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SID 5 Research Project Final Report

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Project identification

1. Defra Project code: HH3101TX

2. Project title:
   Novel strategies to exploit existing natural infections: synergisms between baculoviruses and other toxins.

3. Contractor organisation(s):
   NERC Centre for Ecology & Hydrology
   Mansfield Road
   Oxford
   OX1 3SR

4. Total Defra project costs (agreed fixed price): £ 279,503.00

5. Project: start date ..... 01 January 2003
   end date ..... 31 March 2007

6. It is Defra's intention to publish this form.
   Please confirm your agreement to do so..... **YES** ☒ **NO** ☐

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   Defra recognises that in a small minority of cases there may be information, such as intellectual property or commercially confidential data, used in or generated by the research project, which should not be disclosed. In these cases, such information should be detailed in a separate annex (not to be published) so that the SID 5 can be placed in the public domain. Where it is impossible to complete the Final Report without including references to any sensitive or confidential data, the information should be included and section (b) completed. NB: only in exceptional circumstances will Defra expect contractors to give a "No" answer.
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Executive Summary

7. The executive summary must not exceed 2 sides in total of A4 and should be understandable to the intelligent non-scientist. It should cover the main objectives, methods and findings of the research, together with any other significant events and options for new work.

Baculoviruses are a well studied group of insect viruses which have known advantages as biological control agents of insect pests in agriculture and forestry; strains used are very specific to the insect species they can infect, they can be formulated and sprayed conventionally and they are easily broken down by UV thereby leaving minimal non-toxic residues. They are the major group of viruses which infect insects but they do not replicate in vertebrates, plants or other micro-organisms. However, the very fact that they have a restricted host range and may take several days to kill an insect has meant baculoviruses have not been rapidly taken up or developed as alternatives to synthetic pesticides. The aim of this project was to investigate whether any other pathogens/toxins could synergise with baculoviruses and improve baculovirus efficacy against common *Brassica* pests such as the cabbage moth (*Mamestra brassicae*). This is a viable assumption as quiescent, symptomless baculovirus infections are common in caterpillars in the field and these have been shown to become fully lethal infections when triggered by another baculovirus. In addition, many pathogens and toxins (e.g. antifeedants) act by restricting growth or retarding development. This could act to extend the "window of vulnerability" of many pest caterpillar species to baculoviruses by delaying the onset of developmental resistance. This work contributes directly to Defra's aim of promoting sustainable methods of pest control by reducing inputs of chemical pesticides and the exploitation of biological alternatives.

Prospective pathogens/toxins were identified as potential triggers of persistent baculovirus infections. Representatives from each group of pathogen/toxin were tested in bioassays against caterpillar pests from *Brassica* crops (namely the cabbage moth (*Mamestra brassicae*), the silver Y moth (*Autographa gamma*), the large white (*Pieris brassicae*) and the cabbage looper (*Trichoplusia ni*)). The ability of pathogens/toxins to trigger persistent baculovirus infections in caterpillars and the nature of the interaction of potential triggers with an applied baculovirus *Pafl*NPV (*Panolis flammea* nucleopolyhedrosis virus) were tested in laboratory bioassays in small dishes and on whole plants in microcosms (laboratory insect rearing cages). Although it is known that the addition of a second baculovirus is a consistent trigger of persistent infections in *M. brassicae* our results suggest that other chemicals/pathogens do not exhibit the same effect with any degree of consistency. However, the chemical Spinosad and insect bacterium *Bacillus thuringiensis* (*Bt*) exhibited synergism with applied *Pafl*NPV as they consistently retarded host growth which in turn increased the likelihood that an insect would die of a *Pafl*NPV infection. It was hypothesised that this was due to the well recorded antifeedant effect of both Spinosad and *Bt* delaying the onset of developmental resistance and allowing more insects to become infected with baculovirus. When insects were exposed to Spinosad and *Bt* alongside *Pafl*NPV in microcosm experiments, the relationship between antifeedant and baculovirus became less clear. Difference between the results on whole plants and in contained dishes is suspected to be due to the ability of the caterpillars to avoid contaminated food or perform compensatory

feeding. Results investigating host immune responses indicated that PO activity was not significantly increased in the presence of DETC or *Bt*, despite the fact that DETC has been shown to suppress the host immune system.

The presence of persistent baculovirus infections in several moth species was confirmed using molecular methods developed during the project. Differences in occurrence of persistent infections were shown, both geographically and within species of Lepidoptera; a fact that has previously only been shown for *M. brassicae* and its persistent baculovirus infection. The screening of large numbers of Lepidoptera is essential to answer questions regarding the evolution and maintenance of these infections in nature and the possibility that triggering of persistent infections may occur in non-target caterpillars that are exposed to applied baculovirus could be an important factor in testing of the side effects of baculovirus biocontrol agents. It was not within the scope of this project to answer these questions but the development of protocols within the current project means we are now in a position to advance our knowledge in this area.

The transmission dynamics in *M. brassicae* of an applied baculovirus *Pafl*NPV, the synergists Spinosad & *Bt* and the persistent infection *Mb*NPV were investigated in a series of small plot caged field trials on spinach plants. The transmission of a baculovirus can be modelled using the mass action model which is based on the assumption that infection is acquired at a constant rate, so that eventually all individuals that are exposed become infected with virus i.e there is a linear relationship between infection and viral mortality. However, baculoviruses are known to have a non-linear transmission pattern in the field because some individuals are able to escape infection by being in a pathogen-free refuge. This refuge may be due factors such as a patchy distribution of virus in the field whereby individuals never encounter virus or a proportion of individuals are less susceptible to viral infection. We tested the hypothesis that the addition of *Bt* or Spinosad would reduce the pathogen-free refuge and result in a greater probability of viral infection within field caterpillar populations. There was no difference between applying the virus concurrently with *Bt* or with a 2 or even 4 day delay between applications on the likelihood that insects would die of a baculovirus infection but overall, there was increased host mortality due to virus in the presence of *Bt* suggesting that the toxin enhanced viral mortality. Combined mortality from the two pathogens was double that when only virus was applied and this increase in mortality was almost entirely due to increased viral deaths. The mechanism sustaining this synergism is not clear but further field studies showed there were significant reductions in host mass gain in the presence of *Bt* which in turn may delay the onset of virus developmental resistance, thereby keeping individual larvae in a “susceptible” pool. The slight synergism shown between Spinosad and *Pafl*NPV in laboratory tests did not extend to field conditions. The likelihood that an insect would succumb to a lethal virus infection was not significantly different in the presence or absence of Spinosad.

The ability of a baculovirus to self-perpetuate and cause lethal infections in subsequent generations of larvae is one of the long-term benefits of using baculoviruses for biocontrol. We hypothesised that reduction in host mass gain in the presence of *Bt* may negatively impact on the amount of virus produced per cadaver (i.e. viral yield) but there were likely to be more patches of virus over the plants (i.e. proportionally more insects die of virus) and each patch would be smaller than when *Bt* was absent (i.e. viral yield per cadaver was reduced in the presence of *Bt*). We thought this may be beneficial to viral transmission as an increase in patches of virus may increase the likelihood that an insect would encounter a viral patch.

Within the UK, *M. brassicae* generally have two generations within an average cropping season. To test the hypothesis that the combined application of *Bt* and baculovirus would achieve season long control of *M. brassicae*, we produced predictive models and simulated two generations of larvae in field trials on cabbage plants over a period of 5 weeks. The field experiment evaluated the transmission of virus from applied sprays to insects in the first generation and the subsequent transmission of virus from viral first generation insect cadavers to second generation larvae. First generation larvae were exposed to *Bt*, *Pafl*NPV or a mixture of the two pathogens. When these larvae died or pupated, a second generation was simulated by the introduction of eggs into the crop. The transmission dynamics of the virus in the presence of *Bt* in the first generation of larvae were the same as the previous trial; the proportion of insects dieing due to a viral infection was increased in the presence of *Bt*. The majority of insects died due to an *Mb*NPV infection suggesting this was due to triggering of the persistent infection by the applied baculovirus *Pafl*NPV. As the two trials were done using the same methods but in different years and on different host plants (spinach and cabbage) it suggests that the synergism between *Bt* and the baculovirus may not be affected by abiotic factors or host plant. However, more evidence would be needed to corroborate this implication. However, reductions in host mass gain in the presence of *Bt* in the first generation of larvae incurred a penalty to the virus in terms of reduced viral yield per cadaver and transmission to the second generation. Mortality due to virus in the second generation was lower in plots where first generation larvae had been exposed to *Bt*. This suggested that the effect of Bt on yield in the first generation was detrimental to the ability of the virus to cycle to the second generation. It is probable that insects die in areas where they have been feeding (as was observed in several plots during experiments) and this

increases the likelihood that other feeding larvae will come into contact with virus. Many, small patches would seem to be more beneficial in this case. However, the virus is subject to abiotic factors such as UV and precipitation which can inactivate and remove infectious material from the crop. This is likely to have a more significant effect on the potential infectivity of a patch of virus when it is a small patch compared to larger patches which can afford to lose some infective material. Interestingly, the fact that only two larvae succumbed to *Bt* infection in the second generation suggests that the efficiency of viral transmission was due to baculovirus alone and that the synergist was only acting in the first generation on host mortality. Similarly, the fact that the presence of *Bt* in the first generation had no impact on the weight of larvae in the second generation suggests that even sublethal effects were not occurring. As far as we are aware, this is the first time that the secondary transmission of a baculovirus in the presence of *Bt* has been demonstrated.

Conclusions

This study has shown that there is potential to achieve improved control of caterpillar pests in *Brassica* crops using combinations of insect control agents approved for used in organic crop production and baculoviruses. Our research considered a series of intricate interactions in both the laboratory and field between an applied baculovirus, a persistent baculovirus and potential synergist pathogens/toxins. Persistent baculovirus infections were not reliably triggered by the range of pathogens/toxins tested which has important implications as non-target Lepidoptera tested were shown to carry persistent baculovirus infections and may be exposed to low levels of toxins/pathogens applied for biocontrol. Field trials against the pest *M. brassicae* showed that suppression of first generation larvae could be improved by co-application of a baculovirus (*Pafl*NPV) and the bacterium *Bt*. Experimental evidence suggests that this is due to a delay in the onset of larval developmental resistance, induced by the presence of *Bt*. However, the presence of *Bt* reduced viral yield from first generation larvae and this was detrimental to secondary transmission of the virus, implying that longer term control of pests by the self-perpetuating virus would be reduced. These experiments have been undertaken with a specific life stage of *M. brassicae* and work would be needed to investigate the interactions at different instars and in more than one generation of the pest in order to make any recommendations regarding control of *M. brassicae* with a combination of virus and *Bt*. Overall, this research provides evidence that there is potential to reduce synthetic pesticide use or improved control achieved by organically approved products through the development of combinations of biopesticides as alternatives to, or in conjunction with, currently registered agronomic products for control of agricultural and horticultural pests.

Project Report to Defra

8. As a guide this report should be no longer than 20 sides of A4. This report is to provide Defra with details of the outputs of the research project for internal purposes; to meet the terms of the contract; and to allow Defra to publish details of the outputs to meet Environmental Information Regulation or Freedom of Information obligations. This short report to Defra does not preclude contractors from also seeking to publish a full, formal scientific report/paper in an appropriate scientific or other journal/publication. Indeed, Defra actively encourages such publications as part of the contract terms. The report to Defra should include:
   - the scientific objectives as set out in the contract;
   - the extent to which the objectives set out in the contract have been met;
   - details of methods used and the results obtained, including statistical analysis (if appropriate);
   - a discussion of the results and their reliability;
   - the main implications of the findings;
   - possible future work; and
   - any action resulting from the research (e.g. IP, Knowledge Transfer).

1. INTRODUCTION

Baculoviruses are a group of well studied insect specific viruses which have been developed as bioinsecticides over the last century for caterpillar control (Moscardi, 1999).They have advantages for use as biological control agents to control insect pests; strains used are very specific to the species they can infect, they can be formulated and sprayed conventionally and they are easily broken down by UV thereby leaving minimal non-toxic residues. Baculoviruses produce occlusion bodies (OBs) which are proteinaceous bodies that can survive outside the host for short periods of time but can easily be broken down by UV light if not protected. It is in this form that caterpillars ingest them along with plant material on which they are feeding. These occlusion bodies break down in the insect gut and release virus particles which cross the gut wall into the body tissues. There, they replicate until all body tissues have been converted into virus particles. The caterpillar then dies and liquefies, liberating new OBs over the foliage. Because of this life cycle, a baculovirus may take several days to kill an insect and this fact, alongside their restricted host range, has meant they have not been rapidly taken up by agrochemical companies.

