($P < 0.001$). There was also a significant interaction of molasses with exposure time ($P = 0.007$). Molasses at 1%, included as an extra treatment, lowered the LET_{50} compared to the pure CpGV sample but the difference was not significant.

The possibility of molasses affecting the droplet output from the sprayer and virus deposition was ruled out as a cause for its enhancing effect on CpGV uptake. Neither droplet size, density nor virus deposition were significantly affected (Table 6.7).

Fig. 6.2 Effect of molasses on uptake of CpGV from sprayed leaf discs



ii. Laboratory screening of other potential feeding stimulants:

A number of compounds were tested in a 'rapid-screening programme' in which only three exposure times were used; limiting the precision of the LET_{50} estimates but allowing more samples to be tested within the constraints of time and supply of neonate larvae. Molasses, sucrose, fructose, sorbitol - all at 10% - and apple juice showed dramatic increases in the speed of ingesting a lethal dose of virus (Table 6.5). The two artificial sweeteners containing aspartame and saccharin, at levels equivalent (according to the manufacturers) to 10% sucrose, were unsuccessful, as were pectin, malic acid (a constituent of apples) and lactose. Apple juice is 99% carbohydrate (dry analysis) (Diem & Lentner, 1970), and the sample had a dry weight of 11.5%. Natural "sweeteners" may account for the effect of apple juice; this was considered later. On the basis of these tests, molasses at 10% (v/v) and sorbitol were chosen for inclusion in a field trial investigating the effect of formulation on virus efficacy (sub-section iv; below).

Table 6.4 Effect of molasses on rate of ingestion of CpGV from sprayed leaf surfaces

(1) Effect of molasses

TREATMENTS:	CpGV (10^7/ml)	CpGV + 15% (v/v) molasses
ANALYSIS		
LET_{50} (mins)	268,8	5,29*
Fiducial limits	119-1316	2,8-8,3
Slope	0,812	1,02
Slope heterogeneity	<0,05	-
Control mortality = 0%		

* Treatment significantly different from pure CpGV.

(2) Effect of molasses at three levels of CpGV

TREATMENTS:	Purified CpGV only			CpGV + Molasses (15%)			CpGV+Mol (1%)
	$5x10^7$	10^7	$2x10^6$	$5x10^7$	10^7	$2x10^6$	10^7
LET_{50} (mins)	55	55,4	No response	6,98	11,4	22,0	24,5
Fiducial limits	18,6-147	27,4-895	-	4,34-10,3	8,55-15,6	16,3-35,5	17,9-41,3
Slope	0,71	1,45	-	1,83	2,46	2,56	2,54
Slope heterogeneity	-	-	-	-	-	-	-
Control mortality = 0%							

Analysis of variance (logit transformation):

Source of variation	F, probability	
Virus concentration	<0,001	***
Molasses	<0,001	***
Time	<0,001	***
Virus conc. x molasses	0,626	
Virus conc. x time	0,053	
Molasses x time	0,007	**

Table 6.5 Effect of various spray additives on uptake of CpGV

(1)	CpGV only (10^7/ml)	+ molasses (15%)	+ sucrose (10%)	+ fructose (10%)
LET_{50} (mins)	289,8	24,1	33,8	18,77*
Fiducial limits	23,9-3516	17,9-45,8	23,1-112,1	15,8-23,3
Slope	0,944	2,46	2,30	4,44
Slope heterogeneity?	-	-	-	<0,05
Control mortality = 0%				

(2)	CpGV only (10^7/ml)	"Natrena" (2%)	"Candarel" (10%)	Lactose (10%)	Apple juice (99%)
LET_{50} (mins)	No significant response			73,6	7,79
Fiducial limits	-	-	-	34,3-3x106	5,07-12,0
Slope	-	-	-	1,82	1,03
Slope heterogeneity	-	-	-	-	-
Control mortality = 0%					

(3)	CpGV only (10^7/ml)	+ Pectin 2%	+ Sorbitol 10%	+ Molasses 10%	+ Malic acid 10%
LET_{50} (mins)	145,3	NSR*	3,00*	3,66*	147,2
Fiducial limits	14,0-206	-	0-6,42	1,79-7,48	32,9-659
Slope	1,12	-	1,50	1,14	1,46
Slope heterogeneity	-	-	-	-	-
Control mortality = 1,3%					

*NSR = No significant response
* Treatment significantly different from pure CpGV.

iii. Laboratory assessment of feeding stimulants

In more extensive tests (Table 6.6) formulation additives were compared over five exposure times covering the sensitive range of the assay. Molasses, sorbitol, sucrose, apple skin and apple flesh homogenates all reduced LET_{50}s outside the 95% confidence limits of the pure virus sample. Skimmed milk powder, attributed by Keller (1973) as improving the efficacy of CpGV did not enhance the uptake of virus in these tests. Ten percent molasses and 6% sucrose (the equivalent sugar concentration of 10% cane molasses) were similarly effective at increasing the rate of virus ingestion by larvae (Table 6.6; test 7); the LET_{50} of pure virus being reduced from 33.2 minutes (confidence limits: 20.8-83.2) to 5.67 minutes (4.05-7.29) with molasses and 5.05 (2.32-7.62) minutes with sucrose. The sugar content of molasses could account entirely for its effect on virus ingestion. Similarly, apple flesh homogenates containing 2.5% reducing sugar increased the rate of virus ingestion as did pure sucrose at 2%. By contrast, apple skin homogenates, containing just 0.75% reducing sugar were highly effective whereas pure sucrose at 0.75% had no effect compared to the pure standard. Some other constituent of apple skin was, perhaps, enhancing virus uptake. However, neither organic extracts of apple skin nor synthetically produced α-farnesene - as used in the field trial - reduced the LET_{50} significantly.

Surface sterilising larvae after exposure to sprayed leaves using the cationic surfactant hyamine 1622, did not alter the uptake of CpGV. CpGV was, therefore, unlikely to have been simply sticking to the mouthparts and legs, and ingested later by preening or from contamination of the diet. Similarly, molasses was still highly effective in hyamine-washed larvae and the virus had clearly been ingested during the exposure to sprayed leaf discs.

Impure CpGV homogenate - as used in the 1988 field trial - was at least as effective as pure CpGV. Molasses was still highly effective when added to impure CpGV.

Tables 6.4, 6.5 and 6.6, show considerable variation in the slopes of the exposure-time/mortality response curve. In general, steeper slopes were recorded with additives which caused significant reductions in the LET_{50} values. Although the mode of action of CpGV was unlikely to have

been altered by these formulations, the rate of ingestion has clearly been increased and this is consistent with a steeper response slope.

Larvae consumed more holes on leaf discs sprayed with molasses ($P < 0.05$), though the average hole size was similar in discs with, or without molasses (table 6.8).

Table 6.6 Laboratory analysis of molasses, sorbitol, sugars and apple extracts as "feeding stimulants" or "attractants"

(1)	CpGV only 10^7/ml	Sorbitol 10%	Skimmed milk powder 1%
LET_{50} (mins)	10,1	2,10*	14,52
Fiducial limits	6,3-14,2	0,518-3,55	11,15-8,27
Slope	1,56	1,83	2,94
Slope heterogeneity?	-	-	<0,05
Control mortality = 7,5%			

(2)	CpGV only (10^7/ml)	Apple skin extract	Apple flesh extract
LET_{50} (mins)	277	11,47*	6,83*
Fiducial limits	78,0-10^7	7,29-16,57	2,68-11,28
Slope	0,871	1,43	1,06
Slope heterogeneity?	-	<0,05	<0,05
Control mortality = 3,2%			

/Continued...

Table 6.6 (Continued)

(3)	CpGV only (10^7/ml)	Apple skin extract	Apple flesh extract
LET_{50} (mins)	57.43	21.06*	12.47*
Fiducial limits	34.3-170.7	16.9-26.8	8.82-17.17
Slope	1.20	2.51	1.60
Slope heterogeneity?	-	-	-
Control mortality = 0%			

(4)	CpGV only 10^7/ml	Apple skin extract	Apple flesh extract	Sucrose 2%
LET_{50} (mins)	63.94	16.62*	27.70	14.19*
Fiducial limits	36.3-251.8	12.5-24.3	20.0-43.6	10.7-18.7
Slope	1.10	2.11	1.59	2.00
Slope heterogeneity?	-	-	-	-
Control mortality = 0%				

	CpGV only 10^7/ml (Hyamine wash)	Molasses 10%
LET_{50} (mins)	132.9	9.35*
Fiducial limits	62.1-1317	7.12-11.82
Slope	1.23	2.38
Slope heterogeneity?	-	-

/Continued...

Table 6,6 (Continued)

(5)	CpGV only 10^7/ml	Sucrose 0,75%	Impure CpGV 10^7/ml	Impure CpGV + molasses 10%
LET_{50} (mins)	19,6	44,07	13,6	3,44*
Fiducial limits	13,9-29,4	28,7-96,1	10,2-18,1	1,67-4,89
Slope	1,49	1,29	1,89	2,03
Slope heterogeneity?	-	-	-	-
Control mortality = 0%				

(6)	CpGV only 10^7/ml	Molasses 10%	Farnesene 0,1%
LET_{50} (mins)	103,5	1,067*	40,34
Fiducial limits	51,8-724,4	0,003-3,38	23,5-132,1
Slope	1,12	0,81	1,00
Slope heterogeneity?	-	-	-
Control mortality = 0%			

(7)	CpGV only 10^7/ml	Molasses 10%	Sucrose 6%	LAM 5 129C	LAM 5 132B	LAM 5 132C
LET_{50} (mins)	33,2	5,67*	5,05*	65,3	19,15	24,16
Fiducial limits	20,8-83,2	4,05-7,29	2,32-7,62	35,8-334,6	14,5-25,7	17,3-38,2
Slope	1,10	2,55	1,49	1,11	2,10	1,64
Slope heterogeneity?	-	<0,05	-	-	-	<0,05
Control mortality = 1,1%						

	Farnesene 0,01%	Farnesene 0,1%
LET_{50} (mins)	23,0	20,0
Fiducial limits	16,1-35,6	13,5-32,2
Slope	1,40	1,29
Slope heterogeneity?	-	-

* Treatment significantly different from pure CpGV.

Table 6.7 Effect of molasses on laboratory sprayer output and virus deposition

Droplet density (droplets/cm²)	With attenuator		Without attenuator	
	Mean	S.E.	Mean	S.E.
CpGV (10^7/ml) + 0.02% Agral	244.3	38.9	536.0	102.1
CpGV + Agral + 10% molasses	205.7	39.0	496.8	101.8

Droplet size (µm):	With attenuator						Without attenuator					
	Mean	S.E.	VMD	NMD	Max	Min	Mean	SE	VMD	NMD	Max	Min
CpGV (10^7/ml) + 0.02% Agral	31.0	22.9	76.4	20.2	128.7	3.2	38.3	27.2	96.7	29.1	183.4	3.2
CpGV + Agral + 10% molasses	32.2	25.2	88.3	20.6	147.6	4.7	-	-	-	-	-	-

Virus density (granules/mm²)

(With attenuator):	Mean	Confidence limits
CpGV (10^7/ml) + 0.02% Agral	43.2	7.74-70.4
CpGV + Agral + 10% molasses	61.5	38.2-81.6

Table 6,8 Effect of molasses on consumption of leaf surface

Larvae exposed to leaf discs for 60 minutes.

Treatment	No. Larvae	No. larvae making holes	Mean hole size	SE
No Molasses	37	16	0,098 mm²	0,024
With Molasses	37	26	0,104 mm²	0,014
X^2		P < 0,05		
Student's t			Not sig.	

iv. Field comparison of potential "feeding stimulants" or "attractants" and an impure virus formulation.

In the 1987 spray-formulation trial, CpGV was applied at a low dose of 1 x 10^{12} granules/ha such that unformulated CpGV would give incomplete control as in the 1985 trial. A spray regime of four weekly applications was used since this more frequent treatment strategy gave better overall control by compensating for poor virus foliar persistence (Chapter 4, section III(b)). Differences due to the effect of attractants should still have been apparent and would theoretically be more discernible in the absence of the uncontrolled effect of poor virus persistence in all treatments. Five formulations were tested including an impure homogenate of CpGV.

All apples were collected and examined as in Trial (a)i. The analysis was based on the total fruit, harvest and windfalls, since damage levels in the 1987 trial were too low to warrant further dissection. Both "superficial" (categories I and II, Glen, 1981) and "deep" damage (III and IV) were analysed. The analysis was by analysis of variance of the numbers of damaged apples with a square-root transformation. Analysis of variance on the percentage data produced similar results.

Table 6.9 summarises the data and analysis: damage levels were low but this has been compensated in part by the huge sample sizes involved. The analysis includes the 'block' effect of the IPM programme (see section II(b)v) on two of the four blocks. As expected this programme had no

complicating effects on the control of codling moth damage. Neither the virus treatments nor the IPM programme affected the total fruit yield.

"Superficial" damage was low and no significant treatment effects were observed. There was a major treatment effect on "deep" damage and the contrast of the unsprayed versus CpGV treatments ("control") was highly significant with damage reduction ranging from 44% (pure virus) to 74% (+ molasses). There was a significant "additive" effect demonstrating that the three potential "attractants" (molasses, sorbitol and α-farnesene) gave improved control when compared with pure virus. The "deviation" contrast indicates no significant difference between these additives. The impure homogenate performed at least as well as pure CpGV (63% damage reduction).

Sooty moulds were again abundant on molasses-sprayed foliage but much lower levels were recorded on sorbitol-sprayed plots (Table 6.10).

Table 6.9 A comparison of spray additives and purified and impure formulations of CpGV for control of fruit damage

Treatment	"Superficial" damage (I + II) (Harvest + windfalls) Mean no. per plot	Transformed mean (√)	%	"Deep" damage (III + IV) (Harvest + windfalls) Mean no. per plot	Transformed mean (√)	%	Total no. apples
A. Unsprayed	9,86	3,14	0,291	49,56	7,04	1,571	13480
B. Pure virus	11,63	3,41	0,323	29,05	5,39	0,876	13968
C. Impure homogenate	3,57	2,36	0,147	18,15	4,26	0,578	13160
D. Pure virus + molasses	4,58	2,14	0,116	12,67	3,56	0,416	14020
E. Pure virus + sorbitol	5,20	2,28	0,0799	18,58	4,31	0,484	15228
F. Pure virus + alpha farnesene	4,88	2,21	0,172	11,97	3,46	0,494	8828
Standard error of difference of transformed means (S.E.D.)		0,696			0,685		
IPM blocks	5,10	2,26	0,111	18,58	4,31	0,509	43992
Virus only blocks	8,52	2,92	0,261	25,3	5,03	0,905	34692
S.E.D.		0,053			0,451		

Analysis of variance

Source of variation		F prob.	Sig.	F prob.	Sig.
1. Total fruit yield	Block	0,404			
	Treatment	0,565			
2. Fruit damage			"Superficial"	"Deep"	
	Block		0,073	0,250	
	Treatment		0,488	0,003	**
	Contrasts:				
	a) "Control"		0,286	<0,001	***
	b) "Impure"		0,895	0,881	
	c) "Additive"		0,124	0,016	*
	d) "Deviation"		0,727	0,430	

Key: Block = Effect of "IPM" vs "virus only" blocks.
Treatment = Variance of all treatment effects.

