The Role of Pathogen Diversity on the Evolution of Resistance

by
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B.Sc., Simon Fraser University, 2014

Thesis Submitted in Partial Fulfillment of the Requirements for the Degree of Master of Science

in the Department of Biological Sciences Faculty of Science

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SIMON FRASER UNIVERSITY
Fall 2017

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Abstract

My aim is to determine whether baculovirus diversity affects the rate at which resistance evolves. Using *Trichoplusia ni* as a host, changes in resistance against single versus mixtures of AcMNPV variants were examined in an evolution experiment. AcMNPV variants were isolated using dilution cloning and characterized using RFLP and pathogenicity bioassay. I found that the rate of evolution of resistance to more diverse pathogen infections to be less than that of single variants and the level of resistance was reduced by over 284-fold compared to specific single variants. Identity of the single variants had a major influence on the rate of evolution of resistance. Additionally, I found evidence of higher fitness costs of resistance to more diverse infections. My findings indicate that pathogen diversity should be factored into resistance management strategies for microbial insecticides and provide insight into the role of pathogen diversity on the evolution of resistance to pathogens.

**Keywords:** Pathogen diversity; evolution of resistance; AcMNPV; *Trichoplusia ni*; host-pathogen relationship
Acknowledgements

Thank you to my family for supporting me. I would not be here if it wasn’t for you.

Thank you to Jenny Cory and Mike Hart for the support and guidance during all my time at SFU. You are everything a student could hope for and it’s a true pleasure to be supervised by you.

Thank you to NSERC and SFU for funding me.

Thank you to all my volunteers and lab assistants. It would not be possible to complete the experiment that I did without all your help.

Thank you to all my friends and lab mates at SFU, especially everyone in the Cory Lab. You have made my time here at SFU wonderful and memorable. It wouldn’t be the same without you.
# Table of Contents

Approval ........................................................................................................ ii  
Abstract ........................................................................................................... iii  
Acknowledgements .......................................................................................... iv  
Table of Contents ............................................................................................ v  
List of Tables .................................................................................................... vii  
List of Figures ................................................................................................... viii  

## Chapter 1. General introduction ................................................................. 1  
1.1. Insect pathogens and biocontrol .............................................................. 1  
1.2. Insect resistance ....................................................................................... 4  
1.3. Costs of immunity and resistance ............................................................ 7  
1.4. Diverse pathogen interactions .................................................................. 7  
1.5. Diverse pathogen resistance ..................................................................... 9  
1.6. Baculovirus diversity ............................................................................... 10  
1.7. Pathogen – The baculovirus: AcMNPV .................................................. 11  
1.8. Host – The Cabbage Looper .................................................................... 11  
1.9. Thesis aims ............................................................................................. 12  
1.10. References ............................................................................................ 13  
1.11. Figures .................................................................................................. 24  

## Chapter 2. The role of pathogen diversity on the evolution of resistance ....... 26  
2.1. Introduction ............................................................................................ 26  
2.2. Methods .................................................................................................. 32  
2.2.1. Preparation of the insect colony .......................................................... 32  
2.2.2. Isolation and characterization of virus variants ................................. 33  
2.2.2.1. In vivo dilution cloning ................................................................. 33  
2.2.2.2. Enumeration of occlusion bodies ............................................... 34  
2.2.2.3. Initial Mortality Screening ......................................................... 34  
2.2.2.4. Viral DNA Extraction ................................................................. 34  
2.2.2.5. DNA analysis .............................................................................. 35  
2.2.3. Effect of diversity on virulence ......................................................... 36  
2.2.4. Selection experiment ......................................................................... 36  
2.2.5. Changes in resistance ........................................................................ 37  
2.2.6. Statistical analysis ............................................................................. 38  
2.3. Results ................................................................................................... 39  
2.3.1. Initial Mortality Screening ................................................................. 39  
2.3.2. Effect of diversity on virulence .......................................................... 39  
2.3.3. Selection experiment ......................................................................... 40  
2.3.3.1. Virus mortality ............................................................................ 40  
2.3.3.2. Potential costs ............................................................................. 41
2.3.4. Change in resistance ................................................................. 42
2.3.4.1. Comparison between variants and variant mixtures ......................... 42
2.3.4.2. Comparison between levels of diversity ........................................... 43
2.3.4.3. Mortality differences between selected and unselected lines ................ 44
2.4. Discussion .................................................................................... 45
2.5. References: ................................................................................... 51
2.6. Tables and Figures ......................................................................... 61