Brassica crops within the UK are attacked by a number of pests and the most serious economic damage is caused by caterpillars. The main caterpillar pests are the small white butterfly (*Pieris rapae*), the large white butterfly (*Pieris brassicae*), the cabbage moth (*Mamestra brassicae*) and occasionally the diamondback moth (*Plutella xylostella*) and the Silver Y moth (*Autographa gamma*). Current control methods are based on applications of synthetic pesticides such as Chlorpyrifos. However, through Defra's aim to promote sustainable methods of pest control and the drive by consumers and environmental groups there is a need to reduce the use of toxic chemicals. Under such pressure, the use of baculoviruses as biopesticides is an area that is being re-visited (Szewczyk, 2006). This research project was initiated to develop novel control solutions for caterpillars in UK brassica crops based on baculoviruses. The aim of the project was to extend the use of baculoviruses by exploring potential interactions between them and other pathogens/toxins. The specific intention was to investigate the ability of other pathogens/toxins to synergise with baculoviruses and increase the level of viral kill of common pests in Brassica crops such as *M. brassicae*. There are two principle reasons to believe why they could form synergisms. The first is that quiescent, symptomless baculovirus infections have been shown in the Lepidoptera species *M. brassicae* in the field (Burden, 2003) and another baculovirus may trigger these into fully lethal infections. The second is that many pathogens and toxins (e.g. antifeedants) act by restricting growth or retarding development. This could act to extend the "window of vulnerability" of many pest caterpillar species to baculoviruses, delaying the onset of developmental resistance (e.g. Groove & Hoover, 2007). This will provide new pest control options, with minimal impact on non-targets and with low toxicity to mammals.

2. PROJECT OBJECTIVES

The overall purpose of this project was to extend the use of baculoviruses (insect viruses) as biological insecticides for pest control of caterpillars in UK Brassica crops by exploring potential synergisms between baculoviruses and other pathogens/toxins. The specific objectives of the project were as follows:

1) Investigate the potential of pathogens & toxins to trigger latent baculoviruses in populations of *Brassica* pests.
2) Investigate the potential impact of identified triggers on non-target Lepidopteran species likely to be found in field margins of *Brassica* crops.
3) Investigate synergistic/competitive interactions between baculoviruses and potential triggers.
4) Conduct short term field experiments to characterise the comparative transmission dynamics of baculoviruses and toxins in the initial time period following microbial sprays, and to investigate synergistic/inhibitory interactions in the field.
5) Develop current models of transmission to incorporate pathogen-toxin interactions; and to use these to make qualitative predictions about the control efficacy of pathogen combinations throughout the season.
6) Conduct "whole growing season" experiments to test qualitative predictions about the relative efficacy of pathogen combinations in the field.

There was a high degree of feedback between objectives 1, 3 & 4 during the course of the project. However, for the purposes of this report, objectives are presented separately.

3. POTENTIAL OF OTHER PATHOGENS AND TOXINS TO SUPPRESS THE HOST IMMUNE SYSTEMS AND TO TRIGGER LATENT BACULOVIRUSES IN POPULATIONS OF *BRASSICA* PESTS (Objective 1).

3.1 Candidate Pathogens/toxins as potential triggers (Year 1)

A literature review (Hesketh & Hails; in prep.) of the evidence for synergistic interactions between pathogens and toxins with baculoviruses for invertebrate biocontrol identified a number of primary candidate groups of organisms and toxins for experimentation. A representative of each group of pathogen/toxin was selected, which all had different primary modes of action on caterpillars (Table 1). All the selected pathogens/toxins (from now on referred to as potential triggers) can be applied using standard spraying techniques in the field and some are registered agrochemical products so are potentially immediately acceptable to growers.

Table 1: Pathogens & Toxins evaluated as triggers of persistent baculovirus infections in Brassica pest Lepidoptera

Potential trigger	Product	Active ingredient	Effects on host
Azadirachtin[1]	Neem	Plant limonoid	Potent antifeedant, toxic at high doses & insect growth disruptor
Boric Acid	-	Borate	Stomach poison & abrasive
Bacillus thuringiensis var. *kurstaki*	DiPel® DF[2]	Entomopathogenic bacterium	Toxic & antifeedant effects & dose-dependent mortality
Beauveria bassiana[3]	Isolate originally from Mycotrol® WP	Entomopathogenic fungus	Systemic infection & dose-responsive mortality
Diethyldithiocarbamic acid	DETC or DDCA	Sodium salt	Effects on host immune system
Panolis flammea NPV	-	Entomopathogenic virus	Systemic infection & dose-dependent mortality
Spinosyns A & D (from *Saccropolyspora spinosa*)	Spinosad®[4]	Toxins from actinomycete bacterium	Antifeedant & toxic –neurotoxin with dose-dependent mortality

[1] Kindly supplied by Prof. Steven Ley, University of Cambridge, UK; [2] Valent BioSciences Corporation;[3] Kindly supplied by Dr. Judith Pell, Rothamsted Research, UK; [4] Experimental sample kindly supplied by Dow Agrosciences

3.2 Leaf disc bioassays (Years 1 & 2)

A series of experiments were done to determine whether potential triggers could be used to reliably trigger persistent baculovirus infections present in Lepidopteran larvae into fully overt infections. CEH Oxford maintains lines of Lepidoptera in culture which harbour persistent baculovirus infections and larvae from four of those species were used for leaf disc experiments. In the first year of study, the species *M. brassicae* which carries *M. brassicae* NPV (*Mb*NPV) and *Trichoplusia ni* (the cabbage looper) which harbours the *T. ni* NPV (TniNPV) were used. During the second year, additional cultures of *P. brassicae* and *A. gamma* were established. The culture of *P. brassicae* harbours a persistent granulovirus infection (*Pb*GV) and the culture of *A. gamma* (established from field collected individuals) was also found to contain a persistent NPV infection. We are currently confirming the species of virus forming the persistent infection in *A. gamma* using molecular techniques but early indications are that it has most genes in common with a *Mamestra*-like NPV.

Larvae were reared under until they reached 2nd instar (*M. brassicae* & *A. gamma*) or 3rd instar (*P. brassicae* and *T. ni*) at which time all larvae were actively feeding. Larvae were exposed to increasing doses of potential triggers on small leaf discs of cabbage (*Brassica oleracea*) plants (var. "Greyhound") or perpetual spinach (*Spinachia oleracea*). A small cork borer was used to punch discs from leaf material which were mounted individually in 1% technical agar (weight:volume) in each cell of a 5x5 repli-plate. Each disc had 1µl of the potential trigger applied (diluted in 0.03% Tween 80) and was then allowed to air dry. A single larva was introduced to each compartment to the centre of the leaf disc and incubated for 24h at 23° C. Two replicate plates (i.e. 25 insects per replicate with 50 insects per treatment) were set up for each trigger & dose combination. The following day, larvae were carefully moved to individual 25ml "incubating pots" consisting of small polypots containing approximately $2cm^3$ synthetic diet. Larvae were then reared until death or pupation and monitored daily. Any viral deaths were recorded, collected and frozen for further analysis. The individual weight of 10 surviving larvae from each replicate (i.e. 20 larvae per treatment) was recorded 6 days following inoculation. A total of 21 bioassays were done to assess the effect of the 7 potential triggers on at least one of the four species of Lepidoptera larvae. A total of 3 replicate bioassays were done for *M. brassicae* and *T. ni* exposed to *Bt*. Data for mortality were expressed as a proportion of insects exposed at each pathogen dose and were then analysed using binomial errors in GLIM version 3.77. This allowed for the fact that fewer insects survive in the higher doses through exposure to a pathogen/toxin and hence the number of insects available to succumb to mortality from a triggered viral infection is also reduced. Data for the time taken for insects to die of viral infection were analysed using the general linear models function in MINTAB version 14.1.

The greatest mortality due to triggered virus (12.6% treated insects) was recorded when *M. brassicae* larvae were exposed to *Bt* on spinach and was negatively related to increasing dose (χ^2_1 4.350, p=0.037; Figure 1a). Mortality due to triggered virus was not related to dose in a replicate experiment on spinach (3.03% treated insects; χ^2_1=0.951, p=0.330) or when larvae were exposed to *Bt* on cabbage discs (0.71% treated insects; χ^2_1=1.229, p=0.268). When *M. brassicae* and *T. ni* were dosed with *Bt*, triggering was linked to host species but when the models take account of the fact that fewer insects survive the higher doses of *Bt* this effect became insignificant (χ^2_1=0.0943, p=0.759), indicating that host differences were due to differences in susceptibility to *Bt*. On one occasion, there was an increase in triggered viral mortality related to increasing dose when *T. ni* were exposed to *Bt* (χ^2_1=7.403, p=0.008; Figure 1b) but only 2.24% of insects succumbed to infection and the relationship was not observed in two further replicates of the experiment (Table 2). Tests using the entomopathogenic fungus *B. bassiana* failed to trigger the respective persistent viruses in both *M. brassicae* and *T. ni* (Table 3). Weight of *M. brassicae* larvae was significantly reduced upon exposure to *Bt*, Spinosad and Azadirachtin and was related to increasing dose (Table 2). Similar reductions in weight were found when *T. ni* larvae were exposed to DETC and *Bt* and when *A. gamma* was exposed to Spinosad (Table 2). Mortality due to Spinosad was approximately 50% of treated insects whilst that due to *Bt* differed depending on the species of larva used; *M. brassicae* were relatively resistant with less than 20% of treated individuals over the dose range dieing of *Bt* infections whilst *T. ni* were more susceptible with nearly 50% of insects dieing of *Bt* in the same dose range (0-10 x field rate).

In summary, other than the addition of another baculovirus, a reliable trigger could not be identified as there were no consistent relationships between triggered viral infections in different hosts and the presence/absence or dose of a pathogen/toxin applied. Promising significant reductions in host mass gain were recorded when insects were

exposed to pathogens/toxins with known antifeedant effects.

Figure 1: Probability of triggered viral infection (given that an individual has escaped a lethal *Bt* infection) in the presence of increasing doses of *Bt*. The recommended field rate of Bt is 32 IU's/μl.

a) *M. brassicae* (n=25 insects per repliate) **b) *T. ni* (n=25 insects per replicate)**

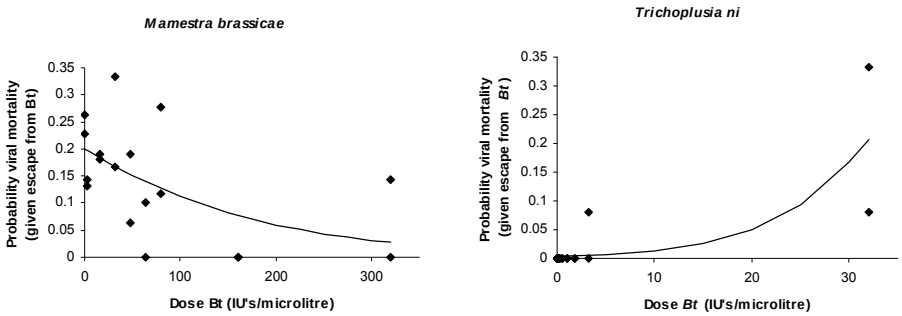


Table 2: Number of larvae of different Lepidoptera species succumbing to overt triggered viral infections and changes in larval weight when exposed to increasing doses of a number of pathogens & toxins identified as potential triggers of persistent viral infections.

Chemical or pathogen	Lepidopteran host	Range of doses tested (total no. doses in parentheses)	Inoculation plant material	Triggered virus* (%)	Pesticide or pathogen mortality* (%)	Effect of dose on triggered viral deaths	Reduction in weight (6 dpi) with increasing dose
Azadirachtin	*M. brassicae*	0-10^{-2}x FR[1] (4)	Spinach	3.23	69.70	Independent of dose χ_1^2 =2.403, p=0.121	Sig.; $F_{1,39}$=23.91, p<0.001
Beauveria bassiana	*M. brassicae*	1x10^3-1x10^7 conidia/ml (5)	Cabbage	0	33.91	-	-
"	*T. ni*	1x10^3-1x10^7 conidia/ml (5)	Cabbage	0	55.75	-	-
Bacillus thuringiensis	*A. gamma*	0-10x FR (9)	Spinach	0.67	30.79	Independent of dose χ_1^2 =3.297, p=0.069	-
"	*M. brassicae*	0-1x FR (4)	Spinach	3.03[2]	17.14	Independent of dose χ_1^2 =0.951, p=0.330	NS; $F_{1,42}$=0.87, p=0.357
"	"	0-10x FR (9)	Spinach	12.60	19.16	Decreased significantly with increasing dose χ_1^2 =4.281, p=0.039	Sig.; $F_{1,157}$=60.94, p<0.001
"	"	0–1x FR (11)	Spinach	1.84	10.13	Independent of dose χ_1^2 =2.725, p=0.099	Sig.; $F_{1,532}$=4.51, p=0.034
"	"	0-10x FR 11	Cabbage	0.71	12.56	Independent of dose χ_1^2 =1.229, p=0.268	Sig.; $F_{1,179}$=31.21, p<0.001
"	*P. brassicae*	0-10x FR (9)	Cabbage	1.62	65.45	Decreased significantly with increasing dose χ_1^2 =7.411, p=0.006	Sig.: $F_{1,114}$=23.75, p<0.001
"	*T. ni*	0-10x FR (9) 3rd instar	Spinach	0.22	45.17	-	Sig.:$F_{1,145}$=47.98, p<0.001
"	"	0-10x FR (9) 2nd instar	Spinach	5.01	48.98	Large no. control deaths	NS; $F_{1,107}$=2.44, p=0.122
"	"	0-1x FR (11) 2nd instar	Spinach	2.24	2.43	Increased significantly with increasing dose χ_1^2 =7.043, p=0.008	NS; $F_{1,515}$=0.24, p=0.624
DETC	*A. gamma*	0-500g/l (10)	Spinach	0	3.91	-	NS; $F_{1,199}$=3.12, p=0.079
"	*M. brassicae*	0-500g/l (9)	Spinach	2.02	8.67	Independent of dose χ_1^2 =0.011, p=0.918	Sig.; $F_{1,179}$=7.00, p=0.009
"	*T. ni* (3rd instar)	0-500g/l (10)	Spinach	0.20	1.81	-	Sig.; $F_{1,199}$=78.30, p<0.001
"	" (2nd instar)	0-500g/l (9)	Spinach	3.84	9.71	Independent of dose χ_1^2 =1.335, p=0.248	NS; $F_{1,179}$=0.43, p=0.512
Boric Acid	*A. gamma*	0-40 μg/ml (10)	Spinach	2.00	4.01	Independent of dose χ_1^2 =0.039, p=0.843	NS; $F_{1,198}$=0.48, p=0.488
"	*M. brassicae*	0-40 μg/ml (10)	Spinach	0.63	18.33	Independent of dose χ_1^2 =0.324, p=0.569	Sig.; $F_{1,198}$=3.87, p=0.050
Spinosad	*A. gamma*	0–1x FR (8)	Spinach	0.75	43.61	Independent of dose χ_1^2 =0.01, p=0.920	Sig.; $F_{1,128}$=70.81, p<0.001
"	*M. brassicae*	0-1x FR (4)	Spinach	3.13	52.78	-	Sig.; $F_{1,30}$=6.21, p=0.019