Contrasts: 1. "Control" = A vs B-F 2. "Impure" = C vs B, D, E, F
3. "Additive" = B vs D, E, F 4. "Deviation" = D vs E vs F

Significance levels: * = P<0.05 ** = P<0.01 *** = P<0.001

Table 6.10 Effect of spray formulation on incidence of sooty moulds (*Cladosporium* spp)

	Mean no. colonies/leaf			
	29 July		19 August	
Treatment	Mean	S.E.	Mean	S.E.
Pure CpGV	0	0	0	0
CpGV + sorbitol	2.32	2.63	3.17	1.80
CpGV + molasses	36.3	10.4	27.1	14.6

c) Laboratory and field evaluation of impure and dry formulations of CpGV

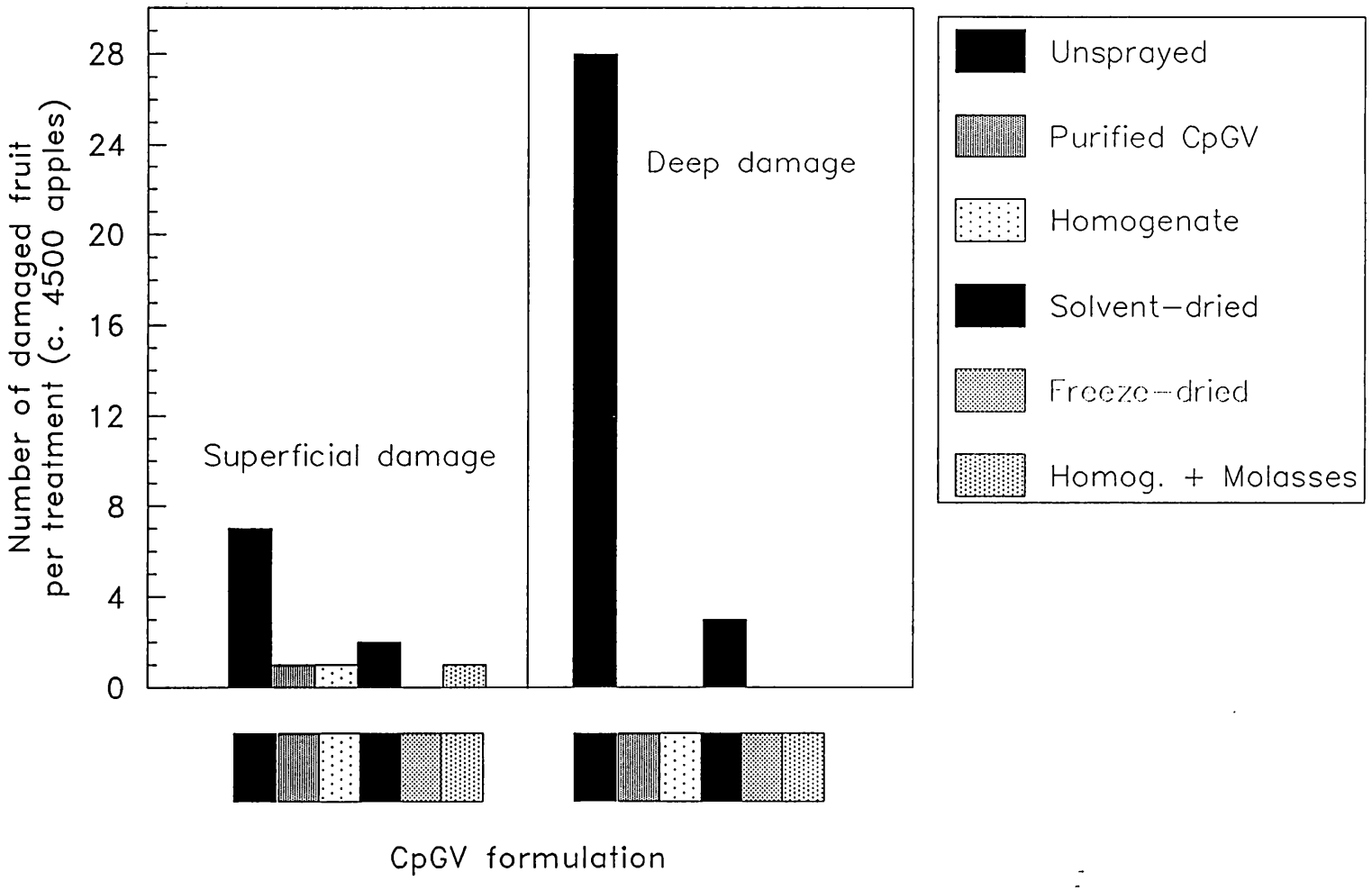
1. Field trial

Despite large numbers of adult males recorded in the pheromone trap - situated only 40 m from the trial plots - only a low level of damage was recorded. Nearly all the damage was confined to the four control plots, with a virtual absence in any of the spray treatments. Figure 6.3 gives the results.

Although, from these results, it is impossible to distinguish any greater or lesser efficacy amongst the treatments, damage has been reduced to zero or a negligible level in each of the CpGV treatments. CpGV has remained active and proved effective in field treatments despite the use of an impure homogenate or prior dry-processing. Laboratory tests (below) give a clearer indication of the suitability of dry and impure formulations for spraying.

Fig. 6.3

Effect of CpGV formulation on control of codling moth fruit damage.



ii. Laboratory bioassay

The rate of uptake of CpGV from sprayed leaf discs was similar in pure, impure and two dry formulations (Table 6.11). Sonication did not significantly increase the response, suggesting that aggregation of virus granules was not a problem in the resuspension of dry powders.

Table 6.11 Pure, impure, and dry formulations: effect on larval ingestion of CpGV

Treatment (CpGV at $5x10^7$/ml)	Pure CpGV	Impure CpGV	Freeze dried	Fre/dry Sonicated	Solvent dried	Sol/dry Sonicated
LET_{50} (mins)	4,99	4,39	5,00	3,54	3,73	1,43
Fiducial limits	2,90-6,85	2,65-5,77	2,99-6,82	0,98-5,74	0,862-6,21	0-3,28
Slope	2,06	2,53	2,06	1,39	1,29	1,34
Slope Heterogeneity?	-	-	-	-	< 0,05	-
Control mortality = 3,8%						

IV. DISCUSSION

CpGV is a highly potent biological insecticide and for a median effective dose young codling moth larvae need only ingest 5 virus granules (Sheppard and Stairs, 1977). The nature of the pest - the habit of larvae boring into fruit - means that they are likely to be exposed to CpGV only for a limited time between hatching and entering the fruit. CpGV can be ingested from both apple and leaf surfaces (Chapter 5, section III(b)). The efficiency of CpGV could be improved, theoretically at least, by improving application (Chapter 4), prolonging the life of active virus on plant surfaces and increasing the amount ingested by young larvae - either by increasing the dose or encouraging its ingestion.

a) Improving the persistence of CpGV

In field trials reported in this chapter, loss of unformulated CpGV was rapid with three-fold losses in just 2-3 days and a 63-fold loss over 23 days. These results are consistent with earlier studies (Jaques and

Laing, 1982; Glen and Payne, 1984; Richards, 1984; Huber, 1988) and, although not tested in these experiments, is likely to reflect virus activation rather than physical loss from leaf surfaces; CpGV is, for example relatively rainfast (Keller, 1973; Charmillot *et al.*, 1984; Richards, 1984; Burgerjon and Sureau, 1986). In an attempt to improve persistence, a number of potential 'protectants' were added, including skimmed milk powder, none of which caused any major improvement in persistence.

Ultraviolet light, between 280 and 320 nm wavelength is the main cause of baculovirus inactivation (Shapiro *et al.*, 1983) and agents have been developed to give protection in this region such as 'Shade' a polyflavonoid (Ignoffo *et al.*, 1972) which gave protection to laboratory irradiated CpGV but, despite increasing field persistence slightly, had no effect on control of codling moth (Richards, 1984). In laboratory tests, claimed efficiencies for various additives were at best three fold (Shapiro *et al.*, 1983) and these results were based on false assumptions where virus activity was analysed by simply comparing the % mortalities of test larvae fed on irradiated and non irradiated virus (Ignoffo and Batzer, 1971). This method is invalid since the relationship between dose and percentage mortality is not linear (Richards and Payne, 1982).

Jones and McKinley (1986), using *Spodoptera littoralis* nuclear polyhedrosis virus (NPV), found that all tested additives gave some protection and that there was no advantage in using specialised protectants rather than readily available materials such as clay minerals and molasses. In a study on cotton foliage, Jones and McKinley (1987) found the greatest protection against UV was the foliage itself with over three fold loss of *S. littoralis* NPV in 4 days from upper leaf surfaces but a negligible loss from lower surfaces.

From the results reported here and from other workers, there seems to have been little progress in achieving dramatic improvements in field control with CpGV using UV protectants. However, by frequent regular application of low doses of CpGV, the persistence problem can be overcome (Chapter 4, section III(b)).

CpGV irradiated with artificial UV light follows a linear deactivation curve (Richards, 1984) whereas the field deactivation rate falls off with time producing a curved response (Richards, 1984; Tatchell and Payne, 1986,

Richards and Payne, 1982). A similar response was observed in this study (Fig. 6.1) and is most likely to result from the shading of a proportion of virus. The possibility of a proportion of the virus being inherently more tolerant (Brassel and Benz, 1977) would not explain the linear deactivation curve produced in the laboratory (Richards, 1984).

b) **Improving the efficacy of CpGV with the addition of molasses**

Using pure CpGV formulated with 0.2% skimmed milk powder, codling moth deep entry damage in fruit was reduced by 79% compared to unsprayed trees at a dose of 1 x 10^{13} granules/ha, with no further improvement at 1 x 10^{14} granules/ha. The addition of 15% cane molasses to CpGV at only 1 x 10^{12} granules/ha gave 81% control, significantly better than the equivalent dose with no molasses ($P < 0.01$), and at least as good as the skimmed milk formulation at a ten times higher virus dose. Molasses did not increase the initial deposition or the foliar persistence of CpGV.

Despite the significant savings on CpGV dose, molasses caused a potentially adverse side-effect in encouraging the growth of sooty-moulds (*Cladosporium* spp.) on the leaf surfaces. Although the sooty moulds did not promote leaf senescence their presence was considered undesirable as they would not favour the use of CpGV by growers.

c) **Development of larval "feeding stimulants" or attractants"**

In order to investigate the 'formulation effect' a laboratory bioassay system was developed that would reflect the 'field' situation; i.e. where larvae ingest CpGV from sprayed foliage. The system allowed larvae only limited time to pick up virus before being transferred to virus-free diet, rather than being fed continuously on infected food. Increased mortality was recorded with increased exposure to sprayed leaves with no threshold minimum time required for ingestion. The time-mortality relationship could be fitted to a straight line by maximum likelihood probit (MLP) analysis. Molasses, sorbitol, and a number of sugars and sugar-containing substances dramatically increased the rate of CpGV uptake. Artificial sweeteners had no effect. The sugar content of apple flesh and apple juice was probably

responsible for the increased rate of virus uptake when formulated with CpGV.

In 1987, molasses was retested in the field along with sorbitol and α-farnesene (a naturally occurring component of apple skin), at a low virus dose of 10^{12} granules/ha. Had higher doses been applied, it may have been difficult to discern the effects of additives. The formulation additives produced a significant reduction in deep damage ($P = 0.016$) from 44% with unformulated virus, to 68% with α-farnesene, 69% with sorbitol and 74% with molasses.

In laboratory leaf bioassays, apple skin homogenate increased virus ingestion despite having a sugar content below that at which sucrose itself increased virus uptake. However, the apple skin derivative, α-farnesene, had no effect in spite of its success in field trials. At the tested concentrations, organic extracts of apple skin were also unsuccessful.

In the experiments reported here, 1% skimmed milk failed to improve virus uptake. This contrasts with Huber (1982), who suggested that 1% skimmed milk may act as a feeding attractant for codling moth. Skimmed milk was also thought to have acted as a feeding stimulant for tortrix larvae; thus accounting for higher tortrix moth damage to fruit after CpGV formulated with skimmed milk was applied (Payne *et al.*, 1983).

CpGV formulated with molasses and sorbitol improved the efficiency of CpGV in both laboratory and field tests. Fructose and sucrose were also successful in laboratory assays. Since molasses had no effect on virus persistence in field trials, or on the laboratory sprayer output and deposition on leaf discs, it seems likely to be acting as a "feeding stimulant" and/or "attractant" (these terms have been defined by Dethier et al, 1960: a "feeding stimulant" is a chemical which elicits feeding, whereas an "attractant" is one which causes insects to make orientated movements towards its source). It seems from earlier work (Chapter 5, section III) that larvae can ingest CpGV from leaf tissue without active feeding. Molasses, sugars and sorbitol probably caused larvae to spend more time, and even feed superficially, on spray deposits as they searched for fruit. Artificial sweeteners, similar to sugar to the human taste, may have no effect on larval taste receptors.

Molasses did cause a significant increase in the number of larvae excavating the surface of sprayed leaf discs in laboratory tests, although the amount of leaf surface consumed by each *feeding* larvae was similar. This result supports the hypothesis that molasses was acting as a feeding stimulant, though the main effect on virus uptake - as explained above - probably occurred without the need for active consumption of leaf tissue.

Codling moth larvae are known to be attracted to apple skin odour (Sutherland, 1972; Subinprasert, 1988) and this attraction is effective up to 1.5 cm from the source (Sutherland, 1972). Organic extracts of apple and the (Z,E)- and (E,E)-isomers of α-farnesene (naturally occurring components of apple skin: Sutherland *et al.*, 1977) - act as larval olfactory attractants (Sutherland, 1972; Sutherland and Hutchins, 1973, Wearing and Hutchins, 1973). Since α-farnesene is volatile, and has an olfactory effect, its failure to increase the rate of CpGV uptake in the laboratory bioassay may have been expected where the leaf discs were evenly sprayed. By contrast, the distribution of spray on foliage in the field would have been patchy: an analysis of the spray deposition and penetration from the mistblower used in these field trials showed variable spray coverage of leaves, with an average 52% coverage of upper leaf surfaces and 37% of lower surfaces (Chapter 4, section III(e)). In the field, an olfactory attractant effect may have encouraged larvae to move to sprayed areas of leaves, or perhaps increased the amount of time larvae spent outside fruit; either by direct attraction to α-farnesene on foliage, or by confusion due to the presence of non-fruit attractant odour disrupting the natural gradients towards fruit. In either case, the more time spent on foliage, the greater the chance of ingesting a lethal dose of virus (Chapter 5, section III(a)). Increased field control could thus, perhaps, be achieved in two ways: (1) increased feeding by a feeding-stimulant effect of sugars and (2) increased exposure to sprayed leaves by an attractant or confusion effect of volatile additives. Molasses, may even have a dual effect, as attractant and feeding stimulant. Volatile compounds present in molasses attract adult moths, a feature used in moth traps (eg. Chapter 2, section IX(f)).