Appendix A: Virulence comparison between the 8 single AcMNPV isolates ..... 73
Appendix B: Virulence (log(LD50)) comparison ............................................ 74
Appendix C: Change in resistance .............................................................. 75
Appendix D: Dose response over time ....................................................... 76
List of Tables

Table 2.1. The origin of individuals in the original mixed *T. ni* population. Individuals were sourced from three cultures: wild, lab, and imported, and combined in different pair combinations. Approximately half of each colony type was male/female (the sex ratio was roughly equal) in all pair combinations. 61

Table 2.2. LD70 (OBs) +/- 95% CI of four single AcMNPV isolates (f,j,r,u), three 4-isolate mixtures (L,M,H), and wild-type (wt) used in the selection experiment in early 4th instar *T. ni*. 62
List of Figures

Figure 1.1. Illustration of the three baculovirus groups. Based on OB (occlusion body) morphology, baculoviruses were originally divided into: Nucleopolyhedrovirus (NPVs) and the Granulovirus (GVs). NPVs occlusion bodies are also called polyhedra and GV occlusion bodies are called granules. SNPVs contained a single nucleocapsid within an enveloped virion while MNPVs contained multiple nucleocapsids. ....... 24

Figure 1.2 Baculovirus life cycle. 1. Insect ingests occlusion bodies (OBs) on foliage. 2. After ingestion of the OBs, the occlusion derived virions (ODVs) are released in the midgut and infect epithelial cells. 3. Virions migrate to the host cell nucleus where replication of viral genes initiate. 4. Nucleocapsids assemble and then bud through the cell membrane acquiring an envelope. 5. Budded virion (BV) spread infection throughout the insect in secondary infections. 6. Virions accumulate in the nucleus and are occluded into OBs until the larva disintegrates. 7. The OBs are released into the environment and the cycle repeats after a new host ingests the OBs. ................................................................. 25

Figure 1.3 The life cycle of T. ni: consists of larval instars followed by a pupal and adult stage. At 25°C, the average generation time is 28 days............ 25

Figure 2.1 Diagrammatic summary of the T. ni – AcMNPV selection experiment and virus resistance assay. Pt I: Selection experiment; T. ni population infected with AcMNPV at LD70 with one of eight treatments: four single AcMNPV isolates (f,j,r,u), three 4-isolate mixtures (Low, Medium, High), and wild-type (wt) for five generations. Pt II: Change in resistance assay; compared selected vs. unselected line after skipping selection for one generation. Both experiments had five replicates............................... 63

Figure 2.2 Comparison of virus mortality in early 4th instar T. ni at 100 OBs between 29 AcMNPV isolates and the mixed wild-type (wt) (proportion mortality +SE: N= 3 repeats with 24 insects per clone in each repeat). The 8 clones used in the selection experiment (red) and the wild-type (black) are highlighted. ........................................................................ 64

Figure 2.3 Gel electrophoresis of 8 AcMNPV isolates and the wildtype (Wt) isolate using (a) EcoRV and (b) HindIII enzymes with a size ladder (M; 0.5-48.5 kb). Gels were run at 35V for 24h with 0.8% agar; 2.9 micrograms of DNA were loaded in each lane. Banding differences between clones are identified with white dots......................................................... 65

Figure 2.4 Dose-response of early 4th instar T. ni to four single AcMNPV isolates (f,j,r,u), three 4-isolate mixture (L,M,H), and the wild-type (wt). Virus mortality is given in logits (log(p/1-p)). 'p' denotes proportion mortality due to virus. Lines are from the final statistical model and jittered mean experimental points are in the same corresponding colour. N=3 repeats, with 22 larvae per treatment per replicate on average. ....................... 66

Figure 2.5 AcMNPV-induced mortality (shown as logits) in early 4th instar T. ni at different levels of virus diversity: 1 (isolates: f,j,r,u,v,i,k,q), 4 (mixes: L,M,H), 8 (all 8 single isolates), 20 (wt) with modeled lines and jittered
mean experimental points in corresponding colour. Virus mortality is given in logits (log(p/1-p)). ‘p’ denotes proportion mortality due to virus. N=3 repeats.