* Total number over all doses
[1] FR = Field Rate
[2] note that these bioassays were done concurrently and there was 23.81% viral deaths in controls

3.3 Whole plant bioassays (Years 1 & 2)

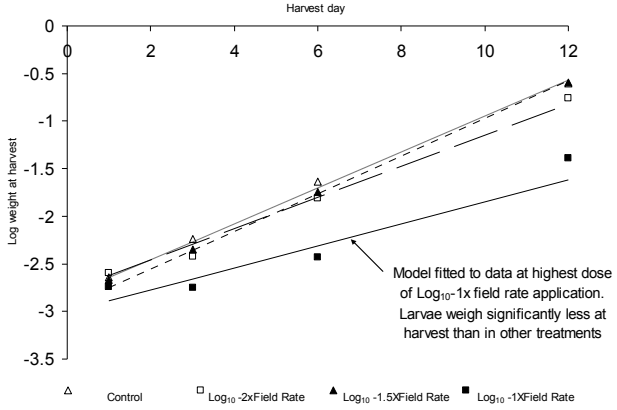
Three experiments were done in microcosms on whole plants to further evaluate the potential interactions between Spinosad and *Bt* with the persistent infection in *M. brassicae* and to investigate the sublethal effects of both potential triggers on the host. Both Spinosad and *Bt* caused a significant reduction in host mass gain in leaf disc experiments. The first experiment evaluated the effect of three doses of Spinosad on host mortality whilst the second expanded on this and evaluated the effect of 6 doses of Spinosad to obtain more detail on the sublethal effects of Spinosad. The third experiment investigated the sublethal effect of 7 doses of *Bt* on larvae (Table 3).

Table 3: Range of doses of the chemical Spinosad and bacterium *Bt* (DiPel) applied to *M. brassicae* 2nd instar larvae on whole spinach plants in experimental microcosms to evaluate sublethal effects and potential triggering of persistent viral infections.

Pesticide	Doses applied (x field rate)	Log dose (x field rate)	Harvest day
Spinosad Microcosm Exp. 1	Water only	0	0,1,3,6,12
	0.1	-1	"
	0.0316	-1.5	"
	0.01	-2	"
Spinosad Microcosm Exp. 2	Water only	0	6 &12
	0.1	-1	"
	0.079	-1.1	"
	0.063	-1.2	"
	0.05	-1.3	"
	0.04	-1.4	"
	0.0316	-1.5	"
DiPel Microcosm Exp. 3	1 (32 IU's/ml)	0	3 & 6
	0.776 (25 IU's/ml)	-0.11	"
	0.631 (20 IU's/ml)	-0.20	"
	0.501 (16 IU's/ml)	-0.30	"
	0.316 (10 IU's/ml)	-0.50	"
	0.174 (5.6 IU's/ml)	-0.76	"
	0.1 (3.2 IU's/ml)	-1.00	"

In each experiment, cabbage plants (5-6 leaf stage) were sprayed with either Spinosad (microcosm exp. 1 & 2) or *Bt* (as DiPel; microcosm exp. 3) at a number of doses below the recommended field rate for the product. Plants were sprayed to run off using a hand held mister sprayer at a rate of 1000L/ha to give full coverage of all leaves. A water control was applied first and then the product was applied to groups of 16 plants, with 2 replicates for each time/treatment combination. Plants were allowed to air dry and were then randomly assigned into groups of 4 with each group placed in a specialized insect rearing cage (BugDorm-1, 30x30x30cm) which have removable lids to allow easy access to experimental plants and larvae. Groups of 30 *M. brassicae* larvae (2nd instar) were weighed together and each group was added to a separate cage onto a leaf near the middle of the cage. Plants were destructively harvested at a number of time points up to 12 days following the introduction of larvae (Table 3). All live larvae were individually weighed and then incubated as in section 3.2.

Figure 2: Weight of retrieved larvae when exposed for different periods to increasing doses of Spinosad and maintained on whole spinach plants in microcosms.



When larvae were exposed to three doses of Spinosad in microcosm exp. 1 and harvested at four time points, there was a significant reduction in growth related to log dose and exposure time ($F_{1,34}$ =15.15, p< 0.001; Figure 2). There was no triggering of persistent virus in any treatment but mortality due to pesticide was recorded in excess of 80% of treated insects at the highest dose of Spinosad (0.1x field rate). In microcosm exp. 2, larvae were exposed to an increased number of doses of Spinosad but harvested on only two occasions following inoculation. The weight of larvae was not related to the dose of spinosad ($F_{1,402}$=1.01, p=0.317) but was significantly different between the two harvest dates ($F_{1,405}$=1390.98, p<0.001). There were no viral deaths recorded in any treatment but similarly, pesticide mortality was large (>47% retrieved insects) at the highest dose of Spinosad. When larvae were exposed to *Bt* at a range of 7 doses in microcosm exp. 3, there was a low amount of viral mortality due to triggering (1.49% exposed insects) but this was not related to dose of *Bt* applied (χ^2_1=0.003, p=0.956) or related to harvest date (χ^2_1=1.81, p=0.179). There was also low mortality due to *Bt* (18.88% retrieved insects) and this was not related to dose of *Bt* ($F_{7,23}$=1.66, p=0.168). There was a significant effect of harvest date on the weight of larvae ($F_{1,23}$=612.57, p<0.001) such that larvae harvested at the later time were heavier than those harvested earlier but this was not related to dose of *Bt* to which larvae were exposed ($F_{7,23}$=2.25, p=0.068).

In summary, the results from whole plant experiments were not as conclusive as those from Petri dish experiments; there were lower levels of triggering of virus recorded and this was not related to dose of synergist. Changes in host mass gain were also not related to dose of *Bt* or Spinosad applied.

3.4 Phenoloxidase activity (Year 1)

Potentially, pathogens/toxins may trigger persistent infections which do not result in an overt infection but in a sub-lethal response in the host. Whilst the insect immune system is relatively crude, there are a variety of antibacterial, antiviral and antifungal proteins that circulate in the haemolymph. The protein phenoloxidase (PO) is thought to be important in the immune response of insects and has been implicated in resistance to a range of pathogens, including baculoviruses, fungi and nematodes. We investigated the expression of PO as an indicator of sub-lethal effects of exposure of larvae to potential triggering pathogens/toxins.

To measure PO activity, a proleg was pierced on a larva using a very fine, sterile needle and the haemolymph was allowed to pool onto Parafilm^{TM}. Five µl of haemoplymph was added to 200µl of ice-cold phosphate-buffered saline (PBS) or to 150µl of PBS with 50µl of phenylthiourea (PTU) added to inhibit PO activity. Immediately, 900µl of L-Dopa (3,4-dihydroxy-L-phenylalanine) was added to each sample and the samples were then incubated for 25 minutes at 25°C. Following incubation, the light absorbance of samples was measured on a microplate reader at 490nm. PO activity was expressed as units/µl haemolymph, where one unit represents the amount of enzyme required to increase the absorbance by 0.001 per minute at 490 nm/minute (Hung & Boucias, 1996). PO activity was measured in 4th instar *M. brassicae* larvae for a total of 54 larvae exposed to increasing doses of *Bt* and a total of 35 insects exposed to increasing concentrations of DETC in leaf disc experiments set up as in section 3.2. PO activity was not significantly raised in the haemolymph of larvae exposed to *Bt* ($F_{1,50}$=0.75, p=0.390) or in those exposed to DETC ($F_{1,26}$=0.31, p=0.585).

3.5 Discussion and Conclusions

The relationship between the number of triggered viral infections and the presence/absence or dose of a pathogen/toxin was inconsistent between host insect and pathogen/toxin applied. The increase in mortality due to triggered virus with increasing dose observed when larvae of *T. ni* were exposed to *Bt* was inconsistent with replicate experiments as the number of triggered viral deaths was too low. There was a contradictory decrease in viral mortality of *P. brassicae* related to increasing doses of *Bt* whilst the relationship between dose of *Bt* and viral mortality in *M. brassicae* was also inconsistent with differences between replicate bioassays. Although it has previously been shown that the addition of a second baculovirus was a consistent trigger of persistent infections in *M. brassicae* (Burden *et al.,* 2003; Hughes *et al.*, 1993), our results suggest that other chemicals/pathogens do not exhibit the same effect with any degree of consistency. However, there were a restricted number of pathogens/toxins tested and a further programme of screening may identify more reliable triggers.

More promisingly, there were generally significant decreases of weight of larvae exposed on leaf discs to increasing doses of the pathogen/toxins which inhibit larval feeding i.e. Azadirachtin, Spinosad & *Bt*. Reducing host mass gain may slow the rate of onset of developmental resistance to an applied baculovirus infection and these individuals would need a lower dose of an applied baculovirus to cause a lethal infection compared to those that had not been exposed to the pathogen/toxin. We hypothesised that this may lead to an increase in the proportion of larvae succumbing to lethal baculoviruses when an antifeedant is present compared to when larvae are exposed to applied virus alone. This hypothesis was tested in the laboratory (objective 3) and field (objective 4).

A limitation of the leaf disc method is that insects are confined to small chambers and are unable to avoid contaminated leaf material. Therefore, further investigations of the relationship between two antifeedants (Spinosad & *Bt*) and host mass gain/viral triggering in *M. brassicae* were done on whole plants. A positive relationship between dose of antifeedant and host mass gain was observed on one occasion with Spinosad (but not in a second experiment). Despite positive results from leaf disc experiments, there was also no effect of *Bt* on host mass gain on whole plants. In addition, although some mortality due to triggered virus was noted, this was not dose related. However, mortality due to *Bt* was lower in microcosm experiments than in leaf disc experiments suggesting that even at high doses of *Bt*, there was little effect on the host. It is difficult to postulate whether sublethal effects such as activation of host immune responses were occurring and whether these were related to pathogen dose. Results investigating host immune responses indicated that PO activity was not significantly increased in the presence of DETC or *Bt*, despite the fact that DETC has been shown to suppress the host immune system (Washburn, 1963). The complexity of the relationship between host and antifeedant is further enhanced when insects are allowed to forage over whole plants. Potentially there is patchiness of antifeedant application which may allow host insects to avoid contact with the pathogen/toxin by moving to non-treated areas of the plant. The feeding rate of the host may vary and there may be compensatory feeding i.e. insects increase their feeding rates on plant material that is of "poor" quality with antifeedant present. In comparison, the dose received on a leaf disc in a confined area will be more constrained and the host has little opportunity to escape the antifeedant or to change its feeding rate.

4. POTENTIAL IMPACT OF IDENTIFIED TRIGGERS ON NON-TARGET LEPIDOPTERA SPECIES LIKELY TO BE FOUND IN FIELD MARGINS OF BRASSICA CROPS (Objective 2)

As work under objective 1 did not yield a consistently reliable trigger, in agreement with the defra project officer, work under objective 2 was purposefully limited for this study. Spinosad has already been tested extensively against non-target predators and parasitoid wasps and a recent review of the scientific literature has reported

results for 52 species of natural enemy (Williams *et al*., 2003). Similarly, the impact of *Bt* on non-target organisms is well recorded (e.g. Boulton & Otvos, 2004; Walker *et al*., 2007).