This is not the first time that attractants have been suggested for use with insect viruses. Potter and Watson (1983) found that extracts of garbanzo beans increased the mortality of tobacco budworm larvae with *Heliothis virescens* NPV in laboratory tests. Formulating *Autographa*

californica NPV (AcNPV) with cottonseed flour, cottonseed oil, and sucrose increased the mortality of *H. virescens* neonate larvae fed on sprayed plants (Bell and Kanavel (1978). Larvae of the pink bollworm, *Pectinophora gossypiella*, were also more responsive to AcNPV formulated with cotton extracts and sucrose: pink bollworm larvae also feed very little on the plant surface and, like codling moth, its control by virus is also limited by the limited period for virus ingestion (Bell and Kanavel, 1975). A cottonseed extract was shown by Smith *et al.* (1980) to improve the efficacy of *H. zea* NPV. However, their laboratory tests were inadequately controlled since the formulation also dramatically increased the size of spray droplets which could have affected virus uptake.

In testing for UV protectants Shapiro *et al.* (1983) may have missed the phenomenon of feeding stimulant or attractants. Tests performed by Shapiro and co-workers were inadequately controlled: in their studies, NPV suspensions with or without additives were applied to leaf or diet surfaces and then irradiated. The only controls were non-irradiated pure virus; with no test for the non-irradiated formulated virus. Larvae were fed on treated leaves or diet for 48 hours prior to transferring to clean diet. Molasses, sucrose and Coax (a cottonseed extract designed for the formulation of microbial insecticides for tobacco budworm) were said to improve resistance to UV irradiation, and yet their results could have been entirely due to a feeding stimulant effect. In more recent studies, workers have used a method of washing virus from treated surfaces and adding the washings to diet (Richards, 1984; Martignoni and Iwai, 1985), thereby diluting-out any attractants.

d) **Efficacy of impure preparations of CpGV**

In both laboratory and field tests, an impure homogenate of CpGV - prepared from infected insects and diet without purification - proved to be at least as effective as purified CpGV. A greater level of control was achieved in the field with 63% control for impure CpGV compared to 44% for purified CpGV (both at the low dose of 10^{12} granules/ha). Protein impurities in impure virus preparation have been reported to improve laboratory and field persistence (David, 1969; Jaques, 1971; Elnagar and Abutivasr, 1980; Jones and McKinley, 1986, Huber, 1988) and this may have contributed to improved field control.

In a separate field trial, pure, impure and two dry CpGV formulations were tested and, although control was excellent, the low level of pest damage prevented any comparison between treatments. A laboratory study of the same materials showed the dry and impure formulations to be equally effective as pure CpGV and all treatments were ingested at similar rates. Aggregation of CpGV in the dry powders did not appear to be a problem.

V. CONCLUSIONS

The assay system developed for this study has proved ideally suited for testing formulations in the laboratory and provides a screening technique prior to field testing. Although the attractant properties of α-farnesene would need further confirmation it is clear from the other tested additives that dramatic improvements in field control and virus-uptake in the laboratory can be achieved using formulation additives with CpGV. These additives appear to act as feeding stimulants. As yet, for 'stimulant' activity in laboratory bioassays, a minimum concentration of 2% (sucrose) was required to enhance virus uptake. This could prove too expensive or impractical unless low volume sprays were used in the field. However, both the principal of feeding stimulants and a method of screening have been firmly established and warrant further study: synthetic compounds or components present in apple skin may have the enhanced activity at low concentration required for their practical use. The method of assay could also be adapted for other insect-pathogens where the pathogen has to be ingested for it to take effect.

Cost of production is a limiting step in the commercialisation of insect pathogens; reducing the doses required by using feeding stimulants or attractants may provide an economic answer.

Chapter 7

Use of Cydia pomonella granulosis virus in an integrated pest management scheme for apple orchards

I. INTRODUCTION

Codling moth can be controlled using conventional broad spectrum insecticides but their adverse effects on predatory insects and mites often result in secondary pest problems such as outbreaks of the fruit-tree red spider mite, *Panonychus ulmi* - resistant to most broad spectrum insecticides (Way, 1978) - which require the additional use of specific acaricides for their control. By contrast, CpGV is highly specific and known to infect only a few closely related tortricid species (Falcon *et al.*, 1968; Huber, 1978, 1982; Payne, 1981) and its selectivity has already been shown to be advantageous by allowing the natural control of pest insects and mites by predators (Dickler and Huber, 1984; Glen and Phillips, 1984, Glen *et al.*, 1984).

Although its selectivity should result in the natural suppression of secondary pest problems such as *P. ulmi*, CpGV is consequently unable to control non-host pest species such as orchard leafrollers (Tortricidae) (Glen *et al.*, 1985) which would otherwise be killed by broad-spectrum insecticides. During a three year research study in South East England, the full impact of CpGV on target and non-target arthropods was tested and its suitability for use in an orchard IPM programme evaluated. Initially CpGV was included in a straightforward comparison with a broad spectrum insecticide deltamethrin. In the two following years other selective control agents were incorporated in CpGV trials in an attempt to control the full complement of orchard pests without disrupting predator populations preserved through application of CpGV. Throughout the trials, attention was paid to minor pest species which, in the absence of broad spectrum control, could perhaps become important.

A comparatively new compound, fenoxycarb (Insegar, Dr R. Maag Ltd) was tested in 1986 against leafrollers. Fenoxycarb has juvenile-hormone

activity interfering with metamorphosis, preventing pupation in leafrollers (De Reede *et al.*, 1984). Owing to its mode of action, spray timing is critical since it is most effective against final instar larvae. Many leafroller species overwinter as larvae and many, such as *Adoxophyes orana*, feed after overwintering, and before pupation. Fenoxycarb is thus applied against these spring larvae rather than against the summer, fruit-damaging, generation (de Reede and de Wilde, 1986). Fenoxycarb has not previously been tested against leafrollers in the UK - although trials have been carried out in Holland (de Reede *et al.*, 1984, 1985a) - and the impact on the UK leafroller species complex and non-target arthropods was of considerable interest in determining the potential of fenoxycarb as a compatible agent for a CpGV-based IPM programme.

II. MATERIALS AND METHODS

Field trials were carried out at the Institute of Horticultural Research, East Malling, and at Eden Farm, West Malling. Trials were designed to evaluate the efficacies of various selective and broad-spectrum insecticides - including CpGV - in terms of their ability to reduce fruit damage and their direct and, ecological, effects on non-target arthropods. In each trial, fruit damage by codling moth and other species was assessed and classified according to the system detailed in Chapter 2 (section VIII(f)). A full sampling programme, to determine the relative densities of a range of pest and beneficial species recorded after spray treatment, was followed in each trial (Chapter 2, section IX). Statistical analysis was by analysis of variance on square-root transformed data. Details of plot layout, spray application and spray timing are included in Chapter 2, section VIII(a, d & e).

a) The impact of CpGV and deltamethrin on non target arthropods

PLot WF103, East Malling, 1985 (Fig. 2.1)

In Chapter 4, the ability of CpGV to reduce codling moth damage to fruit was compared with deltamethrin. During this trial the effects of both CpGV and deltamethrin on non target arthropods was also investigated and the results are reported here. The following treatments were applied in a replicated, randomized block field design (see Chapter 4, section II(a)i for full details).

1. Unsprayed control

2. Purified CPGV at 10^9 granules/litre (10^{12}/ha) + 0.2% (w/v) skimmed milk powder (w/v) + 0.02% Agral (ICI, UK)

3. Purified CPGV at 10^{10} granules/litre (10^{13}/ha) + 0.2% (w/v) skimmed milk powder (w/v) + 0.02% Agral

4. Purified CPGV at 10^{11} granules/litre (10^{14}/ha) + 0.2% (w/v) skimmed milk powder (w/v) + 0.02% Agral

5. Purified CPGV at 10^9 granules/litre (10^{12}/ha) + 15% (v/v) molasses + 0.02% Agral

6. Deltamethrin, 250 ml/ha

Materials were sprayed twice: on 27th June and 17 July to correspond with expected codling moth egg hatch. The abundance of mite species was measured for all six treatments, whereas for other arthropods, the two low concentration CpGV treatments were not included; any effects due to virus application would be most likely at high CpGV dose.

b) CpGV and fenoxycarb: control of fruit damage and impact on orchard fauna

Eden Farm, West Malling and WF103, East Malling, 1986 (Fig. 2.5).

The insect growth regulator fenoxycarb (Insegar) has been tested as a selective insecticide against leafroller species in Holland (de Reede *et al.*, 1984; 1985a). Fenoxycarb was tested on two sites, one with, the other without dual application of CpGV. Its efficacy against target leafrollers was assessed, as was its effect on other species, particularly predatory insects and mites. Its effect on codling moth damage was also measured since, to be compatible with a CpGV-based IPM programme, it should not obviate the need to apply CpGV.

A one hectare orchard of Tydeman's Early Worcester, at Eden Farm, had not been chemically-sprayed for at least three years and had been recently

pruned from a derelict state. Eight plots were completely randomized between two treatments. It was necessary to pay special attention to the location of plots and incorporate a large area of additional spray cover to overcome the problem of unwanted immigration of adult leafrollers into trial plots (see Chapter 2, section VIII(a)iv and Fig. 2.5 for details).

Treatments:

1. Fenoxycarb (Insegar, Dr R. Maag Ltd), 600 g/ha
2. Unsprayed control

The timing of fenoxycarb application followed the manufacturer's recommendation: the first application at petal fall, to coincide with the last instars of *Adoxophyes orana* and *Archips podana*, and again 14 days later (Dr R Maag Ltd, Technical Information). Sprays were thus applied on 29th May and 9th June.

Fenoxycarb was additionally tested in an unreplicated trial at plot WF103, East Malling using two 0.5 ha plots from a one hectare orchard of Laxton's Fortune and Cox. Treatments were applied at 250 l/ha on 29th May and 9th June.

1. Fenoxycarb, 600 g/ha
2. Control

The two plots were part of a replicated trial testing different spray frequencies of CpGV (Chapter 4, section II(a)ii) where the experiment was "blocked" for fenoxycarb application. Although unreplicated, the trial provided additional information on the dual use of CpGV and fenoxycarb.

c) Control of fruit damage, and ecological impact of CpGV-based IPM programme

WF103, East Malling, 1987

A complete IPM programme was applied to an experimental orchard. Limitations on orchard space restricted the design of this trial; an assessment of selective agents was incorporated with a trial of CpGV

formulation (Chapter 6, section II(b)v). Treatments were chosen to complement CpGV and cover the range of expected orchard pests. The chitin synthesis inhibitor, diflubenzuron, was chosen as a pre-blossom spray aimed primarily against early season caterpillars including winter moth, *Operophtera brumata* and the clouded drab moth, *Orthosia incerta*. Summer application of diflubenzuron against leafrollers was avoided since it is also effective against codling moth (Hoying and Reidl, 1980). Pirimicarb was applied in the spring for the control of aphids. Whilst pirimicarb and diflubenzuron are standard pre-blossom orchard insecticides, the microbial agent *Bacillus thuringiensis* (B.t) var. kurstaki is comparatively poorly tested in apple orchards. B.t was included in an attempt to control leafrollers, following success in laboratory bioassays (Huber and Undorf, 1985, Undorf and Huber 1986). Fungicides used on the experimental orchard were chosen for their low toxicity to predators. CpGV was applied at low dose (10^{12} granules/ha) in different formulations as part of a replicated trial (Chapter 6, section II(b)v). A 1 ha orchard of Laxton's Fortune and Cox was divided into twenty-four small plots for the CpGV formulation experiment and four 0.25 ha blocks for the IPM trial (blocking within the CpGV trial, Fig. 2.3). Two spray programmes were evaluated for fruit damage control and impact on orchard fauna:

A. IPM Scheme

Pre-blossom diflubenzuron (Dimilin W.P, 25% a.i.) at 600 g/ha
Pre-blossom pirimicarb (Aphox) at 420 g/ha
Summer application of *Bacillus thuringiensis* (B.t) var kurstaki (Dipel 2X) at 600 g/ha

CpGV (10^{12} granules/ha) (+ some non-virus plots)

Fungicide programme:

Dithianon (Delancol, ICI) at 2.1 l/ha - April 16
Dithianon at 2.1 l/ha - April 24
Thiophanate-methyl (Mildothane, M&B) at 2.2 l/ha - May 8
Dithianon at 2.1 l/ha - May 20
Bupirimate (Nimrod, ICI) at 1.1 l/ha - May 20
Dithianon at 1.4 l/ha - June 15
Nitrothal-isopropyl (Pallitop, BASF) at 0.9 kg/ha - June 15
Nitrothal-isopropyl at 0.9 kg/ha - June 23
Nitrothal-isopropyl at 0.9 kg/ha - July 9

B. Control

Fungicide programme (as above)

CpGV (10^{12} granule/ha) [+ non-virus plots]

Treatments were applied by mistblower at 250 l/ha. Pre-blossom sprays were timed according to the orchard growth stages and sprayed on April 24th.

CpGV and B.t. - timed to control young codling moth and leafroller larvae - were sprayed on July 1, 10, 16 and 24. Spray timing was governed by pheromone trap records (see Chapter 2, section VIII(e)).

III. RESULTS

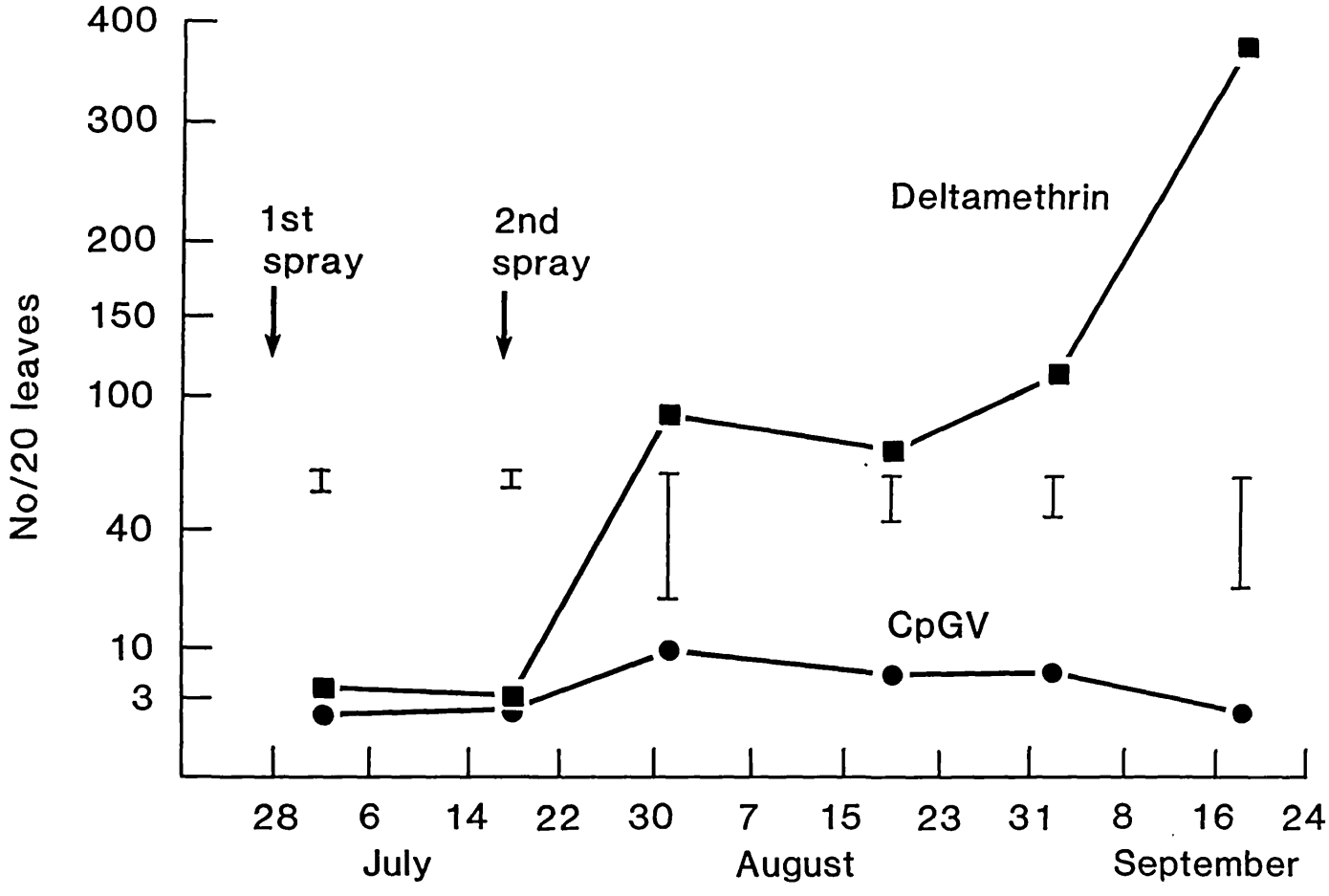
a) Effect of CpGV and deltamethrin on orchard arthropod fauna