4.1 Non-target Lepidoptera collections (Years 2-3)

In order to gauge how widespread sublethal persistent infections are in non-target moths and butterflies, we undertook initial studies of several moth species to determine whether they carry persistent viruses in natural conditions. Samples were obtained from the Rothamsted Insect Survey national light trapping for moths where a series of nearly 100 light traps are positioned throughout the UK and are emptied daily. From the moth samples collected by Rothamsted, we selected a number of indicator species. These are moths that occur commonly throughout the UK, were abundant in moth trap catches from a number of different locations, fed on plant material likely to be found in field margins (e.g. herbaceous and grassy plants) and are not considered to be pests of brassicas or other field crops so can be classified as non-target Lepidoptera. These moths were; *Noctua pronuba* (Large Yellow Underwing), *Abrostola tripartita* (The Spectacle), *Diachrysia chrysitis* (Burnished brass), *Tyria jacobaeae* (the Cinnabar), *Spilosoma luteum* (Buff Ermine) and *Spilosoma lubricipeda* (White Ermine). Samples represented up to 8 different individuals of the same species that had been caught at each of 15 sites so that comparisons could be made between the proportions of individuals found to be carrying a persistent infection

4.2 PCR detection of baculovirus in non-target Lepidoptera (Year 3)

Following removal of wing material, whole moth cadavers were put through a series of surface washes of proteinase K in SDS (Sodium dodecyl sulphate) and water to remove any contaminating baculovirus on the external surfaces. A Nucleoplex^{TM} BAC automated DNA purification system (Tepnel Life Sciences) was used to extract DNA from moth cadavers using the Nucleoplex^{TM} plant DNA kit. Cadavers were macerated following washing in special DNA extraction tubes which contain a metal ball bearing and then tissues were disrupted through a process of lysis, beating in a bead beater and incubation at 65° C. The samples were then placed onto the instrument for processing using an automated protocol and DNA was eluted in 10mM Tris for immediate use or storage. DNA was extracted from a total of; 50 *N. pronuba*, 22 *A. tripartita*, 22 *D. chrysitis*, 22 *T. jacobaeae*, 22 *S. luteum* and 22 *S. lubricipeda*.

Sequences for NPV baculovirus polyhedrin gene were obtained from the National Centre for Biotechnology Information (NCBI). Polyhedrin proteins are major components of baculovirus occlusion bodies and should therefore be present and allow detection of NPVs within host insects. A primer pair was designed to amplify a 325 base pair region and was tested on a number of known Lepidoptera NPV's to confirm baculovirus detection. A second primer set was designed from published sequences of the highly conserved non-coding gene ribosomal 16s RNA to amplify a 420 base pair region. The presence of polyhedrin and 16s rRNA in test insects was confirmed through PCR using the two primer sets separately and PCR products were viewed using standard gel electrophoresis. Confirmation of a successful DNA extraction using detection of 16s rRNA allowed removal of false negatives from the data set. Conclusive results were obtained for all species except *T. jabobaeae* (Figure 3).

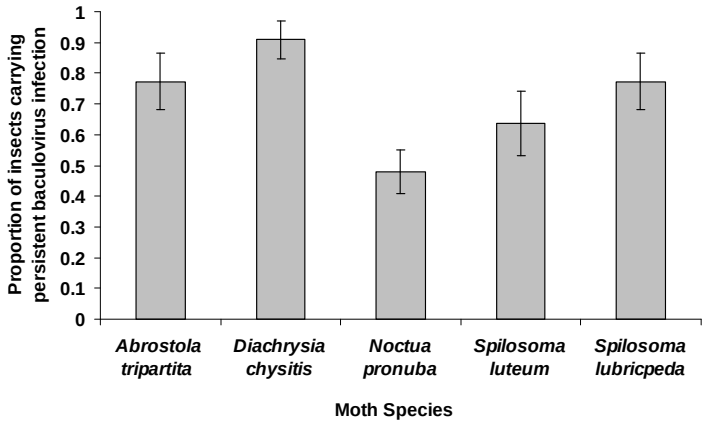


Figure 3: Total proportion of individuals from 5 moth species obtained from Rothamsted Research insect survey moth traps that tested positive for persistent baculovirus infections.

There was a significant difference between the moth species in the proportion testing positive for persistent baculovirus infection ($F_{4,37}$=2.66, p=0.048) but this was not related to the site from which individuals were collected ($F_{1,37}$=0.06, p=0.810) and there was no interaction between baculovirus infection and site ($F_{4,37}$=1.13, p=0.358).

4.3 Discussion and Conclusions

These results are important for several reasons. Firstly, for the first time it suggests that the presence of persistent infections is widespread, both geographically and has been confirmed within several species of Lepidoptera, a fact that has previously been shown in only one species namely *M. brassicae* with a persistent *Mb*NPV infection (Burden *et al*., 2003). This suggests that there is a possibility that persistent infections could be triggered in not only target but also non-target moths that are exposed to synthetic and/or biological pesticides and has very important implications for current practices of host-range testing. The impact of toxins and pathogens should be tested on

populations of Lepidoptera which carry a persistent infection as well as non-persistently infected individuals to obtain information regarding impact on non-targets. This does not imply that triggered baculovirus infections would then move into further susceptible moth populations; the occurrence of sporadic outbreaks of baculoviruses are infrequent (Tanada & Fuxa, 1987) as the transmission of baculovirus between hosts (horizontal transmission) is generally limited by host population density (Anderson & May, 1981). There may be geographical variation in susceptibility of hosts and also in virulence and genotypes of the same NPV which could increase or reduce the likelihood of baculovirus infections in natural populations (Fuxa, 2004). Nevertheless, as these results suggest that baculoviruses occur frequently as persistent infections, the screening of large numbers of Lepidoptera is essential to answer questions regarding the evolution and maintenance of these infections in nature. Other aspects are still unknown for example, whether these positive results indicate different NPV infections or whether is there a specific strain of baculovirus which can commonly persist at low levels and infect multiple species. It was not within the scope of this project to answer these questions but the successful development of protocols within the current project means we are now in a position to advance our knowledge in this area.

5. SYNERGISTIC/COMPETITIVE INTERACTIONS BETWEEN BACULOVIRUSES AND POTENTIAL TRIGGERS IN THE LABORATORY (Objective 3)

Promising results (sections 3.2 & 3.3) suggested that *Bt* and Spinosad could reduce host weight gains and may therefore reduce the onset of developmental resistance. However, the antifeedant nature of these potential triggers could also reduce the amount of virus that larvae consume (the primary method of viral infection) and may subsequently have a negative effect on viral transmission by reducing the number of successful viral infections. Alternatively, compensatory feeding may increase feeding rates and have a positive effect on pathogen transmission as the corresponding quantity of virus consumed is increased (as detailed in Boots, 2000). Fully factorial bioassays were used to investigate these questions and determine whether mortality was additive, synergistic or competitive in the presence of combinations of virus and other pathogen/toxins.

5.1 Synergisms I : leaf disc experiments

Materials and methods were as in section 3.2. Larvae of *M. brassicae* were exposed on spinach leaf discs in a 5x5 repli-plate to a range of doses of the baculovirus *Panolis flammea* NPV (*Pafl*NPV) in combination with a range of doses of Spinosad, *Bt* or DETC in a fully factorial experiment. To identify changes in the relationship between pathogen/toxin and *Pafl*NPV, doses of baculovirus were selected to give a range of final viral mortalities from 0-100% and low doses of the antifeedants represented those that caused significant reductions in weight gain from experiments done in sections 3.2 & 3.3. Within each experiment there were 62 treatment combinations and a control with 25 larvae in each treatment, giving a total of 1575 larvae per experiment.

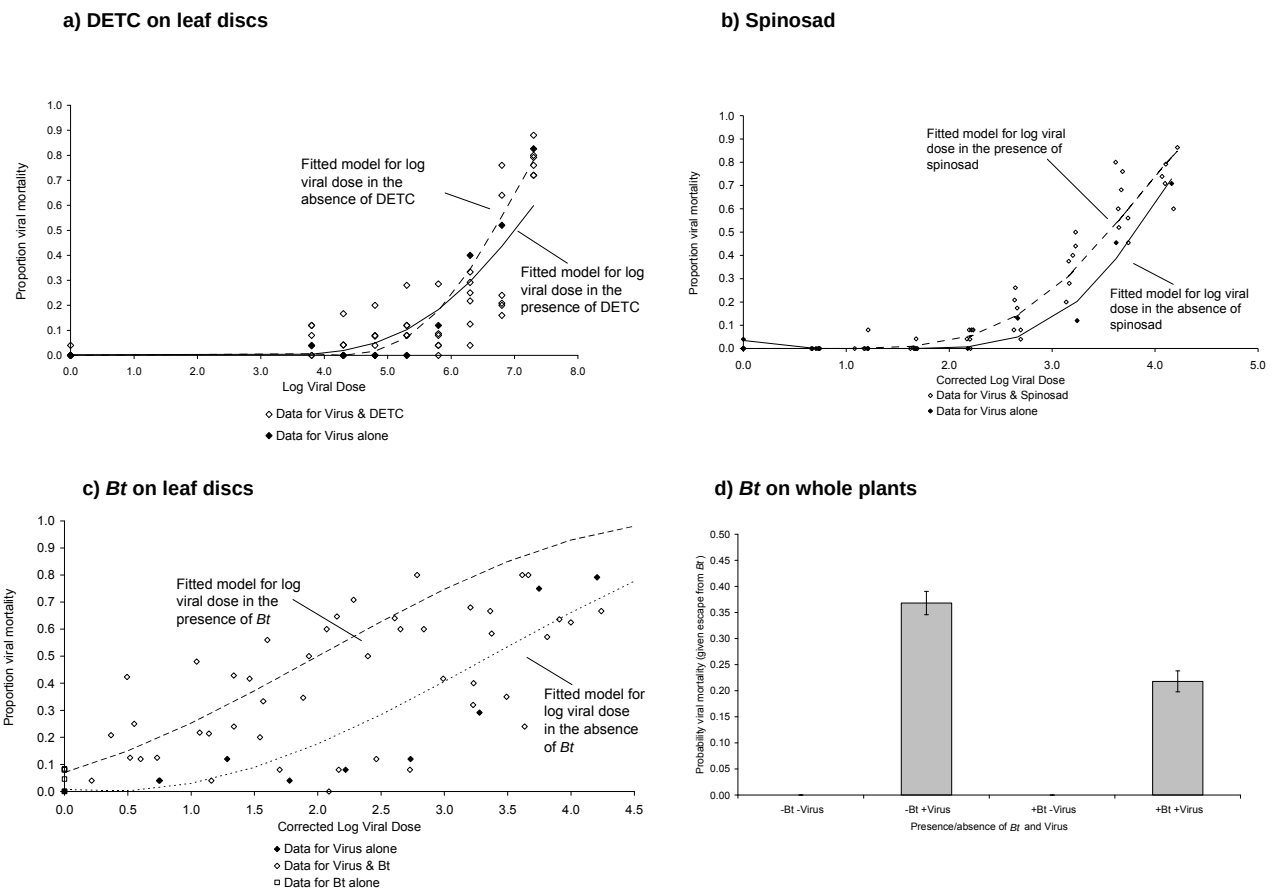
When larvae were inoculated with increasing doses of DETC and *Pafl*NPV, viral mortality was enhanced at low doses when DETC was present but this was not significant and at higher doses, the relationship was reversed ($F_{1,59}$= 0.04; p=0.841; Figure 4a). Total mortality (i.e. chemical and viral mortality) was not related to increasing dose of DETC (χ^2_1=0.104, p=0.747) but was related to increasing viral dose (χ^2_1=43.93, p<0.001). When *M. brassicae* larvae were inoculated with both Spinosad and *Pafl*NPV, viral mortality was enhanced when Spinosad was present, but this was not significant (χ^2_1=3.47, p=0.062; Figure 4b). There was no effect of Spinosad on total mortality (χ^2_1=0.056, p=0.813) but there was a significant effect of increasing dose of virus (χ^2_1=65.39, p<0.001). When *M. brassicae* were exposed to virus and *Bt* on leaf discs, an insect had a significantly greater chance of dying of virus when *Bt* was present than when only virus was present ($F_{1,61}$=13.34, p<0.001). There was a synergistic interaction between *Bt* and the virus in leaf disc experiments (Figure 5c). The total mortality of exposed insects increased significantly both with increasing dose of Bt (χ^2_1=46.13, p<0.001) and with baculovirus (χ^2_1=137, p<0.001) but there was no interaction between the toxins on total mortality

In summary, a synergistic interaction was recorded between *Bt* and *Pafl*NPV when the two pathogens were co-applied to *M. brassicae* larvae in leaf disc experiments. Interactions between Spinosad and DETC with *Pafl*NPV were not synergistic in this situation at the doses tested but equally, there was no evidence of antagonism between the baculovirus and chemicals.

5.2 Synergisms II : microcosm experiment

The interaction between *Bt* (3 doses) and the LD_{50} (lethal dose which kills 50% of treated insects) of *Pafl*NPV was assessed further in a microcosm experiment on whole spinach plants. The experimental set up was as in section 3.3 and larvae were harvested and weighed at two time points (3 & 6 days post exposure). Harvested larvae were incubated separately and any viral and bacterial deaths were recorded. When *Bt* and *Pafl*NPV were co-applied to whole plants the results were less clear than in Petri dish experiments. There was no triggering of persistent infections when *Bt* alone was applied (Fig. 4d). When virus was applied, the proportion viral mortality in the presence of *Bt* was significantly lower than in absence of *Bt* (χ^2_1=16.75, p<0.001).

Figure 4 Proportion of viral deaths in *Mamestra brassicae* dual inoculated with increasing doses of PaflNPV and a) DETC on leaf discs, b) Spinosad on leaf discs c) *Bt* on leaf discs and d) *Bt* on whole plants in microcosms.



5.3 Discussion and Conclusions

There was a synergistic relationship recorded *Bt* and *Pafl*NPV were co-applied in leaf disc experiments; with increasing dose of Bt, there was an increased proportion of larvae succumbing to viral infections. Although not significant, there was also a strong enhancing effect of Spinosad on viral mortality in similar leaf disc experiments which suggested that viral mortality was higher in the presence of Spinosad.

Both *Bt* and Spinosad have strong antifeedant effects and larvae exposed to these toxins on leaf discs consumed less plant material than those exposed to virus or water alone. This suggests that reduced feeding was not detrimental to insect contact with a lethal dose of virus although this may be due to experimental design as the confined space of the Petri dish would force larvae to come into contact with plant material that they may avoid otherwise. The promising synergistic interactions noted in Petri dish experiments were then investigated further on whole plants in experimental microcosms.

Interestingly, results from microcosm experiments when *Bt* and *Pafl*NPV were co-applied showed opposite effects to those recorded in the leaf disc experiments. With increasing doses of *Bt*, the proportion of larvae succumbing to viral infections reduced thereby suggesting that there was an antagonistic effect between the bacterium and virus. It is possible that larvae in microcosm experiments were able to avoid contaminated material and feed on areas of the plant which did not receive a spray of *Bt* & virus and therefore did not consume a lethal dose of virus. Data may have been biased against *Bt* mortality as at the highest doses of *Bt* very few insects were retrieved and although the assumption was made that they had died due to *Bt* before harvest, they were not included as mortality data because confirmation of mortality could not be made. The results may therefore either be due to real antagonistic effects or may be an artefact of experimental design. More detailed experiments to examine the sub-lethal effects of *Bt* in such microcosms may provide more information to explain why the results from the microcosm experiments differed from the Petri dish leaf disc experiments.

6. SHORT TERM FIELD EXPERIMENTS TO CHARACTERISE THE TRANSMISSION DYNAMICS OF BACULOVIRUSES IN THE PRESENCE OF OTHER PATHOGENS (Objectives 4 a), b) & c))

Mathematical models can be useful tools for understanding disease dynamics in insect populations. The transmission of a baculovirus can be modelled using the mass action model which assumes that transmission is strictly proportional to the product of the densities of healthy and infected larvae (Anderson & May, 1981). The assumption is that infection is acquired at a constant rate i.e. there is a linear relationship between infection and

mortality, so that eventually all individuals that are exposed become infected with virus. However, baculoviruses are known to have a non-linear transmission pattern which cannot be described by the mass action model as some individuals are able to escape infection thus forming the pathogen-free refuge (Hails et al., 2002). We hypothesised that the addition of Spinosad or *Bt* would reduce the pathogen-free refuge and result in a greater probability of viral infection within the larval population. These three patterns of transmission are illustrated in Figure 5 and were tested experimentally for Spinosad (Objective 4(a); Year 1) and *Bt* (Objective 6; Year 2 & 3) under field conditions.

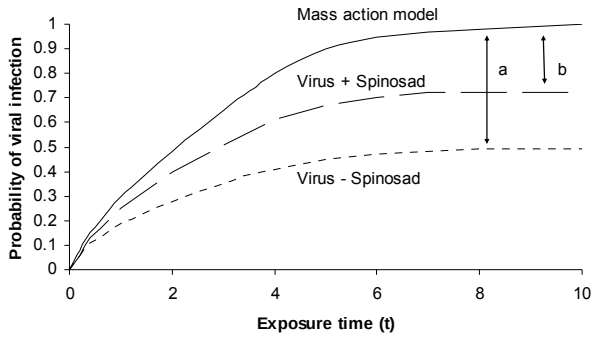


Figure 5: Hypothetical model of transmission rate of virus under three different conditions.
Virus alone (without the addition of Spinosad or *Bt*) has a transmission pattern lower than that of the mass action model and some individuals escape infection thus forming a "pathogen-free refuge" (a). We hypothesised that the addition of Spinosad or *Bt* would reduce the pathogen-free refuge to (b) and result in a greater probability of viral infection within the larval population.

We hypothesised that the addition of Spinosad or *Bt* may also reduce the size of infected larvae in the first host generation due to antifeedant effects. This in turn may reduce the yield of virus from cadavers produced in the first generation which may then reduce the number of larvae which become infected in the next generation. The number of individuals which become infected during secondary transmission from infected cadavers is known to be lower than when a viral spray is applied as the source of inoculum. The transmission of the pathogen requires both the host to be susceptible and for there to be contact between hosts and pathogens. The frequency of contact of insects with sprayed inoculum is higher than when inoculum is patchier as liquefied cadavers. We hypothesised that the number of secondary infections may be further reduced in the presence of Spinosad or *Bt*. The three patterns of transmission are illustrated in Figure 6 and were tested experimentally for Spinosad (Objective 4(b); Year 1) and *Bt* (Objective 4 (c) & 6; Years 2 & 3).

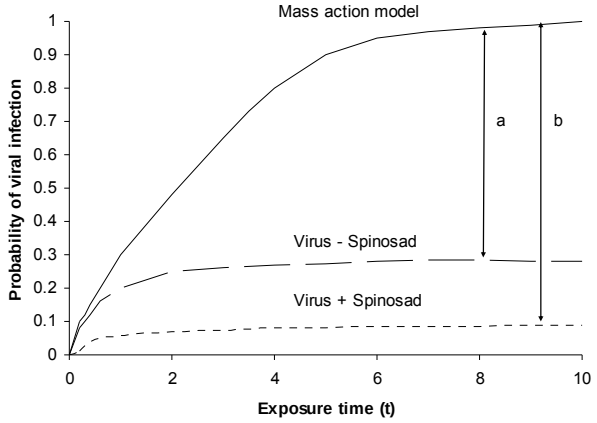


Figure 6: Hypothetical model of secondary transmission rate of virus under three different conditions.
Secondary transmission of virus alone (without the addition of Spinosad or *Bt*) is known to have a transmission pattern much lower than that of the mass action model because of the "pathogen-free refuge" (a). We hypothesised that the addition of Spinosad or *Bt* would increase the pathogen-free refuge to (b) and result in a lower probability of viral infection within the second generation larval population.

There is evidence in the literature that sequential dosing with a virus and bacteria is more effective at increasing viral mortality compared to concurrent application (McVay et al., 1977; Young et al., 1980). If baculoviruses are to be used in integrated pest management with synergists, the impact of application timing on transmission dynamics of the bacuclovirus must be evaluated. We therefore investigated the effect of the timing of application of *Bt* and *Pafl*NPV on the transmission of *Pafl*NPV in the first generation (Objective 4(c); Year 2). We hypothesised that delaying application of *Pafl*NPV following a *Bt* application would increase the proportion viral mortality overall. Those larvae that had recovered from a *Bt* infection or had a sublethal *Bt* infection were likely to be smaller than unaffected larvae and hence may die due to a lower dose of baculovirus.

6.1 Standard field materials and methods

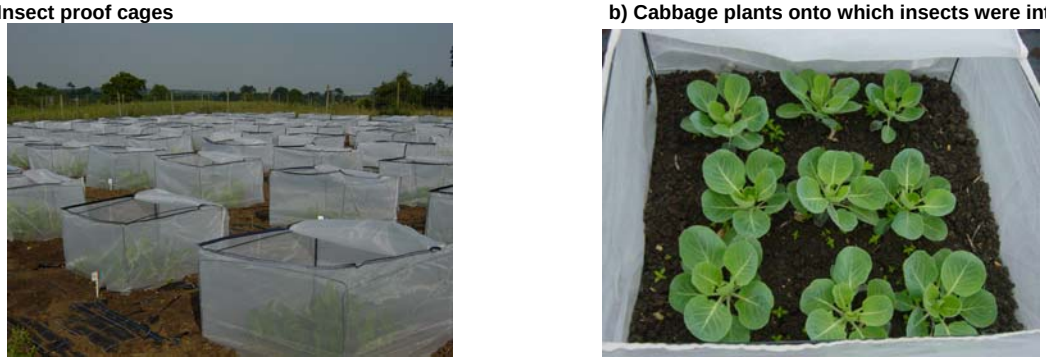
6.1.1. *Field cages and general organisation*

The same methods were used for the general organisation of field trials for all objectives. Specially designed insect proof cages were set up on a field site at Wytham, nr. Oxford, UK (Figure 7). The field trial area was covered with black weed suppressant matting which had plots cut out into which cage frames were sunk (to a height of 60 cm). Frames were covered with a fine insect mesh sleeve and had a Velcro opening along three sides to form a "lid" to allow access for manipulating plants and insects.

Fully randomised block designs were used for all experiments with three replicates of each treatment combination. Nine small plants (spinach or cabbage depending on the experiment) were planted in each cage approximately 2

weeks prior to experiments. Treatments were applied to seedlings using a hand held mister and sprayed until point of run off. All applications were made in 100ml water per plot (=1000L/ha) and plants were allowed to dry before the introduction of experimental insects. The rate of degradation of virus and chemical activity over the course of the experiment was evaluated in one set of plots (degradation treatments) and was used to correct for the amount of virus activity over time in the second set of plots (transmission plots). Control plots only received a water spray. At set time points (specific to each experiment), plots were destructively harvested and retrieved larvae were placed into individual rearing pots with synthetic diet and brought back to the laboratory for incubation. Larvae were monitored daily until death or pupation and any viral deaths were collected and frozen for future analysis.

Figure 7: a) Insect proof cages **b) Cabbage plants onto which insects were introduced**



6.1.2 *Methods for confirming Bt deaths*

A specific ELISA kit (enzyme linked immunosorbant assay; Bt-Cry 1Ab/Cry1Ac ELISA Kit, Abraxis LLC) was used to analyse cadavers that were suspected to have died due to *Bt*. ELISA is a biochemical technique which detects the presence of specific antigens (which produce an immune response) and produces a fluorescent signal which can be quantified. We used the kit to detect the toxins which are present in *B. thuringiensis* var. *kurstakii*, the strain upon which DiPel is based.

6.1.3 *Methods for confirming viral deaths*

A sub-sample of liquefied insect cadavers from viral deaths in each experiment was crudely purified using a series of SDS washes and centrifugation. Total genomic DNA was extracted using the Tepnel Nucleoplex automated DNA extraction machine as previously detailed (section 4.2). Sequences for the polyhedron gene of *Mb*NPV and *Pafl*NPV were obtained from the National Centre for Biotechnology Information (NCBI). A primer pair was designed to amplify a 200 base pair region with 100% identity from both *Mb*NPV and *Pafl*NPV sequences. A second primer pair was designed to be unique and specific for a region on the *Pafl*NPV genome. The proportion of *Mb*NPV and *Pafl*NPV in samples was quantified using the two sets of primers and real time PCR. Real time PCR was done using the appropriate primer pairs to quantify the sum of both *Mb*NPV and *Pafl*NPV or, in a separate run, the quantity of *Pafl*NPV alone in a viral mixture (detecting as few as 16 *Pafl*NPV genomes). The proportion of *Pafl*NPV in a mixture of *Pafl*NPV and *Mb*NPV could then be calculated.

6.1.4 *Quantifying viral yield*

For simple quantification, an estimate of yield of virus was obtained from crudely purified viral samples by counting the number of occlusion bodies per cadaver in an improved Neubauer haemocytometer. For more precise quantification of viral yield, larval cadavers were collected just before the larvae died of viral infection to ensure no virus was lost and the liquefied larvae were suspended in 1ml of sterile distilled water. The number of occlusion bodies was again estimated by counting in a haemocytometer. Viral yield was expressed as OB's per cadaver and differences between treatments were evaluated by comparing square root transformed data in MINITAB v 14.1.

6.2 Interactions between *Pafl*NPV and Spinosad in the field (Objective 4 (a); Year 1)

A randomised block design with three replicates of 7 treatments by 5 harvest dates was set up which gave a total of 105 cages. Treatments of Spinosad and/or *Pafl*NPV were applied as detailed in Table 4. Within each cage, 45 early second instar larvae of *M. brassicae* were released following spray applications by placing them at the centre of the plant at a density of 5 larvae per plant. Larvae were harvested at set time points (0,1,2,3 & 5 days post spray) and monitored until death or pupation.

Table 4: Treatments evaluated to determine whether Spinosad and *Pafl*NPV actsynergistically to increase viral mortality in M. brassicae exposed under field condition.

Treatment No.	Plot type	Virus	Spinosad
1	Transmission (Virus only)	$4.67x10^7$ OBs/plot	-
2	Transmission (Spinosad only)	-	0.01 x Field Rate
3	Transmission (Both)	$4.67x10^7$ OBs/plot	0.01 x Field Rate
4	Control Water	-	-
5	Degradation (Virus only)	$4.67x10^7$ OBs/plot	-
6	Degradation (Spinosad only)	-	0.01 x Field Rate
7	Degradation (Both)	$4.67x10^7$ OBs/plot	0.01 x Field Rate

A total of 3435 larvae were retrieved from plots, representing a return of 74% of introduced individuals. Of these larvae, a total of 984 (48.23% insects treated with virus) succumbed to a viral infection. There were no identifiable deaths due to Spinosad. When infection curves were fitted to data the transmission of virus was shown to be non-linear indicating that the likelihood of an insect sustaining a lethal viral infection was increased as exposure time was increased (Figure 8). The presence of Spinosad did not significantly affect the rate of transmission or the size of the pathogen-free refuge (Figure 8).