CpGV and deltamethrin differed markedly in their effects on non-target species. Pest and predatory mite numbers were monitored from July until September and their densities are expressed in Figures 7.1-7.5 (for clarity, only the high concentration CpGV treatment has been included in these figures since no virus dose effect was observed). Table 7.1 gives the analysis of variance and orthogonal comparisons between unsprayed and CpGV plots; deltamethrin against all plots; and the effect of virus formulation. Compared to unsprayed plots, CpGV had no effect on mites ($P > 0.05$) and the balance between the predatory phytoseiid mite *Typhlodromus pyri* and pest fruit-tree red spider mite *Panonychus ulmi* and rust mites Eriophyidae (mainly *Aculus schlechtendali*) was maintained throughout the season with pest mite numbers remaining low. However, in deltamethrin treated plots, predatory mite numbers were very low and this was accompanied by a rise in both *P. ulmi* (egg and mites) and Eriophyidae, reaching massive numbers by the end of the season. There were highly significant differences ($P < 0.01$) between deltamethrin and non-chemical plots for all mites on most sampling dates. Formulating CpGV with molasses caused no impact on *P. ulmi*, *T. pyri*, or Eriophyidae. There was a curious effect of molasses in the first month after spraying on the population of the phytophagous (though non-pest) Tydeid mites which were significantly reduced in numbers. Significant effects were observed for *P. ulmi* eggs between CpGV and unsprayed plots, though the differences were comparatively small.

Table 7.2 gives partial correlation coefficients between the various mites. Numbers of *T. pyri* and *P. ulmi* were highly correlated (coef. = 0.899) as were *T. pyri* and Tydeidae. *T. pyri* feeds on both *P. ulmi and Tydeid mites (Solomon, 1987)*, but there was also an unexpected relationship between Tydeidae and *P. ulmi* eggs. There is insufficient evidence to favour any direct interaction between the species in question and, furthermore, the existence of a correlation does not necessarily imply a causal link; a third factor may have acted on both populations.

Fig. 7.1 **Numbers of *Panonychus ulmi* after application of CpGV or deltamethrin**

Mean number of mites recorded from four 20-leaf replicates (√x+1 scale). Bars represent standard errors of the difference of √x+1 transformed means.



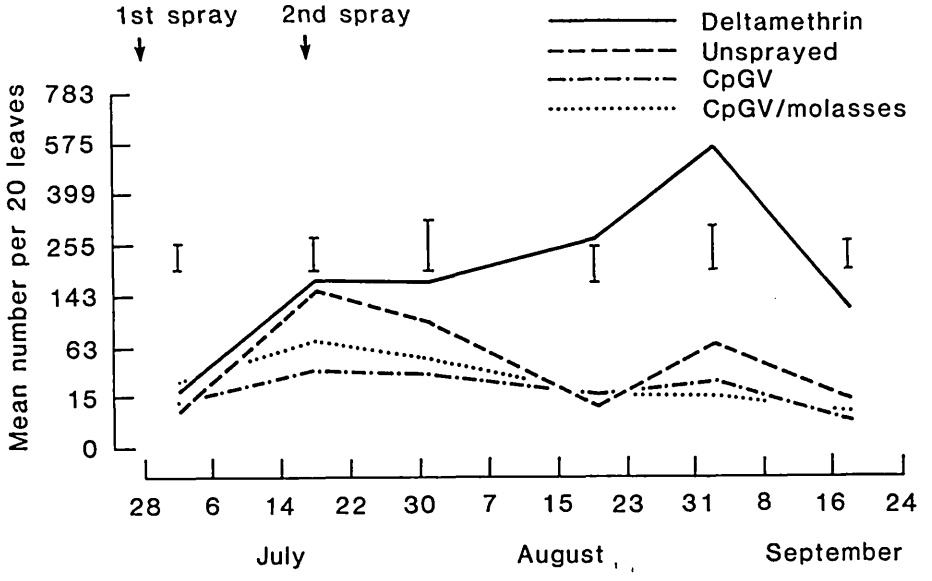


Fig. 7.2 **Numbers of *Panonychus ulmi* eggs after application of CpGV or deltamethrin**

Mean number of mites recorded from four 20-leaf replicates (√x+1 scale). Bars represent standard errors of the difference of √x+1 transformed means.

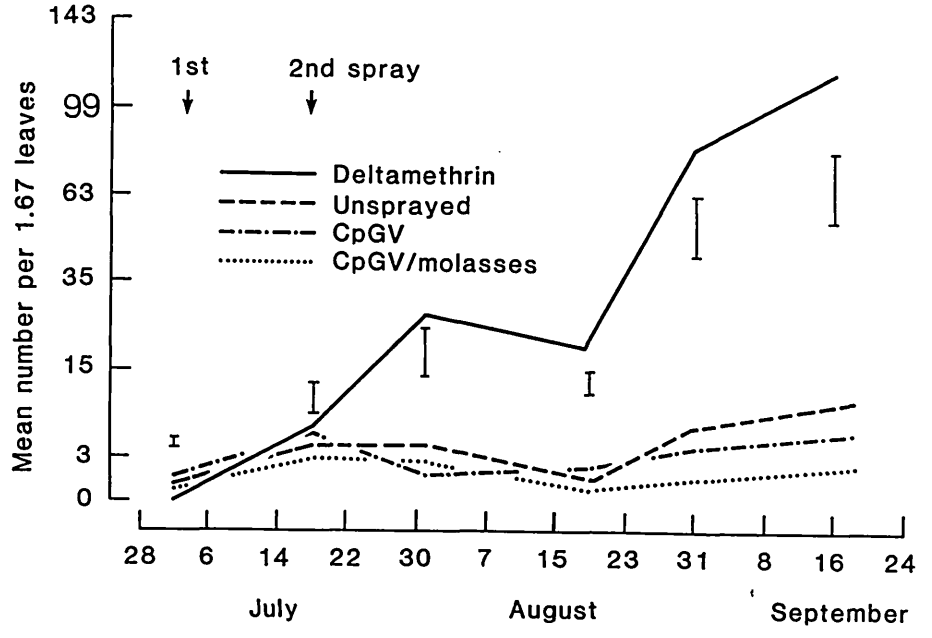


Fig. 7.3 **Numbers of rust mites (Eriophyidae) after application of CpGV or deltamethrin**

Mean number of mites recorded from four 20-leaf replicates (√x+1 scale). Bars represent standard errors of the difference of √x+1 transformed means.

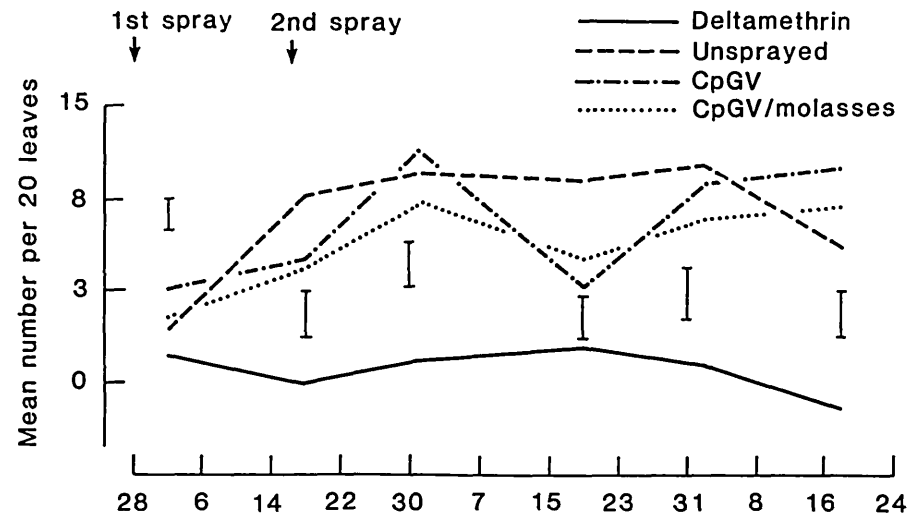


Fig. 7.4 **Numbers of *Typhlodromus pyri* after application of CpGV or deltamethrin**

Mean number of mites recorded from four 20-leaf replicates (√x+1 scale). Bars represent standard errors of the difference of √x+1 transformed means.

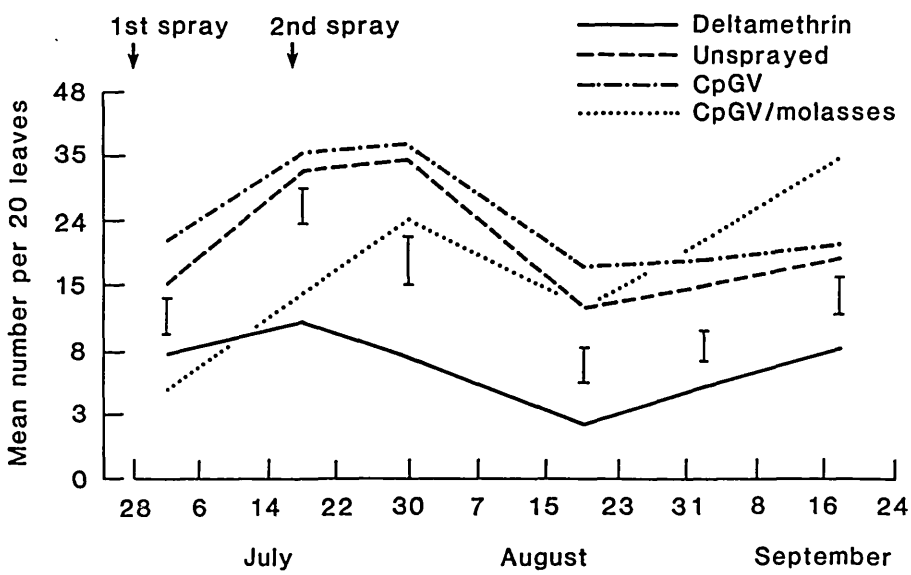


Fig. 7.5 **Numbers of Tydeidae after application of CpGV or deltamethrin**

Mean number of mites recorded from four 20-leaf replicates (√x+1 scale). Bars represent standard errors of the difference of √x+1 transformed means.

Table 7.1 Effect of CpGV and deltamethrin on mites: summary analysis of variance

Analysis	2 July	18 July	31 July	19 Aug	2 Sept	18 Sept
a, *P. ulmi*						
Treatment	-	-	-	***	***	***
Orthogonal comparisons*:						
Unsprayed vs CpGV	-	*	-	-	-	-
Deltamethrin vs non chemical	-	-	-	***	***	***
CpGV/skim milk vs CpGV/molasses	-	-	-	-	-	-
b, *P. ulmi* eggs						
Treatment	-	*	-	***	***	**
Orthogonal comparisons:						
Unsprayed vs CpGV	-	**	*	-	*	-
Deltamethrin vs non chemical	-	**	-	***	***	***
CpGV/skim milk vs CpGV/molasses	-	-	-	-	-	-
c, *T. pyri*						
Treatment	-	**	***	**	**	**
Orthogonal comparisons:						
Unsprayed vs CpGV	-	-	-	-	-	-
Deltamethrin vs non chemical	*	***	**	***	***	***
CpGV/skim milk vs CpGV/molasses	-	-	-	-	-	-
d, *Tydeidae*						
Treatment	**	***	***	**	**	***
Orthogonal comparisons:						
Unsprayed vs CpGV	-	-	-	-	-	-
Deltamethrin vs non chemical	*	***	***	***	***	**
CpGV/skim milk vs CpGV/molasses	***	**	**	-	-	-
e, *Eriophyidae*						
Treatment	-	-	*	***	***	***
Orthogonal comparisons:						
Unsprayed vs CpGV	-	-	-	-	-	-
Deltamethrin vs non chemical	-	-	*	***	***	***
CpGV/skim milk vs CpGV/molasses	-	-	-	-	-	-

Significance: * = $P < 0,05$
** = $P < 0,01$
*** = $P < 0,001$

* See Chapter 4 (section III(a) for details of orthogonal comparisons.

Table 7.2 Mite numbers: Partial correlation coefficients

Species/family	*P. ulmi*	*P. ulmi* eggs	*T. pyri*	Tydeidae
P. ulmi eggs	0,0548	-	-	-
T. pyri	0,899***	0,100	-	-
Tydeidae	0,257**	0,872***	0,513***	-
Eriophyidae	0,0837	0,207*	0,0316	0,134

Significance levels; * = P < 0,05 ** = P < 0,01 *** = P < 0,001

Neither CpGV nor deltamethrin adversely affected the earwig *F. auricularia*; numbers remained high and comparable to unsprayed plots (P > 0.05) (Table 7.3). The numbers of woolly aphid colonies *E. lanigerum* observed on two sampling dates were also similar for all treatments (P > 0.05) (Table 7.4).

Table 7.3 Earwigs: numbers per plot

	12 July		25 July		19 August	
Treatment	Mean	√ Mean	Mean	√ Mean	Mean	√ Mean
Unsprayed	7,67	2,77	28,6	5,35	40,2	6,34
CpGV	7,51	2,74	30,6	5,53	52,1	7,22
Deltamethrin	12,53	3,54	58,5	7,65	46,6	6,83
S.E.D.	-	0,915	-	1,242		0,853
F prob.*	-	0,915	-	0,232		0,229

* F prob. = probability of treatment effect in Analysis of variance (F test).

Table 7.4 Woolly aphids: colonies per plot

	24 July		8 August	
Treatment	Mean	√ Mean	Mean	√ Mean
Unsprayed	29,38	5,42	14,52	3,81
CpGV	24,21	4,92	8,00	2,83
Deltamethrin	19,45	4,41	17,06	4,13
S.E.D.	-	0,850	-	0,872
F prob		>0,05		>0,05

The effect of deltamethrin and CpGV on other arthropods was monitored by vacuum samples taken from July to September. Table 7.5 shows the mean numbers recorded per plot and analysis of variance for the total numbers recorded in the season. Where there was a significant overall treatment effect, orthogonal contrasts for unsprayed vs CpGV, and deltamethrin vs non-chemical have been shown in the table.