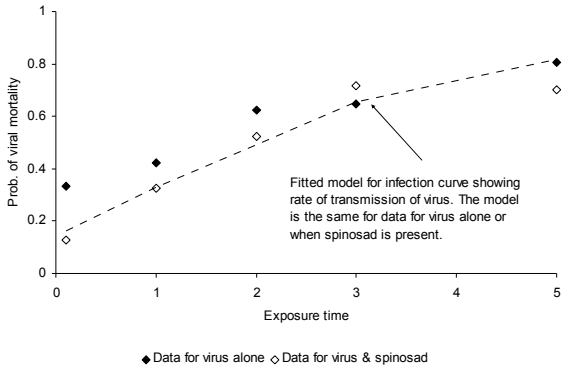


Figure 8: Transmission rate of virus in *Mamestra brassicae* in a field trial in the presence and absence of virus and/or Spinosad sprays.
Larvae were collected from plants between 0-5 days following introduction into plots. The 4 parameter model is not significantly different from the means model (χ_1^2 =10.6, d.f.=6, p=0.103) so the model can be constrained further to one infection curve which is not significantly different from the means model (χ_1^2 =12.2, d.f.=8, p=0.142). The probability of being susceptible to virus given survival is given by the expression $e^{-\upsilon W_0 t}$ where ν =transmission parameter, W_0 =density of pathogen at time zero and *t*= exposure time (Hails *et al.,* 2002). When a plot was harvested, the time was effectively stopped at time *t* and larvae were retrieved to the laboratory and monitored to determine whether they were susceptible or infected at that point. Some insects will remain uninfected by being in the pathogen free refuge for example and the probability of being uninfected given survival is given by the expression $\pi_R + (1-\pi_R)e^{-\upsilon W_0 t}$ where π_R =the fraction of the population in the pathogen-free refuge and is likely to be a function of W_o (Hails *et al.,* 2002). The fitted model for this experimental data is such that the probability of infection given survival at time t = $(1-0.836)e^{-0.174*t}$.

6.3 Secondary transmission of *Pafl*NPV in the presence of Spinosad in the field (Objective 4(b); Year 1)

A randomised block design with three replicates of 7 treatments by 5 harvest dates was set up which gave a total of 105 cages. Treatments were applied to spinach plants as detailed in Table 5. The same day, 1392 larvae of *M. brassicae* were inoculated in the laboratory by feeding them overnight with a lethal dose of *Pafl*NPV. To produce cadavers in the field, infected larvae were introduced to field plots the following day at a rate of two larvae per plant (18 larvae per plot) and were subsequently allowed to die *in situ,* whilst being exposed to Spinosad or water alone. Healthy non-infected larvae were used as controls. The time at which infected larvae died was assessed in a sub-sample maintained in containers in the field alongside plots. This marked the beginning of the experiment as time point 0. At this point, healthy larvae were introduced to all plots at a rate of 5 per plant (45 larvae per plot). The rate of degradation of virus and chemical activity over the following 5 days was evaluated in degradation plots and was used to correct for the amount of virus activity over time in the transmission plots. Larvae were harvested at each of 5 time points (0,1,2,3 & 5 days post infected insect death) and reared until death or pupation.

Table 5: Treatments evaluated to determine the interaction between Spinosad and *Pafl*NPV on secondary transmission of the virus from *M. brassicae* infected with *Pafl*NPV and exposed to Spinosad under field conditions.

Treatment No.	Plot type	Virus	Spinosad
1	Transmission (virus only)	18 infected larvae/plot	-
2	Transmission (Spinosad only)	-	0.01 x Field Rate
3	Transmission (both)	18 infected larvae/plot	0.01 x Field Rate
4	Control Water	-	-
5	Degradation (virus only)	18 infected larvae/plot	-
6	Degradation (Spinosad)	-	0.01 x Field Rate
7	Degradation (both)	18 infected larvae/plot	0.01 x Field Rate

The total number of larvae retrieved was 2490, representing a return of 53% of introduced experimental larvae and of these larvae, a total of 118 (4.74% insects treated with virus) succumbed to a viral infection. There were no identifiable deaths due to Spinosad. The fitted infection curves indicated that whilst secondary transmission occurs at much lower rates than primary transmission, it is also non-linear and not significantly different between treatments (Figure 9).

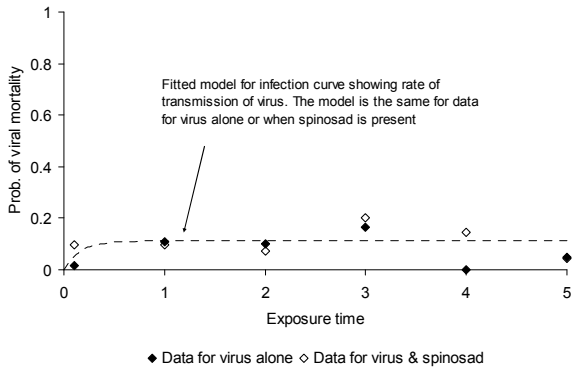


Figure 9: Secondary transmission of virus in *Mamestra brassicae* in a field trial in the presence and absence of Spinosad.
Larvae were collected from plants between 0-5 days following introduction into plots. The data can be constrained to a single infection curve which is not significantly different from the means model (χ_1^2 =8.63, d.f.=10, p=0.568). Fitted model: Probability of infection given survival at time t = $(1-\pi_R)e^{-\upsilon W_0 t}$ with $\pi_R = 0.888$ and $-\upsilon W_o$ =6.25.

6.4 Effect of application timing of *Bt* on transmission and nature of interaction with baculovirus (Objective 4 (c); Year 2)

A randomised block design was set up with 168 plots. Spray applications were made during July 2004 as detailed in Table 6. The dose of *Bt* (DiPel) used was 1kg/ha at an application spray volume of 1000L/ha. Application of *Pafl*NPV (LD_{50} dose $4.67x10^7$ OBs/plot) was made at the same time as *Bt* sprays or after a delay of either 2 or 4 days (Table 6). Plots were destructively harvested at set time points (0, 1, 2, 3 & 5 days post spray) and retrieved larvae were monitored until death or pupation. Any bacterial and viral deaths were collected and frozen for future analysis. Simple quantification of viral yield (total of 54 insects) was done for a sub-sample of viral deaths from transmission plots from each experimental block that had been treated synchronously or with a 4 day delay with virus alone or *Bt* & Virus.

Table 6: Treatments evaluated to determine whether *Bacillus thuringiensis* (DiPel) and *Pafl*NPV act synergistically to increase viral mortality of *M. brassicae* exposed under field conditions.

Treatment No.	Treatment	Date *Bt* sprayed	Date *Pafl*NPV sprayed
1	Transmission (Virus only)	-	14 Jul
2	Transmission (Virus only)	-	16 Jul
3	Transmission (Virus only)	-	18 Jul
4	Transmission (Virus & Bt – applied together)	14 Jul	14 Jul
5	Transmission (Virus & Bt – 2d delay)	14 Jul	16 Jul
6	Transmission (Virus & Bt – 4d delay)	14 Jul	18 Jul
7	Degradation (Virus only)	-	14 Jul
8	Degradation (Virus only)	-	16 Jul
9	Degradation (Virus only)	-	18 Jul
10	Degradation (Virus & Bt)	14 Jul	14 Jul
11	Degradation (Virus & Bt – 2d delay)	14 Jul	16 Jul
12	Degradation (Virus & Bt – 4d delay)	14 Jul	18 Jul
13	Control (Water)	-	-

The total number of larvae retrieved was 4623, representing a return of 61% of introduced experimental larvae and of these, a total of 881 (21.96 % insects treated with virus) succumbed to a viral infection. There were 96 suspected *Bt* deaths collected and analysis showed a total of 86% were positive for *Bt*. Data were corrected for this before further analysis. Infection curves were fitted to viral mortality data for each spray delay date i.e. data for simultaneous application, 2 day delay and 4 day delay applications of virus. Analysis showed that the three sets of data could be described by the same model and so all data were combined for further analysis. The fitted infection curves indicated that whilst the rate of infection was no different when *Bt* was present or absent, significantly more larvae succumbed to viral infection when *Bt* was present than when virus was applied alone i.e. the number of insects that were able to escape infection was reduced when *Bt* was applied with the virus (Figure 10). This was evident when considering total mortality due to pathogens which was higher in all treatments when Bt and PaflNPV were co-applied compared to when Bt was absent (Table 7).

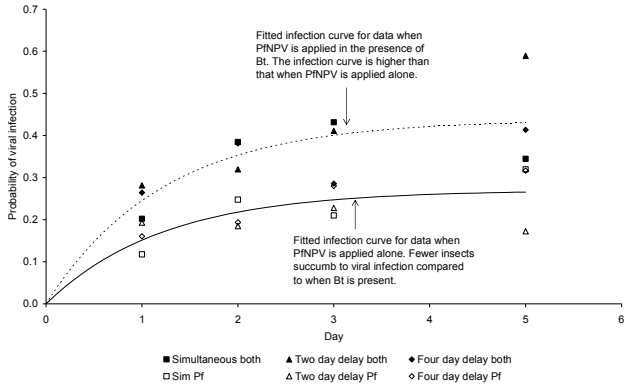


Figure 10: Probability of infection with a virus in *Mamestra brassicae* in a field trial in the presence and absence of *Bacillus thuringiensis* (DiPel).

Larvae were collected from plants between 0-5 days following introduction into plots. Delaying application of *Bt* either 2 or 4 days following viral applications had no significant effect on viral mortality. A model with 2 refuges and 1 transmission parameter was not significantly different from the means model (χ_1^2 =14.1, d.f.=21, p=0.864) but those two refuges are significantly different from each other (χ_1^2=26.0, d.f.=1, p<0.001). Fitted model: Probability of infection given survival at time t = $(1-\pi_R)e^{-\upsilon W_0 t}$. In the presence of *Bt* $\pi_R = 0.562$ and $-\upsilon W_o$ =0.822. and in the absence of *Bt* $\pi_R = 0.730$ and $-\upsilon W_o$ =0.822. The pathogen free refuge was therefore reduced by 33% in the presence of *Bt*.

Table 7: Mortality causes of larvae retrieved from transmission field plots treated with *Pafl*NPV, *Bt* or both pathogens

Treatment	No. Larvae retrieved	No. larvae exposed (-missing & handling deaths)	No. viral deaths	No. bacterial deaths	% viral deaths (of exposed)	% bacterial deaths (of exposed)	% total mortality due to pathogens (of exposed)
Virus	326	318	72	0	22.64	0	22.64
Virus (2d delay)	308	312	61	0	19.55	0	19.55
Virus (4d delay)	337	322	76	2	23.60	0.62	24.22
Bt & virus (co-applied)	390	377	129	24	34.22	6.37	40.58
Bt & virus (2d delay)	301	289	113	18	39.10	6.23	45.33
Bt & virus (4d delay)	249	240	83	9	34.58	3.75	38.33

The proportion of *Mb*NPV and *Pafl*NPV was calculated for a total of 235 cadavers representing approximately 25% of all viral deaths. There was a large proportion of *Mb*NPV in the insects tested with a mean proportion *Mb*NPV per cadaver of 79.67% (SE Mean=2.31). The proportion of *Mb*NPV present in any single cadaver ranged from as low

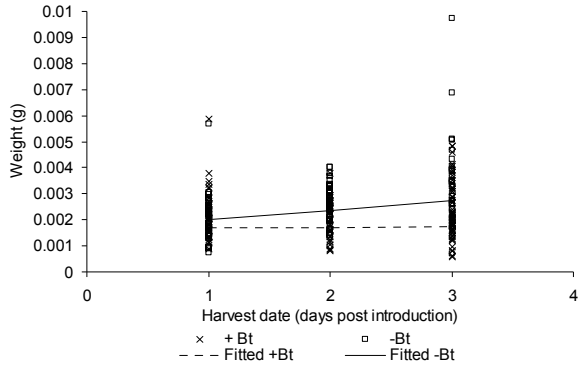
as 0.80% to 100%. However, there was no significant difference between blocks ($F_{1,119}$=0.10, p=0.748), treatment ($F_{5,119}$=1.03, p=0.405) or the harvest time ($F_{1,119}$=0.21, p=0.649) on the occurrence of *Mb*NPV within cadavers. Viral deaths were therefore a mixture of triggered and applied virus but it was not possible to predict from the treatments applied which virus would occur.

Viral yield was not related to treatment ($F_{3,49}$=0.18, p=0.906; Figure 11) but was related to the time at which an insect died ($F_{1,49}$=45.90, p<0.001). Larvae that took longer to die produced a significantly greater yield of virus but these differences were the same whether *Bt* was present or absent ($F_{1,50}$=0.14, p=0.714).

6.5 Effect of *Bt* on host mass gain in the field (Objective 4 (c); Year 2)

To evaluate the effect of *Bt* on host mass gain, a separate experiment was run alongside that detailed in section 6.4. A total of 20 cages were set up in a randomised block experiment with two blocks with a single replicate plot for each treatment within a block. Larvae of *M. brassicae* were introduced to plots at a rate of 45 per plot (5 per plant) and the following day, spray applications of *Bt* (DiPel) were made to plants using 1kg/ha chemical at an application spray volume of 1000L/ha. Larvae were harvested for 3 successive days following inoculation and retrieved larvae were weighed individually. When larvae were exposed to *Bt* they were significantly smaller compared to larvae exposed only to water ($F_{1,250}$=7.18, p=0.008; Figure 12). There was a significant interaction between *Bt* and harvest date ($F_{1,250}$=20.30, p<0.001); those larvae which were not exposed to *Bt* were heavier at each successive harvest than those exposed to *Bt.* Larvae exposed to *Bt* maintained a lower weight over the three harvest dates.

Figure 12: Weight of M. brassicae larvae at harvest when exposed to Bt on spinach plants in field trials for 1-3 days



6.6 Discussion and conclusions

The slight synergism shown between Spinosad and *Pafl*NPV in laboratory tests did not extend to field conditions; the likelihood that an insect would succumb to a lethal virus infection was not significantly different in the presence or absence of Spinosad. However, there was no detrimental effect on secondary transmission of the virus in the presence of Spinosad suggesting that the two methods of control could be co-applied successfully in an integrated approach to insect control.

When *Bt* was co-applied with *Pafl*NPV, there was increased host mortality due to virus in the presence of *Bt* suggesting that the bacterium enhanced viral mortality. Viral mortality of larvae was due to a mixture of triggered virus (*Mb*NPV) and applied virus (*Pafl*NPV) but it was not possible to predict from the treatments applied which virus would occur. The mechanism sustaining the synergism between *Bt* and baculoviruses is not clear; some authors suggest that there may be positive interactions at the insect gut membrane (Granados *et al.,* 2001). Other studies have shown that a positive interaction between *Bt* and a baculovirus is dependent on the sequence in which the inoculations are made (McVay *et al.,* 1977; Young *et al.,* 1980). However, in our field trials there was no difference between applying the virus concurrently with *Bt* or with a 2 or even 4 day delay between applications. This increases the opportunities for control of Lepidoptera pests as the two control agents can be applied in single tank mixes.

The ability of the virus to self-perpetuate and cause lethal infections in subsequent generations of larvae is one of the fundamental benefits of using baculoviruses for biocontrol. Despite larvae being smaller in the presence of *Bt*, the yield of virus per cadaver was not adversely affected. Although differences in host size may have a significant effect on the number of insects that succumb to infections within the first generation, this would suggest that there is no impact on the quantity of virus in the field that may be available to the next generation. However, the data on the size of insects was collected in a different trial to that in which viral yield was estimated and in addition, virus may have been lost during crude purification of viral samples. There is generally a lot of statistical error in viral yield data so it is possible that our data were not sufficiently powerful to detect differences between treatments. Further field trials investigating the effect of *Bt* on cadaver size and viral yield were therefore done under Objective 6.

7. DEVELOPMENT OF WITHIN-SEASON MODELS TO DESCRIBE CONTROL EFFICACY OF PATHOGEN COMBINATIONS (Objective 5).

The aim of this part of the work was to develop models that could be used to describe the pathogen and host dynamics within a single field season, such that the model would encapsulate information regarding the transmission of the baculovirus in the presence of another pathogen or toxin. A paper relating to this work is currently in press in the Journal of Applied Ecology (Raymond *et al.,* 2007) and contains additional details of the mixed and rotational spray models which are summarised below.

7.1 Simulation modelling of mixed & rotational sprays of *Bt* and baculovirus (Years 1-3)

Simulation modelling was used to investigate a range of different biocontrol strategies which may be used in an IPM scenario. The strategies investigated were; simultaneous sprays of an NPV and *Bt* toxin Cry1Ac and alternating sprays of NPV and *Bt* where the virus was treated either as a separate application on each occasion or allowed to persist to create an epizootic within the host population (i.e. a function of secondary transmission was included). The model also incorporated frequency of resistance to *Bt*, pest population size and different sized pathogen free refuges. Within the model there was no migration in or out of the modelled population and there was no density of planting taken into account. The generation of pests were discrete and non-overlapping and an arbitrary density-dependent spray threshold was set. Models were run for 200 generations of the pest.

7.2 Further development of a within-season model of pathogen-synergist dynamics (Year 3)

Based on results from field trials in sections 6 & 8, further model development is proposed. The within-season model was designed to evaluate the dynamics in a host (N)-pathogen (V) relationship as induced by the presence of a synergist (S). There are two main components of the model. The first relates to the individual host mass gain of a larva, i.e. the change in weight of larvae relevant to the presence and absence of the synergist and virus. The second component relates to the population dynamics of the host, pathogen and the synergist. This model can be represented by a series of equations.

Individual host mass gain (m) increases through the acquisition of resources i.e. host feeding at a rate (a) according to an algometric scaling (e.g. $m^{3/4}$). However, mass must be lost through insect catabolism (the metabolic process that breaks down molecules into smaller units) at a set rate (b) and also through infection with a synergist which in this case we have considered to be a non-lethal synergist ($\eta \cdot S(t)$).

$$\frac{dm}{dt} = a \cdot m^{\phi}(t) - b \cdot m(t) - \eta \cdot S(t)$$

The dynamics of the host-pathogen interation follow a modifield Lotka-Volterra (predator-prey model) framework. Host numbers increase at a rate p but will also decline due to infection with the lethal pathogen (i.e. the baculovirus). Overall, transmission of the baculovirus can be enhanced by the effects of the synergist ($\beta + \theta \cdot S(t)$).

$$\frac{dN}{dt} = p \cdot N(t) - (\beta_0 + \theta \cdot S(t)) \cdot N(t) \cdot V(t)$$

The cadavers produced by a lethal infection with a baculovirus will produce a yield of virus which may infect other individuals. This yield γ will be a function of the mass of the individual insect (m) and the decay that will occur as the free-living baculovirus is in the environment and subject to UV degradation. This decay will be at rate d_v .

$$\frac{dV}{dt} = \gamma(m) \cdot (\beta_0 + \theta \cdot S(t)) \cdot N(t) \cdot V(t) - d_v \cdot V(t)$$

The dynamics of a non-lethal synergist i.e. a *Bt* infection for example that causes host mortality will follow a simple logistic population growth.

$$\frac{dS}{dt} = r \cdot S(t) \cdot \left[1 - \frac{S(t)}{K} \right]$$

7.2 Discussion and Conclusions

Results from the strategy model suggested that the strategy of involving rotation of NPV and *Bt* but allowing for the development of an epizootic was effective over a range of conditions. By allowing persistence of the baculovirus, *Bt* spray frequency could be reduced by approximately 20% relative to the rotational models. This has important implications for biocontrol strategies as it suggests that providing a replicating pathogen such as a baculovirus can persist within a crop sufficiently, it may be combined effectively with another toxin/pathogen to provide effective pest control but with additional benefits of preserving natural enemy populations. The mixed spray strategy with varying doses of NPV showed that resistance to *Bt* could quickly become a problem when the survival of resistant insects exceeded that of susceptible ones.

In the within-season model, the synergist is capable of replication but this will be adapted further to include cases when the synergist is applied as a control spray either once or repeatedly. Using this framework, we are exploring the conditions under which a) a synergist and baculovirus persist within the host population, b) the synergist and baculovirus may act synergistically and antagonistically to achieve improved pest control, c) when the synergist is applied as a spray, the optimal regime for host pest control and d) the vertical transmission of persistent infections (i.e. transovarial transmission to the offspring) and their reactivation within the field (linking to models by Bonsall et al., 2005). The effects of persistent baculoviruses converting from persistent to overt infections and cycling within a host population have not been included. Data obtained in laboratory and field experiments (section 3 & 6) suggested that there was a significant effect of *Bt* on host mass gain both in the laboratory and under field conditions. The model takes account of host mass and the impacts of viral yield changes (see section 8.1) in the presence of the synergist *Bt*. The model may be used to investigate the interactions between an applied baculovirus and a synergist such as *Bt*. To further progress this area, more field manipulation experiments would be required and the results fed back to further develop the theoretical model identified above.

8. Transmission dynamics of baculovirus in the presence of *Bt* in field simulated season long control (Objective 6)

Within the UK, *M. brassicae* generally have two generations within an average cropping season. To investigate the impact of combining *Bt* and baculovirus to achieve season long control of this pest, we simulated two generations of larvae over a period of 5 weeks in summer 2006. The first generation were introduced as second instar larvae to the crop and were allowed to develop until death or successful pupation. Following this, the second generation was simulated by the introduction of eggs into the crop. The experiment was designed to evaluate the transmission of virus from applied sprays to insects in the first generation and then the subsequent transmission of virus to the second generation from viral cadavers of first generation larvae. The field trial therefore consisted of two sets of plots; one set to evaluate primary transmission in the first generation and one set to evaluate subsequent secondary transmission to the next generation.

8.1 Transmission of baculovirus in presence of *Bt*; first generation *M. brassicae* larvae (Objective 6; Year 3)

A randomised block design with three replicates was set up which gave a total of 252 cages for both first and second generation studies. Cabbage seedlings (var. Castello) were planted in each cage as the host plant and treatments were applied as detailed in table 7. For the first generation of larvae, 45 early second instar larvae of *M. brassicae* were released into each plot by placing them at the centre of each plant at a density of 5 larvae per plant, giving a total over the experiment of 117 plots and 5265 introduced larvae. Plots were harvested and larvae retrieved at 6 time points (Table 8). From each plant, 1-2 retrieved larvae were weighed individually at harvest.

Table 8: Treatments evaluated to determine whether *Bt* and baculovirus act synergistically under field conditions and the impact of *Bt* on secondary transmission of the baculovirus.

1st Generation			ALL LARVAE INTRODUCED TO PLOTS ON 26TH JUNE	
Treatment No.	Treatment		Spray date	Harvest date
1	Transmission	Virus	27th Jun	1,2,3,5,7,10 days post spray
2	Transmission	Bt	"	"
3	Transmission	Virus & Bt	"	"
4	Control Water	Water only	"	"
5	Degradation	Virus	"	"
6	Degradation	Bt	"	"
7	Degradation	Virus & Bt	"	"
2nd Generation			ALL EGGS INTRODUCED TO PLOTS ON 11th JULY	
Treatment No.	Treatment		Spray date	Harvest date
1	Transmission	Virus	27th Jun	20,23,25,27,29,31 days post spray (=6,9,11,13,15,17 days following egg introduction)
2	Transmission	Bt	"	"
3	Transmission	Virus & Bt	"	"
4	Control Water	Water only	"	"
5	Degradation	Virus	"	"
6	Degradation	Bt	"	"
7	Degradation	Virus & Bt	"	"

The total number of larvae retrieved from all plots was 3441 which represents a return of 63.20% of introduced individuals. Of these larvae, a total of 278 succumbed to viral infection of which 181 were in transmission plots. Mortality due to *Bt* was confirmed in a total of 264 larvae making the total mortality due to pathogens 15.75% of retrieved larvae. The total mortality due to pathogens was increased to 44.06% when Bt & PaflNPV were co-applied, compared to when Bt alone (23.43% mortality) or PaflNPV alone (20.53% mortality) were applied (Table 9). The proportion of *Mb*NPV and *Pafl*NPV was calculated using molecular methods in a total of 100 cadavers. There was a large proportion of *Mb*NPV in the insects tested with a mean proportion *Mb*NPV per cadaver of 99.88% (SE Mean=0.071). The proportion of *Mb*NPV present in any single cadaver ranged from 93.58% to 100%.

Table 9: Mortality causes of first and second generation larvae retrieved from experimental field plots treated with *Pafl*NPV, *Bt* or both pathogens

Treatment	No. Larvae retrieved	No. larvae exposed (-missing & handling deaths)	No. viral deaths	No. bacterial deaths	% viral deaths (of exposed)	% bacterial deaths (of exposed)	% total mortality due to pathogens (of exposed)
1st Generation							
Virus + Bt	390	379	66	101	17.41	26.65	44.06
Bt	374	367	2	84	0.54	22.89	23.43
Virus	576	565	113	3	20.00	0.53	20.53
2nd Generation							
Virus + Bt	844	816	111	0	13.60	0	13.60
Bt	1115	1087	7	1	0.64	0.09	0.74
Virus	508	488	122	0	25.00	0	25.00

There was no significant difference between blocks ($F_{1,84}$=0.60, p=0.439), treatment ($F_{4,84}$=0.75, p=0.564) or the harvest time ($F_{5,84}$=0.84, p=0.524) on the occurrence of *Mb*NPV within cadavers.

When infection curves were fitted to data the transmission of virus was shown to be non-linear indicating that the likelihood of an insect sustaining a lethal infection was increased as exposure time increased (Figure 13). Whilst there was a similar increase during the first 4 days following inoculation, the rate continued to increase when *Bt* was present compared to when only virus was applied where the rate reached a plateau after approximately 4 days harvest. Overall, significantly more larvae succumbed to viral infection when *Bt* was present than when virus was applied alone (Figure 13).