Deltamethrin application resulted in significantly lower numbers of the phytophagous apple sucker *Psylla mali*, cicadellid species (leaf hoppers) and spiders. The predatory mirid bug *Blepharidopterus angulatus*, the black kneed capsid, was apparently unaffected, though any deleterious effects of deltamethrin may have been masked by immigration of alate adults. *Chrysopa* adults (lacewings) were also unaffected, but numbers were low and, once again, migration between plots may have masked any major effects. Spraying with CpGV did cause a significant reduction in leaf hoppers and apple sucker, but this is probably attributable to the physical action of spraying on these volatile bugs.

A number of other predatory species were recorded but the numbers were too low to warrant analysis. The predatory bugs *Himacerus apterus* (Nabidae) and *Dereocorus lutescens* (Miridae) were recorded in all treatments, whereas *Pilophorus perplexus* (Miridae) and *Phytocoris tiliae* (Miridae) were absent in deltamethrin plots

Table 7.5 Effect of CpGV and deltamethrin on non-target arthropods: vacuum sampling

Treatment	Mean numbers per plot					
	Psylla mali	*Psylla pyricola*	Cicadellidae	*Blepharidopterus angulatus*	*Chrysopa* spp. (adults	Spiders
Unsprayed:						
Mean	311,90	33,76	14,29	6,66	3,65	7,02
√ Mean	17,66	5,81	3,78	2,58	1,91	2,65
CpGV:						
Mean	200,50	9,12	6,60	4,93	4,24	3,72
√ Mean	14,16*	3,02	2,57*	2,22	2,06	1,93
Deltamethrin:						
Mean	33,18	5,66	1,54	4,93	3,20	0,36
√ Mean	5,76***	2,38	1,24**	2,22	1,79	0,60***
S.E.D √ Mean	1,95	1,30	0,63	0,608	0,30	0,40

Significance levels: * = P < 0,05 ** = P < 0,01 *** = P < 0,001
S.E.D. = standard error of the difference of means

b) Use of fenoxycarb

Fenoxycarb was tested at two sites, one fully replicated (Eden Farm) and the other with no replication but with large plots and where dual application of CpGV was tested (East Malling, PLot WF103). Fruit damage due to leafrollers was low in both trials but this was partly offset by the very large numbers of apples on which the results were based (total harvest and windfalls). Codling damage was much higher at WF103 than Eden Farm. The results are given in Table 7.6. Fruit yield was unaffected by fenoxycarb applied at 'petal fall' and 'fruit set'. Although codling moth damage was smaller in fenoxycarb plots, in both trials, the differences were not significant and fenoxycarb, applied at 'petal-fall' was clearly not a suitable control agent for codling moth.

At Eden Farm, 'superficial' tortrix damage was only slightly, and non-significantly, reduced by fenoxycarb, though the incidence of damage was very low. Superficial damage was attributed to the summer generation

of leafroller larvae such as *Adoxophyes orana* and *Archips podana*. Incidence of the damage due to the fruitlet mining tortrix, *Pammene rhediella* was much higher and unaffected by fenoxycarb. In contrast to *A. orana* and *A. podana*, *P. rhediella*, - like codling moth - does not resume larval feeding activity in the spring (Anon., 1984) and consequently has no opportunity of ingesting fenoxycarb prior to pupation. Fenoxycarb had no effect against *Blastobasis decolorella* or the mussel scale, *Lepidosaphes ulmi*.

Table 7.6 Effect of fenoxycarb on fruit damage

A, Eden Farm (windfalls and harvest)

Treatment	Mean fruit yield no./plot	Fruitlet mining % damage	'Superficial' tortrix % damage	Codling (deep entry) %	Mussel scale % damage
Unsprayed	731,7	1,474	0,694	1,978	2,25
Fenoxycarb	786,2	1,107	0,438	0,648	2,706
F prob,	0,805	0,916	0,366	0,101	0,993

B, Rocks Farm (windfalls and harvest)

Treatment	Fruitlet mining %	'Superficial' tortrix %	Codling moth (deep entry) %		*Blastobasis decolorella* %	Total apples
			No CpGV	+CpGV		
Unsprayed	2,05	0,161	5,36	0,846	0,628	22373
Fenoxycarb	1,22	0,172	3,41	0,350	0,451	34412

Mite numbers were sampled on two dates, in July and August, and in both, the numbers of predatory mite, *T. pyri*, were high for the unpsrayed and fenoxycarb plots. *P. ulmi* were kept at very low numbers (less than 0.5 per leaf) in both treatments though rust mite numbers were fairly high

in both treated and unsprayed plots. No significant differences were observed for any of the mites recorded (Table 7.7).

Table 7.7 Effect of fenoxycarb on orchard mites

	Mean no. mites per 20 leaves (S.E in parentheses)			
	July		August	
	Control	Fenoxycarb	Control	Fenoxycarb
Panonychus ulmi	1.25 (0.47)	2.75 (1.37)	6.00 (1.87)	8.75 (1.37)
P. ulmi eggs	1.50 (0.65)	3.25 (2.62)	1.75 (1.43)	2.25 (0.48)
Typhlodromus pyri	8.00 (2.02)	7.50 (1.85)	16.0 (2.41)	20.7 (4.13)
Tydeidae	5.50 (3.07)	10.2 (6.4)	10.7 (4.77)	14.5 (8.3)
Eriophyidae	1308 (255)	1155 (95)	4917 (1370)	5178 (1596)
Calvolia transversotriatus	4.50 (2.87)	1.50 (0.64)	26.5 (13.2)	12.5 (2.73)

Although a variety of species were recorded by tree-beating and leaf-cluster analysis, the total number from any one species was generally low preventing meaningful statistical analysis. However, the totals recorded have been given in Table 7.8.

Table 7.8 Effect of fenoxycarb on orchard arthropods

A, Tree-beating; total numbers recorded from four sampling points per treatment and three sampling dates; July/August 1986

Treatment	B.a. adult	B.a. nymph	M.c.	P.t.	Other predatory mirids	Anthocorids	C.c.	P.m.	H.r.
Unsprayed	19	16	13	12	11	6	14	1484	884
Fenoxycarb	11	10	0	0	8	0	8	954	1134

B.a. = *Blepharidopterus angulatus*
M.c. = *Malacocoris chlorizans*
P.t. = *Phytocoris tiliae*
C.c. = *Chrysopa carnea*
P.m. = *Psylla mali*
H.r. = *Humerobates rostrolamellatus*

B, Leaf clusters; Total numbers recorded from four plots, three sample dates July 1986 = 2400 clusters/treatment

Treatment	Total tortrix larvae	*Adoxophyes orana*	*Yponomeuta malinellus* (no. colonies)	*Dysaphes platinagea* (no. colonies)	*Himacerus apterus*
Unsprayed	25	5	27	8	17
Fenoxycarb	10	0	27	16	25

Predatory mirids including *B. angulatus*, *Malacocoris chlorizans*, *Phytocoris tilae*, and anthocorid species were more numerous on the unsprayed plots (77 against 29 in total) and the differences may warrant further study to assess if the differences were due to fenoxycarb or merely by chance. The predator *Himacerus apterus* (Nabidae) was common in both treatments. Fewer summer generation leaf roller larvae were recorded in fenoxycarb sprayed leaf-clusters whereas the phytophagous *P. mali* and rosy apple aphid (*Dysaphes platinagea*), the defoliating small ermine moth *Yponomeuta malinellus*, and the non-pest cherry beetle mite *Humerobates rostrolamellatus* were similarly numerous in both treatments.

Earwigs, *A. forficularia*, monitored in trap-bands in July and August, were equally abundant in the unsprayed and fenoxycarb plots (Table 7.9) ($P > 0.05$). Woolly aphids, *E. lanigerum* were not present.

Table 7.9 Effect of fenoxycarb on earwigs (*F. auricularia*)

Treatment	Mean numbers per plot					
	7 July	24 July	12 August	Mean	S.E.D.	F.prob
Unsprayed	44.2	59.5	67.5	57.1	12.3	0.874
Fenoxycarb	40.5	78.3	46.5	55.1		

Adult tortrix moths collected in bait-pans were present in similar numbers in both sprayed and untreated plots (Table 7.10).

Table 7.10 Adult leafrollers (Tortricidae) recorded at Eden Farm, West Malling: July/August 1986

Treatment	C.p.	A.p.	H.n.	P.h.	P.c.	C.t.	B.a.	S.o.
Unsprayed	4	17	128	182	3	34	6	42
Fenoxycarb	0	18	134	196	6	30	6	32

C.p. = *Cydia pomonella*
A.p. = *Archips podana*
H.n. = *Hedya nubiferana*
P.h. = *Pandenis heparana*
P.c. = *Pandenis cerasana*
C.t. = *Cnicocolana tripunctana*
B.a. = *Batodes angustiorana*
S.o. = *Spilonota ocellana*

c) The effects of a full CpGV-based IPM programme on apple damage, pest and non-target arthropods

Limited orchard space restricted the field design to two replicates, since large plots (0.25 ha) were desirable to prevent migration of predatory and pest species between plots. Fruit damage was generally low - the poor summer weather may have been responsible - but the analysis was based on over 79,000 apples; windfalls and harvest (Table 7.11). Fruit damage has been analysed by analysis of variance on square-root transformed count data, though the overall % damage incidence has also been given. Analysis was divided into windfall and harvested apples. Windfall analysis was restricted to early season 'caterpillar' damage (e.g. by winter moth, *Operophtera brumata*), fruitlet mining tortrix, *Pammene rhediella*, and early codling moth.

Codling moth damage was lower in the full-IPM plots - both with and without the application of CpGV - but the overall reduction between the two regimes was not significant. Pre-blossom application of diflubenzuron (which has some residual activity against codling moth (Glen *et al.*, 1982)) and summer application of B.t. var kurstaki, clearly did not obviate the need to use CpGV for codling moth control.

For all the other species, the mean % damage recorded was lower in the IPM plots but this was only significant in caterpillar damage in windfalls ($P = 0.05$). Reduced incidence of *P. rhediella* damage was not significant but perhaps masked by the restricted replication. Incidence of summer leaf-roller damage was small and the effects of the IPM programme were thus not fully tested. However, with the exception of B.t., the compounds used were accepted orchard pesticides and their combined effect on non-target species was of greater interest (in this study) than their efficacy.

Table 7.11 CpGV/IPM programme: fruit damage

	Control		IPM		S.E.D. (√trans)	F prob. (IPM vs con)	Sig.
	%	No. damaged/ plot* √ mean	%	No. damaged/ plot* √ mean			
1. Windfalls							
Pammene rhediella	1.903	7.25	0.775	5.05	1.02	0.16	
"Caterpillar"	1.677	6.88	1.085	5.76	0.271	0.05	*
Deep codling moth							
+ CpGV	1.93	3.078	1.87	2.91	0.705	0.13	
- CpGV	9.85	6.04	3.95	4.33			
Total fruit	5040		5052				
2. Harvest							
Pammene rhediella	0.337	7.30	0.171	6.07	0.674	0.210	
"Caterpillar"	1.11	13.30	0.624	11.29	4.901	0.722	
Archips podana	0.055	4.02	0.054	4.07	0.255	0.852	
Adoxophyes orana	0.038	3.90	0.0016	2.70	0.458	0.121	
Blastobasis decolorella	0.133	5.22	0.015	3.33	1.092	0.224	
Lepidosaphes ulmi	1.11	13.25	0.730	12.25	6.116	0.883	
Deep codling moth							
+ CpGV	0.443	3.32	0.251	2.82	0.851	0.346	
- CpGV	1.32	5.38	0.467	4.33			
Total fruit	29652		38940				

Significance level; * = P < 0.05

* Note; Analysis of codling moth damage based on small plot trial within IPM trial (see Chapter 6, section III(b)iv) for details)

Table 7.12 gives mean earwig numbers for three sampling dates in July and August, the data analysed by analysis of variance on square-root transformed data. More earwigs were caught later in the season, but this probably reflects the duration of trap placement. More importantly, the IPM strategy had no effect on earwig numbers. Summer application of diflubenzuron against codling moth severely decreases earwig numbers (Ravensburg, 1981; Glen and Phillips, 1984). Pre-blossom application of diflubenzuron is, by contrast, apparently harmless. Numbers of woolly aphid colonies were fairly low and similar in both IPM and control plots (Table 7.13).

Table 7.12 Effect of IPM programme on earwigs, *F. auricularia*

Treatment	Mean earwig numbers per trap					
	9 July		28 July		24 August	
	Mean	√ mean	Mean	√ mean	Mean	√ mean
Control	15,83	3,82	104,7	10,14	303,8	13,10
IPM	12,17	3,46	95,00	9,69	138,8	9,71
S.E.D.		0,39		0,438		1,519
F. prob.		0,456		0,407		0,156

Table 7.13 Effect of IPM strategy on woolly aphids, *Eriosoma lanigerum*

Treatment	Mean no. colonies per 4 tree plot	
	9 July	28 July
IPM	41,00	16,00
Control	50,5	10,0

Numbers of predatory insects recorded in tree-beating samples were too low for formal analysis but have been presented in Table 7.14. Predatory anthocorids were sparse, but mirids, particularly the important *B. angulatus* were more abundant and similarly numerous in both IPM and control plots. The phytophagous *P. mali* was abundant in both regimes.

Table 7.14 Effect of IPM programme on non-target species. Tree-beating samples: totals for season

	Species		Treatment	
			Control	IPM
Predatory mirids	*Atractonomus mali*		8	4
	Blepharidopterus angulatus	Adult	16	15
		Nymph	12	15
	Malacocoris chlorizans		6	5
	Phytocoris reuteri		5	3
	Phytocoris tiliae		7	3
Predator anthocorids	*Anthocoris nemorum*		2	0
	Orius minutus		1	1
Lacewings	*Chrysopa carnea*	Adult	12	15
		Larvae	2	1
Phytophagous Homoptera	Cicadellidae		22	17
	Psylla mali		803	623
	Zygina flamigera		5	3

Predatory mites, *T. pyri*, were abundant in both IPM and control plots, and *P. ulmi* were maintained at low levels. Rust mites were more numerous but similar in both treatments. Only tydeid mites were recorded in lower numbers on the IPM plots, and their distribution was in fact quite patchy and unlikely to have been caused by the IPM programme. No other significant differences were observed (Table 7.15).

Table 7.15. Predatory and pest mites in CpGV/IPM strategy

Mean number per 20 leaf sample; July/August 1987

	Treatment			
	Control		IPM	
	Mean	SE	Mean	SE
Panonychus ulmi	6,0	1,7	6,0	1,7
Panonychus ulmi eggs	12,2	5,3	13,6	5,2
Typhlodromus pyri	8,1	2,1	11,0	2,4
Tydeidae	8,0	4,3	1,0	0,6
Eriophyidae	2034	983	1750	674

IV. DISCUSSION

Application of CpGV had no adverse effect on the populations of any predatory species and, as a consequence, in CpGV-treated trees, the pest mites, *P. ulmi* and Eriophyidae (probably all *Aculus schlechtendali*) were maintained at low levels, apparently by the phytoseiid mite *T. pyri*, which remained numerous in both CpGV and unsprayed plots. By contrast, in the same trial, application of deltamethrin depleted the local population of *T. pyri* and both *P. ulmi* and Eriophyids rose to high numbers within two months.

The rapid increase in pest mites in deltamethrin-sprayed plots is most likely due to the death of many *T. pyri*. An analysis of all the leaf samples from all plots, revealed a significant correlation between *P. ulmi* and *T. pyri* numbers (partial correlation coefficient = 0.899) with an

inverse relationship existing between the two species; high numbers of *P. ulmi* corresponding to few *T. pyri*.

In the UK, *T. pyri* is the most effective natural enemy of *P. ulmi* and the apple rust mite *A. schlechtendali*. Under suitable IPM regimes, *T. pyri* is able to maintain its population at low non-damaging levels (Cranham and Solomon, 1981). Due to its low powers of dispersion *P. ulmi* is restricted to its host thus making it ideal for stable density-dependent control by a K-strategist predator such as *T. pyri* (Way, 1978). K-strategists tend to be adapted to a perennially suitable habitat, not over-exploiting their food supply, having limited powers of dispersion and population increase, forming relatively stable relationships with natural controlling factors and maintaining fairly stable populations (Way 1978). However, unlike many k-strategist predatory species, T. pyri has three or four generations per year and is thus able to respond rapidly to changes in its prey population and is stable even at low numbers - as low as 0.01 *T. pyri* and 0.05 *P. ulmi* per leaf (Solomon, 1982). *T. pyri* becomes active in March, a month before *P. ulmi* hatches, but is able to feed on alternative prey such as rust mites and Tydeidae (Solomon, 1982; Kapetanakis *et al.*, 1986).

By comparison, the predatory mirid, *B. angulatus*, is univoltine and unable to exert density dependent control on the multivoltine *P. ulmi* in a single season (Collyer, 1953; Muir, 1965b; Solomon, 1982). Although *B. angulatus* is capable of significantly reducing the population of P. ulmi (Muir, 1965a) it was unable to prevent rising numbers of *P. ulmi* in deltamethrin plots caused by the sudden death of *T. pyri*, since numbers of *B. angulatus* would already have reached a peak for the season. The anthocorids *Anthocoris nemorum* and *A. nemoralis* have two generations per year, overwintering as adults, and are thus more able to respond to rising numbers of *P. ulmi* (Solomon, 1982). Predatory anthocorids were, however, relatively sparse in these trials.

Deltamethrin significantly reduced spiders compared to the effects of CpGV and unsprayed plots. Earwigs, *F. auricularia* - important predators of aphids, mites, insect eggs (including codling moth), and the mussel scale *L. ulmi* (Karsemeyer, 1973; Glen and Phillips, 1984; Alford, 1984) - were unaffected by either CpGV or deltamethrin. Depletion of earwig numbers is associated with a rise in the number of woolly aphids *Eriosoma lanigerum* (Ravensburg, 1981; Glen and Phillips, 1984). In this trial woolly aphid

numbers remained low in chemical, virus and unsprayed plots. Earwigs are often considered as fruit pests but primary damage to fruit is rare and their numbers can, in any case, be reduced by cultural methods (Alford, 1984).

CpGV had no effect on the other apple pests monitored: leafrollers (Tortricidae); early season caterpillars; aphids; psyllids and cicadellidae (although the two latter groups are normally of no economic significance on apple). In order to prevent crop damage additional agents are required for use with CpGV and they must fulfil the following criteria:

1. Control target pests
2. No damage to beneficial insects and mites
3. Ineffective against codling moth

Any agent failing the third criterion would compete commercially with CpGV rather than complement it!

For the main part then, non-significant effects against non-target species were desirable in the CpGV/IPM trials. In 1986 and 1987 the insect growth regulator (IGR) fenoxycarb, *Bacillus thuringiensis*, pre-blossom application of diflubenzuron, pirimicarb and a full selective fungicide programme used alongside CpGV, caused no major reduction in the important predators *T. pyri*, *B. angulatus* and earwigs. Some reduction in certain mirid and anthocorid species was observed with fenoxycarb though this could not be statistically verified.

Diflubenzuron is recommended as a summer spray for codling moth but pre-blossom sprays were neither deleterious to earwigs (contrasting the adverse effects of its summer application (Ravensburg, 1981; Glen and Phillips, 1984)), nor gave adequate control of codling moth. Fruit damage by codling moth was, however, lower in the IPM plots and at harvest, was reduced by 64% compared to the control plots, with no CpGV applied. In CpGV sprayed plots, damage was also reduced a further 43% by the IPM programme. Chemical insecticides seldom exert density-dependent control, killing a similar proportion irrespective of the population (Way, 1978). Diflubenzuron and CpGV, both acting density-independently, may have an additive effect giving greater control, particularly since they differ in their modes of action; CpGV acting on neonate larvae, diflubenzuron acting

exclusively as an ovicide against codling moth eggs (Hoying and Riedl, 1980). Incomplete control of codling moth by pre-blossom diflubenzuron was consistent with trials reported by Glen *et al.* (1982) where pre-blossom application gave 46% control of deep damage, thus supporting diflubenzuron as the causative agent for reduced damage in the IPM plots.

The pre-blossom application of diflubenzuron at only 250 l/ha (not to run-off) may have allowed earwigs to escape harm owing to the different behaviour of earwigs in the spring and summer. Young earwig nymphs remain in, and on, the ground until June, only after which the third and fourth nymphal stages climb into the tree canopy (Glen and Phillips, 1984).

Incidence of fruit damage due to leafrollers was low in all trials, consequently the selective agents, fenoxycarb and B.t. were not fully tested. *P. rhediella* was the most damaging leafroller pest causing over 1% damage at Eden Farm in 1986. Damage was reduced - though not significantly - by the full IPM programme tested in 1987, but fenoxycarb was ineffective. The overwintering larvae of *P. rhediella* do not feed in the spring prior to pupation - unlike *A. orana* and *A. podana* - and would therefore not ingest fenoxycarb which acts by preventing pupation of the spring larvae. A 'petal fall' application of diflubenzuron is recommended for control of *P. rhediella* (Alford (1984), and its long persistence probably explains the reduced damage in the IPM plots after pre-blossom application. An additional application of diflubenzuron might, therefore, have been more effective - and warrants testing - though post-blossom application is more likely to compete with CpGV for codling moth control and have a more marked effect on earwigs. *P. rhediella* thus remains a problem, though its pest status is generally localised and restricted to Kent and East Anglia (Anon., 1984) and was, in these trials, more prevalent on the previously derelict orchard at Eden Farm. *P. rhediella* can be reduced by cultural methods such as the planting of smooth stemmed spindle-bush trees which also decrease codling moth incidence by providing less favourable sites for overwintering (Gruys, 1982).

Adult leafrollers were caught in bait-traps designed to sample from a localised area (David Glen, pers. comm.). A variety of tortricids were caught at Eden Farm, but numbers were unaffected by fenoxycarb. The number of different orchard leafroller species makes selective control more

complex since not all will be affected by a specific agent; in German trials nine species were recorded from a single orchard (Neuffer, 1984).

Fenoxycarb has an ovicidal effect on codling moth (Charmillot *et al.*, 1985) but fruit damage was only slightly, and non-significantly, reduced despite spray timing which should have corresponded approximately to egg laying in codling moth. In Germany, fenoxycarb was reported to decrease codling moth damage, but only to 5.3% (from 22%) and poorly in comparison with the development inhibitor triflumerone (Bayer) (Boscheri and Mantinger, 1986). Fenoxycarb is recommended against the San Jose scale (*Quadraspidiotus perniciosus*, Diasapididae) in European orchards (Dr R Maag, Technical Information) but had no effect in these trials on mussel scale, *L. ulmi* (Diasapididae), which can cause downgrading by the presence of scale on the fruit. Nymphs ("crawlers") hatch in late May and June - at the time of fenoxycarb application - and this stage was not susceptible at the rates applied. Numbers of the non-economic apple sucker, *P. mali* were reduced by 35% though the fenoxycarb sprays were not timed specifically for this insect. In the same season, Solomon & Fitzgerald (1987) treated a pear orchard at East Malling with fenoxycarb in mid July and achieved 98% reduction in nymphs of the pear psyllid *Cacopsylla pyricola*.

In these UK trials, fenoxycarb was ineffective against most pest species, though, in fairness the main target species, *A. orana* and *A. podana* were scarce, and the trials showed fenoxycarb to be suitable in terms of its non-toxicity to most predators, including *T. pyri*, and its incomplete effect as an ovicide for codling moth. German trials showed fenoxycarb to be relatively ineffective against leafrollers (Dickler, 1984), though Reede *et al.* (1985a) claim effective control of *A. orana* in Holland. The Dutch trials were, however, neither replicated nor included unsprayed controls, and the success of the IGR programme is concluded merely from the effect of discontinuing IGR application in one whole season. The requirement for orchard isolation, either physically or chemically, may discourage growers from using fenoxycarb.

Diflubenzuron is also unsuitable for leafroller control where a variety of species are present: in trials in the Netherlands, diflubenzuron failed to control *A. orana* and, despite good control of *H. nubiferana, A. podana* and *S. ocellana,* the overall level of damage attributed to leafrollers was only reduced by half (Reede *et al.*, 1985a). Control of

A. orana using the viral insecticide *A. orana* NPV reduced damage by only 50-70% and was considered too costly to be feasible (Peters *et al, 1983; 1984); it would, in any case, be ineffective against other leafrollers.*

B.t. was inadequately tested in these trials due to the low incidence of leafrollers. In bioassays, B.t. var kurstaki was found to be highly toxic to A. orana and *P. heparana* (Huber & Undorf, 1985, Undorf and Huber 1986). However, in European orchard trials B.t. was unable to prevent fruit damage by these two species (de Reede et al., 1985b) and even in combination with another biological agent, *Adoxophyes orana* nuclear polyhedrosis virus (AoNPV), damage by *A. orana* was not prevented (Dickler, 1984; Dickler & Huber, 1983).

Four selective agents were used alongside CpGV in these trials: pirimicarb, diflubenzuron, fenoxycarb and B.t., as well as a selective fungicide programme. All were compatible with CpGV in terms of their lack of effect against beneficial species and ineffectiveness against the codling moth. Pirimicarb and diflubenzuron are both established control agents, fenoxycarb however requires further testing against the target leafrollers *A. orana* and *A. podana* and would be unsuitable where unaffected species such as *P. rhediella* were prevalent. B.t. has yet to be proved useful in orchard IPM but may prove effective against leafrollers particularly if new strains are tested (Gruys, 1982).

The advantage of CpGV in terms of its selectivity has been clearly demonstrated: in particular, applications of acaricides to control outbreaks of pest mites would be unnecessary following introduction of a CpGV-based IPM programme. How quickly predatory insects and mites recolonise an orchard when CpGV replaces an existing broad spectrum programme will depend on sources of reinfestation such as neighbouring unsprayed orchards, windbreaks (hedges, trees and associated plants) (Solomon, 1981) and the number of predators surviving previous chemical treatments: in trials in S.E England, *T. pyri* was fully established in only the second year of an IPM programme (Easterbrook, 1984). Predatory mites could be introduced into a new IPM orchard by importing infested shoots from mass-culturing sites (Solomon and Fitzgerald, 1984) and if necessary a selective acaricide could be used in the first season if mites reached pest status. Careful monitoring of predatory and pest mites would indicate if action was necessary.

Although codling moth is a pest on most apple and pear growing regions worldwide, the complement of other orchard pests is variable and the choice of selective agents for an IPM programme would depend on the pest complex. Within a season, careful monitoring would determine if pests such as leaf-rollers had reached threshold levels and in many cases additional summer sprays may prove to be unnecessary.

Even in an "organic" orchard, the full benefits of CpGV may not be attained if orchard practice is not modified. In a trial at an organic Farm in Kent, excellent control of codling moth was achieved using CpGV (Chapter 8, section IIIi and ii). However, in order to control scab (*Venturia inaequalis*) and mildew (*Podosphaera leucotricha*) dispersible sulphur was applied as an acceptable "organic" fungicide. Dispersible sulphur is toxic to *T. pyri* (Collyer and Kirby, 1954; Cranham and Solomon, 1981) and, as a result, *P. ulmi* numbers were high.

Orchards are mature K-type ecosystems with a complex fauna closely adapted to their host and, as such, suitable for biological control (Way, 1978). Successful orchard IPM can be achieved if the ecosystem is only minimally disrupted allowing predator prey relationships to remain stable. Dutch experiments have shown that more than half of the twenty-four species found damaging to apple orchards could be controlled by cultural or biological means (Gruys, 1982). Of the remaining pests, it is imperative that selective agents be used: in this study CpGV has proved ideal for incorporation in a full orchard IPM programme and other selective agents can be used alongside the virus.

Chapter 8

Field trials on commercial orchards

I. INTRODUCTION

CpGV has performed well in replicated experimental field trials and an optimum application strategy has been developed (Chapter 4, section III(b)). In small plot trials the level of pest control has varied according to the formulation, dose and spray timing, with the best treatments giving 74-89% control of codling moth damage to fruit over the three seasons of trials on the 1 ha orchard WF103 at East Malling. The achievable level of control on small plots is likely to be limited by the difficulty in obtaining full spray coverage of a small plot whilst avoiding spray drift onto neighbouring plots. Furthermore, the close proximity of unsprayed plots, and guard rows, could act as a source of late infestation in the event of a small second generation of codling moth larvae. These problems should be eliminated by spraying larger plots with conventional machinery.

In 1986 and 1987, field trials were carried out on commercial orchards where larger plots were sprayed and, in 1987, orchards were treated with an impure formulation of CpGV - designed to reduce production costs - sprayed by the commercial growers using conventional machinery. The trials on commercial orchards encompassed both replication and untreated plots, and farms employing 'conventional', 'supervised', and 'organic' methods of pest control.

II. MATERIALS AND METHODS

a) Formulation of CpGV

Purified CpGV was used for the first of the tests on commercial farms in 1986, whereas in 1987, growers were supplied with impure formulations containing 40% glycerol (Chapter II, section II(d)i; Plate 2.1).

b) Field Trials

i. Congelow Farm, Yalding, Kent 1986.

Congelow Farm (proprietor: Mr Donald Cooper) is an organic farm following Soil Association guidelines, and supplying the supermarket chain Safeways with organic produce.

Trial site: 1.5 ha orchard of apple variety Edward VII: Unreplicated.

Treatments:

1. Purified CpGV at $1x10^{13}$ granules/ha + 5% molasses (v/v).

2. Unsprayed control.

CpGV was applied at 300 l/ha using a Solo Minor mistblower. A wetting agent was not included in the spray to avoid the use of synthetic agents. Molasses was included to enhance the efficacy of CpGV (Chapter 6, section III).

Spray dates: four applications: June 30th; July 9th; 16th; 28th.

ii. Congelow Farm, 1987.

Trial site: 2 ha orchard of variety Laxton's Fortune: eight plots, four treated and four untreated (Fig. 2.7).

Treatments:

1. Impure CpGV homogenate at $5x10^{12}$ granules/ha.

2. Unsprayed control.

CpGV was applied at weekly intervals, using a tractor-drawn mistblower at 450 l/ha.