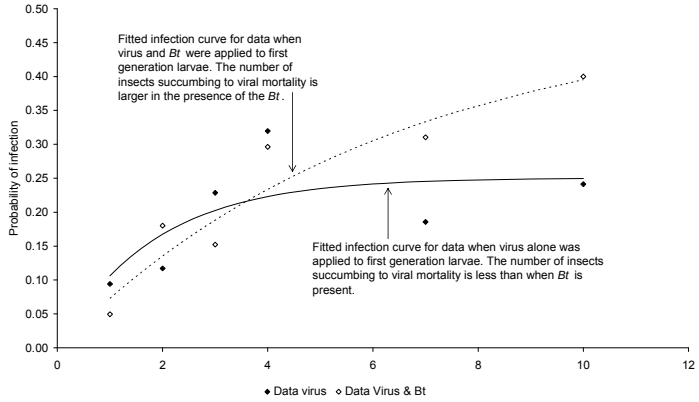
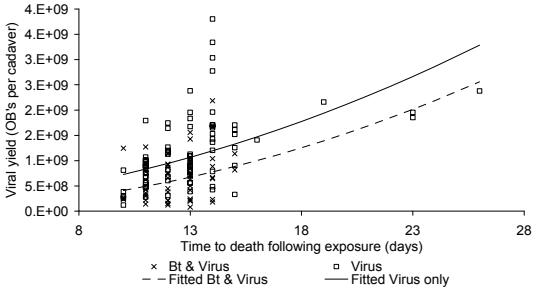


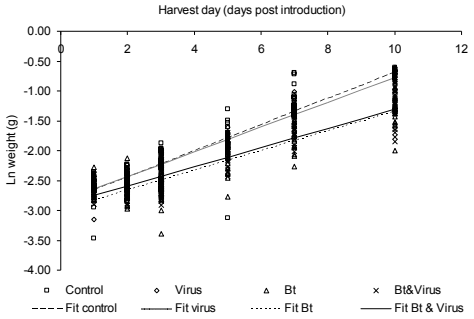
Figure 13: Probability of infection with a virus in *Mamestra brassicae* in a field trial in the presence and absence of *Bacillus thuringiensis* (DiPel).
Larvae were collected from plants 0-10 days following introduction into plots. A refuge with four parameters was not significantly different from the means model (χ_1^2=13.2, d.f.=8, p=0.105). Constraining the model to having a single infection curve was marginally significant (χ_1^2=17.8, d.f.=10, p=0.059). Fitted model; Probability of infection in the presence of Bt given survival at time t= $(1-0.503)e^{-0.551*t}$ and in the absence of Bt at time t= $(1-0.749)e^{-0.551*t}$ .The pathogen free refuge was therefore reduced by 29% in the presence of *Bt*.

In contrast to the results from the previous year, the virus yield from cadavers in the presence of *Bt* was significantly reduced compared to the yield from cadavers produced in the presence of virus alone ($F_{1,138}$=21.34, p<0.001; Figure 14 (a)). Yield of virus per cadaver was significantly affected by the time at which an insect died ($F_{1,138}$=35.25, p<0.001). The weights of larvae retrieved at harvest were significantly smaller in the presence of *Bt* compared to when it was absent ($F_{1,640}$=211.93, p<0.001; Figure 14 (b)). As expected, weights of larvae recorded at each harvest time increased significantly ($F_{1,640}$=4574.72, p<0.001). There was a significant interaction between treatment and harvest date ($F_{3,640}$=28.64, p<0.001); those larvae which were not exposed to *Bt* were heavier at each successive harvest than those exposed to *Bt.*

Figure 14:
a) Yield of viral cadavers of *M. brassicae* from field plots which were treated concurrently with virus or Bt & virus.



b) Weight of M. brassicae at harvest when exposed to Bt, virus & *Bt* or virus alone on cabbage plants in field trials for 1-10 days.



8.2 Transmission of baculovirus in presence of *Bt*; second generation *M. brassicae* larvae (Objective 6; Year 3)

Preliminary experiments in 2006 established that 8-10% of neonate larvae from eggs subsequently survived to 3rd instar on cabbage seedlings under field conditions. Therefore 45 eggs were introduced per plant to simulate a second generation of larvae giving a total of 405 eggs per plot and allowing a harvest of approximately 45 larvae to be achieved. Eggs were introduced to 131 plots, giving a total of 53055 eggs. The average number of eggs oviposited per adult female *M. brassicae* moth was calculated in the laboratory as 90 and therefore the second generation larvae represented an egg lay from approximately 1 adult moth per 2 plants. At each of 6 time points (Table 7), plots were destructively harvested and larvae were retrieved and incubated as previously described.

The total number of second generation larvae retrieved from plots was 5352 which represents a return of 10.09% of introduced individuals or an average of 40.85 larvae per plot. Of these larvae, a total of 271 succumbed to viral infection (5.06% retrieved larvae) of which 234 were in transmission plots. Mortality due to *Bt* was only confirmed in 2 individual larvae. The proportion of *Mb*NPV and *Pafl*NPV was calculated using molecular methods in a total of 100 cadavers. There was a large proportion of *Mb*NPV in the insects tested with a mean proportion *Mb*NPV per cadaver of 98.69% (SE Mean=0.614). The proportion of *Mb*NPV present in any single cadaver ranged from 55.93%

to 100%. There was no significant difference between blocks ($F_{1,79}$=0.12, p=0.726), treatment ($F_{6,79}$=0.22, p=0.969) or the harvest time ($F_{6,79}$=0.1.15, p=0.344) on the occurrence of *Mb*NPV within cadavers. There was no effect of treatment on the weight of larvae when they were harvested ($F_{2,149}$=2.66, p=0.073) but there was a significant effect of harvest date such that larvae harvested at a later date were heavier ($F_{1,149}$=1667.76, p<0.001). When infection curves were fitted to data the transmission of virus was shown to be non-linear indicating that the likelihood of an insect sustaining a lethal infection was increased as exposure time increased. Significantly more larvae succumbed to a viral infection in plots where first generation larvae had been exposed to only virus. When *Bt* was applied to first generation larvae, there was a reduction in the transmission of virus to the second generation (Figure 15).

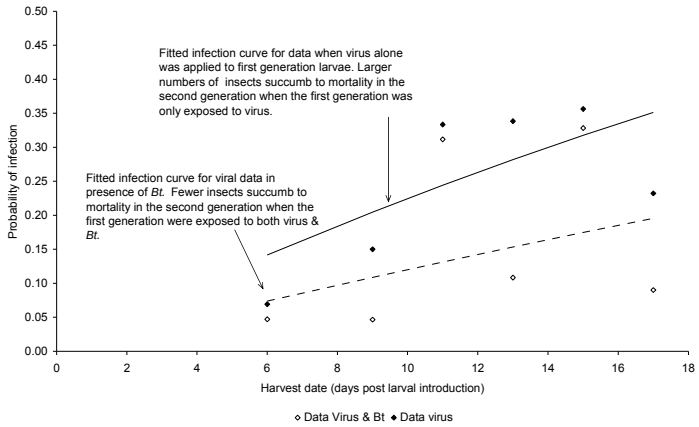


Figure 15: Probability of secondary infection with a virus in *Mamestra brassicae* in a field trial where the source of inoculum was viral cadavers from larvae which had been exposed to *Pafl*NPV in the presence and absence of *Bacillus thuringiensis* (DiPel).
Larvae were collected from plants 0-10 days following introduction into plots. A two parameter mode was not significantly different from the means model (χ_1^2=15.8, d.f.=10, p=0.105). Fitted model; Probability of infection given survival at time t in presence of Bt= $e^{-0.013*t}$ and probability of infection given survival at time t in absence of Bt= $e^{-0.025*t}$. The rate of viral transmission was higher in the absence of *Bt*.

8.3 Discussion and conclusions

The transmission dynamics of the virus in the presence of *Bt* in the first generation of larvae were very similar in trial 6 (section 8.1) to those recorded in field trial 4(c) (section 6.4). As with trial 4(c), the proportion of insects dieing due to a viral infection was increased in the presence of *Bt* as there was a reduction in the pathogen-free refuge. The majority of insects died due to an *Mb*NPV infection suggesting this was due to triggering of the persistent infection by the applied baculovirus *Pafl*NPV. The fact that this did not vary between any treatment suggests that differences were due to other interactions with the applied synergist, *Bt*. As the two trials were done using the same methods but in different years and on different host plants (spinach in 4(c) and cabbage in 6), it suggests that the synergism between *Bt* and the baculovirus is robust and is not affected by abiotic factors or host plant. However, more evidence would be needed to corroborate this implication.

The reductions in host mass gain in the presence of *Bt* in the first generation incurred a penalty to the virus in terms of reduced viral yield per cadaver. This suggested that in the crop, in the presence of *Bt* there were likely to be more patches of virus (i.e. proportionally more insects die of virus) but that each patch would be smaller than when *Bt* was absent (i.e. the yield per cadaver was reduced in the presence of *Bt*). This may have been beneficial to viral transmission as an increase in patches of virus may increase the likelihood that an insect would encounter a viral patch. However, viral transmission dynamics in the second generation suggested that the increased number of patches but reduced virus density per patch was detrimental to the ability of the virus to perpetuate through the host population. It is probable that insects die in areas where they have been feeding (as was observed in several plots during experiments; Figure 16) and this increases the likelihood that other feeding larvae will come into contact with virus. Many, small patches would seem to be more beneficial in this case. However, the virus is subject to abiotic factors such as UV and precipitation which can inactivate and remove infectious material from the crop. This is likely to have a more significant effect on the potential infectivity of a patch of virus when it is small patch compared to larger patches which can afford to lose some infective material.

Interestingly, the fact that only 2 larvae succumbed to *Bt* infection in the second generation suggests that the efficiency of transmission was due to viral yields and that the synergist was only acting in the first generation on host mortality. Similarly, the fact that the presence of *Bt* in the first generation had no impact on the weight of larvae in the second generation suggests that even sublethal effects were not occurring.

Figure 16: Cadaver of *M. brassicae* prior to liquefaction having succumbed to viral mortality within an experimental cabbage plot.

9. Overall Conclusions

This study has shown that there is potential to achieve improved control of caterpillar pests in *Brassica* crops using combinations of insect control agents and baculoviruses. Our research considered a series of intricate interactions in both the laboratory and field between an applied baculovirus, a persistent baculovirus and potential synergist pathogens/toxins. Persistent baculovirus infections were not predictably triggered by the range of pathogens/toxins tested which has important implications as non-target Lepidoptera tested were shown to carry persistent baculovirus infections and may be exposed to low levels of toxins/pathogens applied for biocontrol. The only consistent trigger known of persistent baculovirus infections is the addition of another baculovirus. We have demonstrated for the first time that different populations of Lepidoptera harbour persistent baculovirus infections and this has important implications for improved host range testing of biological control agents and other toxins. Field trials against the pest *M. brassicae* showed that suppression of first generation larvae could be improved by co-application of a baculovirus (*Pafl*NPV) and the bacterium *Bt*. Experimental evidence suggests that this is due to a delay in the onset of larval developmental resistance, induced by the presence of *Bt*. However, the presence of *Bt* reduced viral yield from first generation larvae and this was detrimental to secondary transmission of the virus, implying that longer term control of pests by the self-perpetuating virus would be reduced. These experiments have been undertaken with a specific life stage of *M. brassicae* and work would be needed to investigate the interactions at different instars and in more than one generation of the pest in order to make any recommendations regarding control of *M. brassicae* with a combination of virus and *Bt*. Overall, this research shows that there is potential to reduce synthetic pesticide use or enhance the use of organically approved insecticides through the development of combinations of biopesticides as alternatives to, or in conjunction with, currently registered agronomic products for control of agricultural and horticultural pests.

References to published material

9. This section should be used to record links (hypertext links where possible) or references to other published material generated by, or relating to this project.

10. Outputs & Technology Transfer

10.1 Research Papers

Bonsall, M.B., Sait, S.M., Hails, R.S. (2005). Invasion and dynamics of covert infection strategies in structured insect-pathogen populations. *Journal of Animal Ecology* 74, 464-474

Burden, J.P., Possee, R.D., Sait, S.M., King, L.A., Hails, R.S. (2006). Phenotypic and genotypic characterisation of persistent baculovirus infections in populations of the cabbage moth (*Mamestra brassicae*) within the British Isles. *Archives of Virology* 151, 635-649.

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10.2 Technology Transfer

Hesketh, H. & Hails, R. S. (2006). Recent developments in exploiting interactions between biocontrol agents. *Advances in Pest Management 2006. Association of Applied Biologists. Peterborough, UK, 21st September 2006.*

Hesketh, H. & Hails, R. S. (2006). Synergistic interactions between an insect baculovirus and *Bacillus thuringiensis. Proceedings of the British Ecological Society Annual General Meeting, University of Oxford, 5th-7th September, 2006*.

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Hesketh, H. (2004). Exploiting interactions between baculoviruses and other pathogens/toxins for pest control. *CEH Seminar Series. Centre for Ecology & Hydrology, Oxford. 30th April 2004.*

Presented outputs of this Defra funded project at Defra project reviews, to visiting speakers at CEH and to CEH internal reviews.

Represented CEH and this project at the HRI Warwick, Rural Economy & Land Use meeting "Biopesticides: the Way Ahead", October 2006.

10.3 Literature cited in report

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