Spray dates: Four sprays at weekly intervals, commencing July 7th.

iii. Folly Fruit Farm, Eastergate, West Sussex, 1987.

A trial was carried out at Folly Fruit Farm, at which 'conventional' insecticides are used for pest control; unsprayed plots and replication of treatments were agreed with the farm owner, Mr Philip Weatherdon.

Trial site: 1.5 ha orchard of Cox with Laxton's Fortune, Tydeman's Early Worcester, and James Grieve pollinators: eight plots; three CpGV treated (0.8 ha), three unsprayed (0.4 ha) and two additional plots (0.3 ha) sprayed under the standard chemical programme used on the farm (Fig 2.6).

Treatments:

1. CpGV at $5x10^{12}$ granules/h + 0.02% Agral.

2. Unsprayed control.

3. Standard chemical programme (cypermethrin, 960 g/ha, two treatments).

Spray dates: four CpGV sprays at weekly intervals, two cypermethrin sprays a fortnight apart, both commencing June 26th.

iv. Little Barton Farm, Canterbury 1987.

Little Barton Farm is one of the largest fruit farms in Kent with mechanised fruit sorting and packing facilities on site. The farm management, S. W. Mount, practices 'supervised' pest management which though, not fully integrated, avoids the overuse of pesticides by pest monitoring and careful application. It was not possible to include any replication or unsprayed areas, but some indication of codling moth infestation was gained from pheromone trap data, and useful information was obtained about the populations of predatory insects and mites in the first season of CpGV application after switching from a chemical programme.

Trial site: 'Pigeon Field', 2 ha of variety Cox divided into two equally sized plots.

Treatments:

1. CpGV at $5x10^{12}$ granules/ha.

2. Grower's chemical programme:
   Azinphos-methyl (third week, June)
   Phosalone (first week, July)

Spray dates (CpGV): four sprays at weekly intervals from July 1st.

The fungicides dodine (pre-blossom), dithianol, bupirimate and triadimefon (post-blossom) were applied equally to both plots.

c) Data analysis

Data from the replicated trials were analysed by analysis of deviance with a fitted binomial model to account for the total number of fruit in each sample.

III. RESULTS

1. Congelow Farm, 1986; var Edward VII

Windfalls were sampled on five dates between July 28th and October 24th, and the level of damage and total numbers sampled are given in table 8.1. A misunderstanding with the contract-pickers meant that a harvest analysis was not possible, but the windfall results were supplemented by an on-tree analysis of 900 fruits sampled on four dates from July 28th to September 26th (table 8.1).

Table 8.1 Fruit damage at Congelow Farm, 1986

Treatment	Superficial codling damage %	Deep codling damage %	*P. rhediella* damage %	Total apples sampled
a. Windfalls				
CpGV	0,070	0,140	1,05	1432
Unsprayed	0,342	4,651	1,33	1462
b. On-tree fruit				
CpGV	0,333	0,222	1,33	900
Unsprayed	0,333	2,22	4,00	900

Control of deep-damage by CpGV was excellent with 97% control in windfalls and 90% control in on-tree fruit. Superficial codling moth damage was low in both treatments. P. rhediella damage was high at 1 - 4%. Numbers of pest mites *P.ulmi* were high in both CpGV and unsprayed plots with only one predatory *T.pyri* found in three samples (table 8.2). Dispersible sulphur used to control scab is the most likely cause of the sparsity of *T. pyri* (Collyer and Kirby, 1954) and also explains the extremely low numbers of rust mites (Easterbrook, 1984).

Table 8.2 Congelow Farm: Pest and Predatory mites

Treatment	*P. ulmi* eggs	*P. ulmi*	*T. pyri*	Tydeidae	Eriophyidae
	Number/20 leaves				
a. 28th July					
CpGV	259	147	0	1	2
Unsprayed	570	303	1	3	0
b. 2nd September					
CpGV	61	66	0	0	0

ii. Congelow Farm, 1987

Following the success of the 1986 trial, the owner of Congelow Farm agreed to a second, replicated, trial with unsprayed controls. With large plots (ca 0.25 ha) control of both deep and superficial damage in windfalls and harvested apples was highly significant with 96 and 97% control of deep damage in windfalls and harvest, respectively, and, 79 and 96% control of superficial damage (table 8.3).

Table 8,3 Codling moth damage at Congelow Farm, 1987

Treatment	Superficial damage %	Deep damage %	Total apples sampled
a, Windfalls			
Untreated	1,46	4,38	548
CpGV	0,31	0,15	654
% control	78,8	96,6	
Mean deviance ratio (6+1 df)	9,57	16,0	
Significance ?	*	**	
b,Harvest			
Untreated	1,20	1,20	2000
CpGV	0,05	0,05	2000
% control	95,8	95,8	
Mean deviance ratio (6+1 df)	26,2	41,0	
Significance ?	***	***	

* = P<0,05 ** = P<0,01 *** = P<0,001

iii. Folly Fruit Farm

Fruit damage at Folly Fruit Farm was high in untreated plots with 20.9% deep damage in windfalls and 2.4% at harvest (table 8.4). Replication was restricted to three plots with only two plots for the chemical comparison (Fig. 2.6); a design chosen to fit the available space and the farm programme. The analysis of deviance was thus limited with only three degrees of freedom for the residual deviance. Significant effects were however recorded for the control of deep and superficial damage at harvest with CpGV accounting for a significant proportion of the recorded deviance ($P<0.05$). The performance of CpGV was comparable to that of cypermethrin, with both treatments reducing both superficial and deep damage to low levels.

Table 8.4 Codling moth damage at Folly Fruit Farm, 1987

Treatment	Superficial damage % Mean	S.E	Deep damage % Mean	S.E	Total apples sampled
a. Windfalls					
Untreated	1,53	0,44	20,90	1,42	924
Cypermethrin	0,48	0,35	2,53	0,65	258
CpGV	0,18	0,13	4,98	0,66	1290
% control; Cyper	68,6		87,9		
% control; CpGV	88,2		76,2		
b. Harvest					
Untreated	1,02	0,22	2,43	0,33	2045
Cypermethrin	0,07	0,07	0,26	0,13	1500
CpGV	0,09	0,05	0,55	0,14	3362
% control; Cyper	93,1		89,3		
% control; CpGV	91,2		77,4		

Analysis of deviance	Source	Superficial damage Mean dev. ratio	d.f	sig.	Deep damage Mean dev. ratio	d.f	sig.
Windfalls							
	Cyper. vs all	0,73	1		2,15	1	
	CpGV vs Untr.	9,66	1		3,86	1	
	Residual	-	3		-	3	
Harvest							
	Cyper. vs all	4,33	1		8,73	1	
	CpGV vs Untr.	12,77	1	*	10,45	1	*
	Residual	-	3		-	3	

Level of significance; * = P<0,05

iv. Little Barton Farm

The trial at Little Barton Farm was an unreplicated trial with only a chemical insecticide comparison and no unsprayed control. Although statistically valid assumptions cannot be drawn, it was encouraging to record a virtual absence of codling moth damage in the CpGV sprayed plots despite-above threshold pheromone trap catches (table 8.5).

Table 8.5 Codling moth damage at Little Barton Farm, 1987

Treatment	Superficial damage %	Deep damage %	Total apples sampled
a. Windfalls			
Chemical	0	0	800
CpGV	0	0	800
b. Harvest			
Chemical	0	0	764
CpGV	1	0	785

Threshold pheromone trap catches (> 5/week) :
3rd June = 6 ; 1st July = 11 ; 8th July = 18

The numbers of predatory insects isolated from both the CpGV and chemical plots by tree beating were very small with no records of the common orchard mirid *Blepharidopterus angulatus*, the predatory nabid *Himacerus apterus*, or the phytophagous psyllids and cicadellids (table 8.6). Predatory anthocorids and two lacewing species, *Hemerobius humilinus* and *Chrysopa carnea* were present but very sparse.

Table 8.6 Little Barton Farm: Tree beating samples: Totals for four sampling points in each plot, 10 beats per sample.

	Chemical	CpGV
Blepharidopterus angulatus	0	0
Himacerus apterus	0	0
Anthocorid species	2	4
Chrysopa carnea	1	3
Hemerobius humilinus	1	0
Psylla mali	0	0
Cicadellidae	0	0

Mites were counted from leaf samples in August and September (table 8.7). *T. pyri* were present in all samples with moderate numbers of *P. ulmi*.

Table 8.7 Little Barton Farm: Pest and predatory mites: n° per 20 leaves

	August		September	
	Chemical	CpGV	Chemical	CpGV
P. ulmi	80	126	15	12
P. ulmi eggs	576	433	159	186
T. pyri	5	1,5	3	6
Tydeidae	0	0	0	0
Eriophyidae	870	426	84	84

IV. DISCUSSION

In four large-plot trials on commercial orchards, suppression of codling moth damage to fruit was excellent. In 1987, an impure CpGV preparation was used at 5 x 10^{12} granules/l (four sprays) without the inclusion of feeding stimulants or protectants. In two seasons of trials at a commercial organic orchard, deep-damage was reduced by 97% compared to untreated plots. At the 'chemical' Folly Fruit Farm in West Sussex, control was slightly poorer with around 77% control of deep damage in both harvest and windfall apples with 88 and 91% control of superficial damage. Fruit damage in the control plots was high at Folly Fruit Farm with 21% deep-damage in windfalls and 2.43% damage at harvest. However, the effectiveness of CpGV in reducing crop damage - at least in percentage terms - is reportedly independent of pest intensity (Huber and Dickler, 1977), and this was reflected in the three years replicated trials on the same site, WF103, at East Malling where the degree of damage reduction appeared to be unrelated to the three very different levels of pest infestation observed (Chapter 4, section III(a) and (b), and Chapter 6, section III(b)iv).

Virtually no damage was recorded at Little Barton Farm in either CpGV or the standard insecticide plots. Unfortunately, in this trial untreated plots were not authorised by the grower and this limited the value of these findings. However, above-threshold pheromone trap catches were recorded and pest damage would normally have been expected without treatment.

The 97% control of deep damage - achieved in replicated 0.25 ha plots - was better than most of the small-plot experimental trials undertaken at East Malling where control levels were around 80%. Large plots ought to give greater levels of control because of the ease in which all trees can be sprayed without concern of contaminating neighbouring plots. In the event of a second generation - unlikely in most UK years - large plots would also minimize the risk of immigration of second generation adults from untreated areas. In French trials, surrounding orchards were also treated to prevent immigration and only four applications were said to be sufficient to control both codling moth generations (Burgerjon 1986).

Despite good control of codling moth damage to fruit, the trials on commercial sites have highlighted potential problems. The possibility of developing a full IPM programme based around CpGV has been discussed in Chapter 7. However, the success of an IPM system, where economic control of certain pest species depends on the integration of natural enemies with selective control agents, relies on successful invasion of predatory species into the orchard. The trial at Little Barton Farm has demonstrated a scarcity of predatory insects in the first year of CpGV application. By contrast, predatory phytoseiid mites, *T. pyri*, were reasonably abundant in both CpGV and insecticide plots, which, in this case, would allow successful control of *P. ulmi*. Endemic resistance to organophosphorus (OP) insecticides in *T. pyri* at this site could explain the presence of predatory mites in spite of the OP programme (phosalone and azinphos-methyl) used in the insecticide plots. Ironically, in the 'organic' orchard *P. ulmi* were abundant in both CpGV and unsprayed plots and this is attributable to the virtual absence of predatory mites. Unfortunately, there are no available naturally occurring selective fungicides and in an effort to control scab, *V. inaequalis*, and powdery mildew, *P. leucotricha*, dispersible sulphur was used and this has probably damaged populations of *T. pyri* (Collyer and Kirby, 1954).

The development of a full 'organic' IPM system is unlikely with the agents currently available. However, the premium gained from selling 'organic' apples should overcome the slight losses incurred from non-codling pest damage and, as a control agent for the premier fruit pest, the codling moth, CpGV is unrivalled in the organic market.

Chapter 9

Summary

In the course of this research programme, CpGV was shown to be an effective control agent for codling moth. An optimum strategy for its use in UK orchards was designed and a pilot product suitable for commercial development has been produced. Work on virus uptake and formulation has opened an exciting field for future development; namely, the potential of incorporating feeding stimulants to improve virus efficacy.

A dose of 10^{13} granules/ha was sufficient to give 79% control of deep-entry fruit damage in a small-plot trial, with no further improvement achieved with a ten-fold increase in the applied dose (Fig. 9.1). CpGV persistence on foliage is poor - chiefly due to the action of ultra-violet light (Keller, 1973) - and the addition of certain spray additives did not prolong the lifetime of virus on apple leaves (Fig. 9.2). However, more frequent application of CpGV largely overcame this problem without the need to increase the total dose of applied virus. The same total dose of CpGV, applied over a season, gave significantly improved control of codling moth damage when applied in weekly small doses, than when applied over two large doses a fortnight apart (Fig. 9.4). Increasing the number of spray applications should not result in significantly increased application costs since CpGV is compatible with a range of chemicals, and can be tank-mixed with fungicides applied frequently throughout the season (Fig. 9.3). CpGV was also compatible with medium and ultra-low volume (25 l/ha) application (Fig. 9.5).

CpGV could be ingested by neonate codling moth larvae from leaves or apple surfaces (Fig. 9.6) and ingestion of a lethal dose, at normal spray concentrations, was relatively rapid - in as little time as three minutes on sprayed leaves. Incorporating molasses with CpGV improved control significantly, with the same reduction in fruit damage achieved with only one tenth of the standard virus dose (Fig. 9.1). Molasses, and a number of other agents, were shown to increase the speed of virus uptake from leaf surfaces (Fig. 9.7) and a 'realistic' bioassay system was developed to test new formulations. A number of additives were shown to be effective in field trials, where CpGV was applied at a low rate to allow maximum discrimination between more and less effective treatments (Fig. 9.8).

Fig. 9.1 Effect of CpGV dose and formulation on deep-entry apple damage

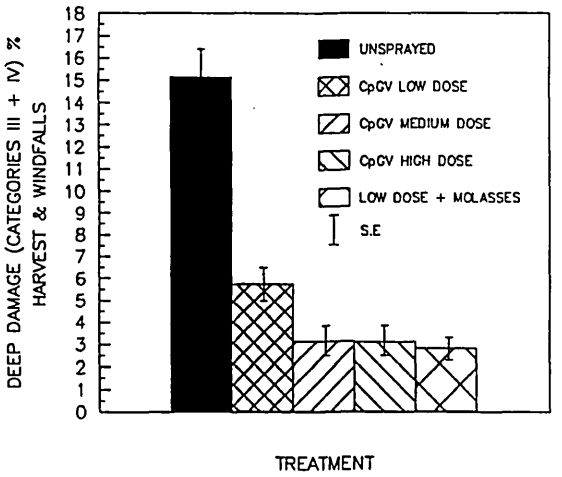


Fig. 9.2 Effect of spray additives on foliar persistence of CpGV

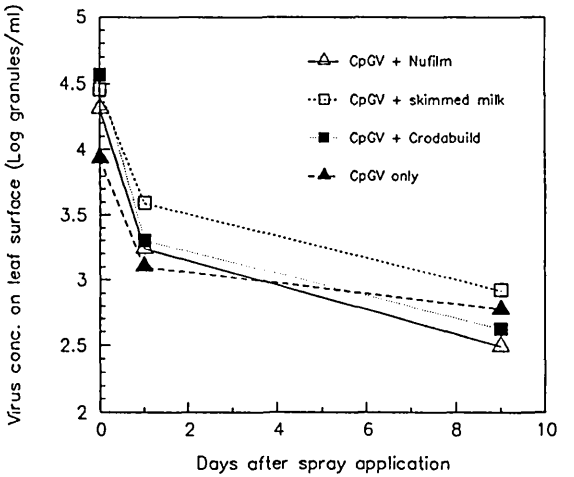


Fig. 9.3 Effect of Tank-mixing CpGV with fungicide on control of deep-entry apple damage

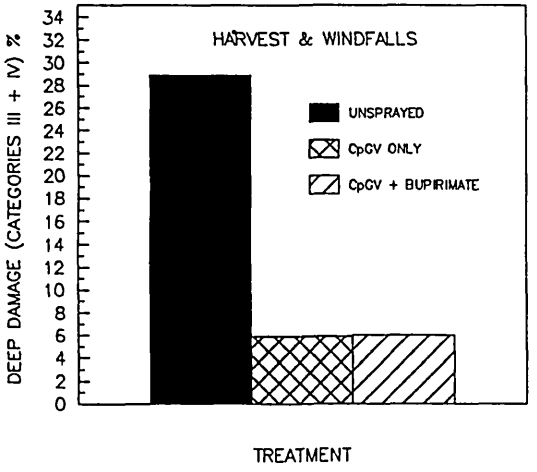


Fig. 9.4 Effect of CpGV spray frequency on control of deep-entry apple damage

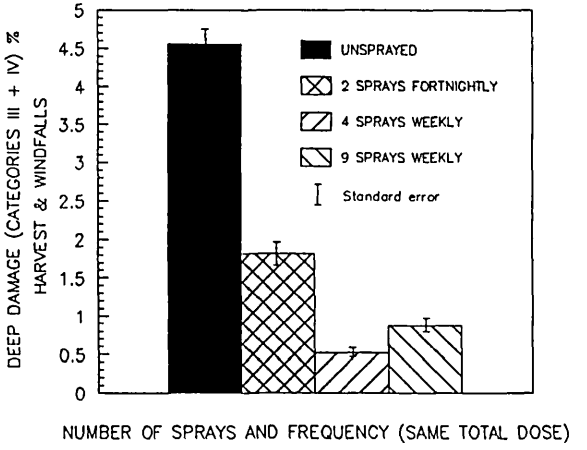


Fig 9.5 Effect of CpGV spray volume on control of deep-entry apple damage

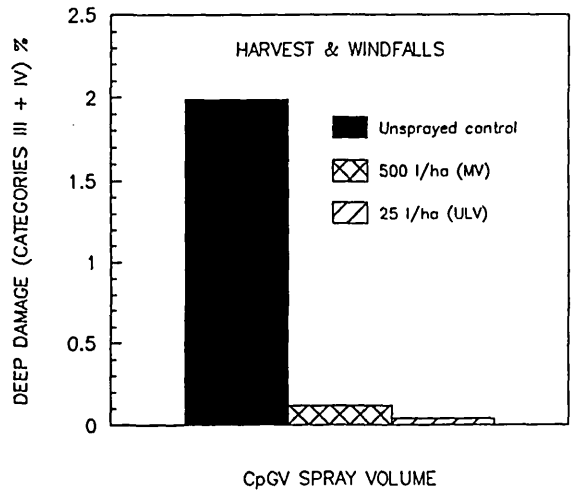


Fig 9.6 Virus acquisition from selectively sprayed apple foliage and fruit

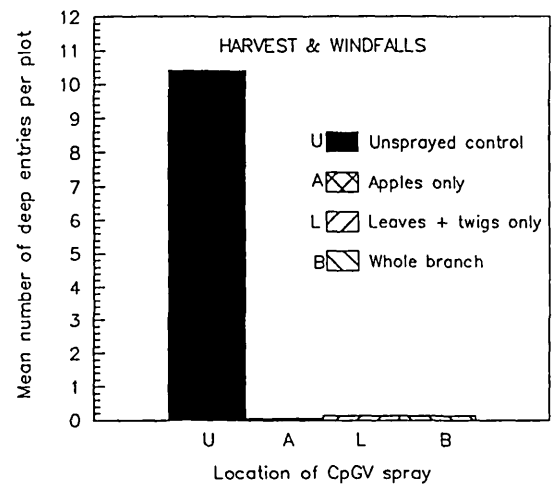


Fig 9.7 Effect of spray additives on uptake of CpGV from sprayed leaves

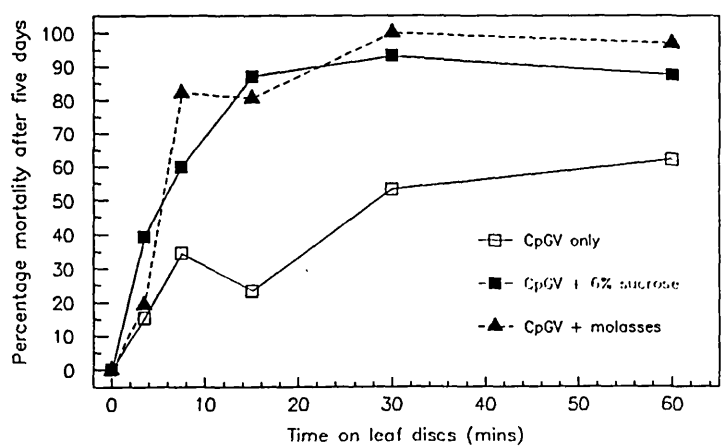
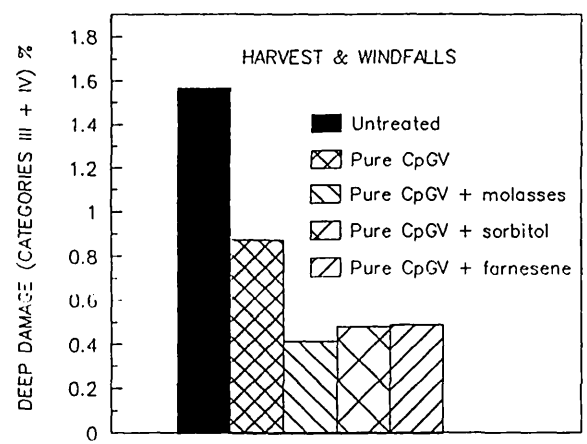


Fig 9.8 Effect of CpGV spray additives on control of deep-entry apple damage (CpGV at low dose)



Note: Anovar based on numbers – see table 6.9 for Standard errors

CpGV was compatible with an orchard IPM programme and had no deleterious effects on populations of beneficial insects and mites, allowing the natural control of secondary pests such as rust mites and *P. ulmi* by the predatory mite *T. pyri* (Fig. 9.9). Other selective agents were compatible with CpGV by being non-disruptive to beneficial insects and mites and, themselves, failing to control the codling moth itself. The selective agent fenoxycarb was not fully tested in trials owing to the sparsity of target pest species. However, problems for its use were identified, and reports of successful trials in the Netherlands were shown to be unreliable. Excellent results were achieved in field trials of CpGV on commercial orchards with control levels of up to 97%; the benefits of large-scale field trials by allowing better spray coverage probably explained the better control as compared to small-plot research station trials (Fig. 9.10).

Virus mass-production was, by necessity, by infection of host codling moth larvae. However, codling moth larvae can be reared on semi-synthetic diet and, provided that a healthy stock can be maintained, they can be reared *en masse* in open trays. Larvae reared from eggs imported from a French laboratory culture were larger and less prone to virus infection when mass-reared than larvae from an existing stock maintained at IHR Littlehampton. When reared in small compartments, interference or cannibalism generally prevented more than one larvae surviving in each chamber. The physical nature of the diet effected larval development; rough, cut surfaces were preferred to a smooth surface from poured diet. Ascorbic acid was also an essential ingredient for development. Maximum levels of occluded virus in infected larvae were realised prior to larval death, hence harvesting at this stage reduced the risk of high levels of unwanted microbial contamination from saprophytic bacteria. An impure preparation, based on homogenized virus-infected insects, and diet, proved to be at least as effective as purified CpGV (Fig. 9.11) and this material had adequate storage life if kept frozen, or if formulated with 40% glycerol (Fig. 9.12). Various drying processes were not advantageous in terms of storage. The quantification of the impure preparation could be achieved using a modified ELISA technique whereby background interference from impurities could be removed by low-speed centrifugation (Fig. 9.13), although the time-consuming use of bioassays could never be entirely eliminated, since these are the only way in which infectivity can be evaluated. Material produced for field trials was diluted to give $5x10^{12}$

granules/litre, sufficient for a single spray application per hectare of orchard (without additional feeding stimulants). Batches of impure CpGV for field trials were each tested for levels of bacterial contamination and contained 3-9x10^{6} cfu/ml, which compared very favourably with production of 'Gypchek' (*Lymantria dispar* NPV, for gypsy moth control) in the U.S.A. where the average bacterial count was 6x10^{8} cfu/g (Podgwaite *et al.*, 1983).

CpGV has proved to be highly effective as a control agent, suitable for integration with other selective orchard pesticides. Of paramount interest, is that its efficacy can be improved by formulation with feeding stimulants. CpGV has proven potential, further work must now concentrate on lowering the cost of production and developing other feeding stimulants which are inexpensive and could be used at lower concentrations.

Fig. 9.9 Effect of CpGV and deltamethrin on red spider mites, P. ulmi

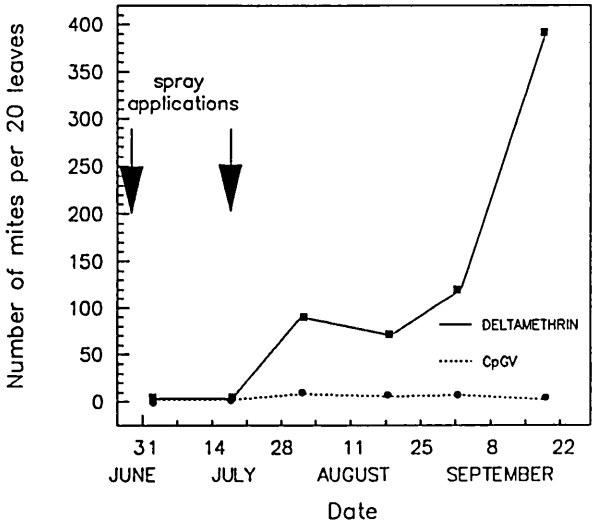


Fig. 9.10 Control of fruit damage on a commercial organic farm

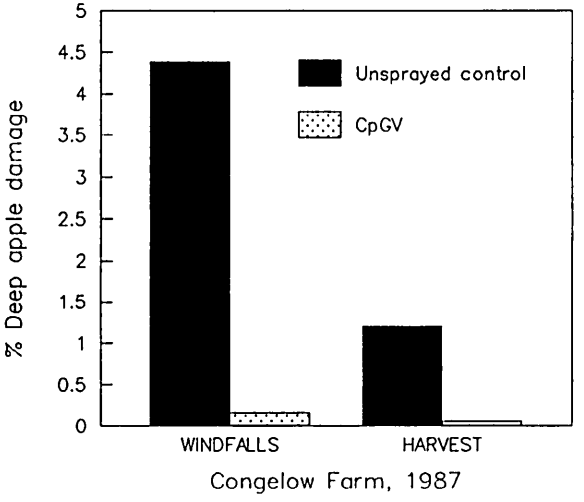


Fig. 9.11 Comparison of purified and impure preparations of CpGV at low dose

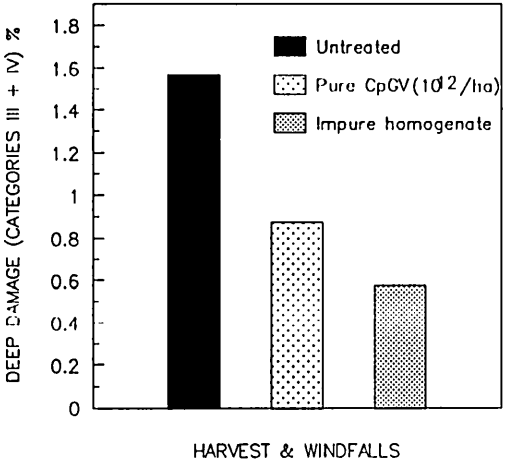


fIG. 9.12 Long term storage of CpGV

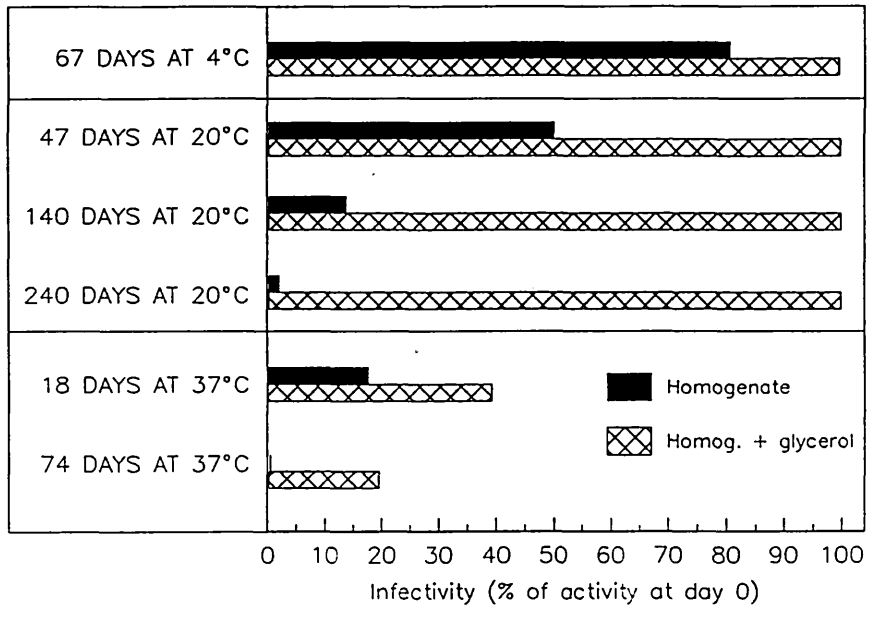
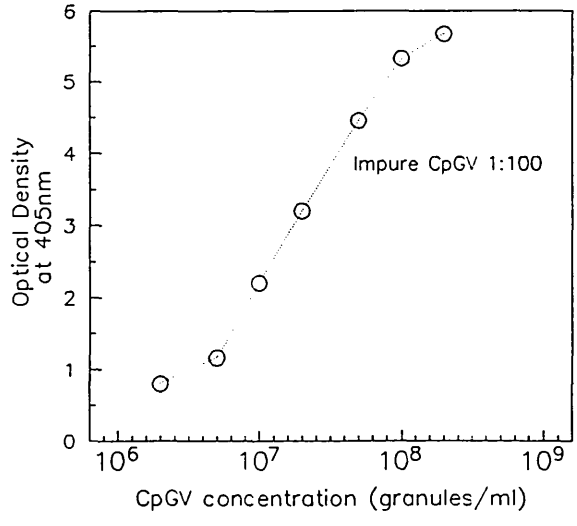


Fig. 9.13 Quantification of CpGV in impure homogenates by ELISA after low-speed centrifugation



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