Figure 2.6  AcMNPV mortality in early 4th instar T. ni after challenge with an LD70 dose over five generations. Lines from the final statistical model are shown and mean virus mortality for each replicate in each treatment is represented with a jittered data point in the corresponding colour (N=5). a) Comparison between the three levels of diversity: single isolates (1), 4-isolates mixtures (4), and wild-type (20). b) Comparison between the four single isolates (solid line), three 4-isolates mixtures (dashed lines), and wild-type (dotted line).

Figure 2.7  The impact of virus (AcMNPV) diversity on a) T. ni pupal weight (back-transformed LS-mean +/- SE) and b) eggs laid in the first three days of oviposition (+/- SE) averaged for all generations based on Tukey-Kramer HSD test. Replicate lines were subjected to varying levels of AcMNPV diversity (1, 4, 20) and control (0) over five generations. Significant differences (p<0.05) are shown by letters. N=16631 for pupal weight and N=3375 for egg count.

Figure 2.8  Comparison of log10(LD50) of early 4th instar T. ni to AcMNPV between a) unselected and b) selected lines across generations with +SE for all treatments: single (u,r,f,j), 4-isolates mixtures (L,M,H), and wild-type (wt).

Figure 2.9  Comparison of mortality due to AcMNPV in early 4th instar T. ni between selected and unselected lines across generations. Modeled lines and jittered data points are shown for all levels of diversity: singles (green), 4-isolates mixtures (red), and wild-type (black).

Figure 2.10  The difference in percent AcMNPV mortality in early 4th instar T. ni between selected and unselected lines across generations with model line and data points for a) all treatments: single (u,r,f,j), 4-isolates mixtures (l,m,h), and wild-type (wt); b) shows average difference for diversity levels: single (1), 4-isolates mixtures (4), and wt (20). N=5 repeats.
Chapter 1.

General introduction

Pathogens can be found in almost every species and can have major impact on their hosts (Keesing et al., 2010). Hosts usually show variation in resistance to pathogens, which can be a combination of genetic and environmental factors. If there is genetic variation in host susceptibility, hosts can be selected to evolve resistance against pathogens (Tabashnik, 1994). It is important to understand what factors alter the evolution and expression of resistance because that will allow for better prediction and potential mitigation of future disease outbreaks. While most of our focus is in preventing disease outbreaks in humans, and domesticated and natural plant and animal populations, pathogens are also used to manage pest species, which share similar dynamics. This includes invasive species and other pests in horticulture and forestry and vectors of human and livestock diseases (Thomas, 1999). Microbial pest control is becoming more common due to increasing pressure to find alternatives to synthetic chemical pesticides as a result of health and environmental concerns, in addition to the evolution of resistance to many widely-used insecticides (Szewczyk et al., 2005; Gupta and Dikshit, 2010). The mode of action of insect pathogens was thought to be too complex for resistance to evolve (Heckel, 1994), however there have been examples of resistance to microbial insecticides (Tabashnik et al., 1990; Asser-Kaiser et al., 2007). Understanding the circumstances under which resistance is more likely to evolve and the factors that modulate the rate of evolution are crucial if we want to extend the life of these important pest control products and develop appropriate application and resistance mitigation strategies, in addition to providing insights on how disease resistance evolves and how host and pathogen interact.

1.1. Insect pathogens and biocontrol

In insects, there are several main groups of insect pathogens (entomopathogens): bacteria, fungi, nematodes and viruses that are used in pest management applications. Here I will focus on the three micro-parasites: bacteria, fungi and viruses. Biological control is a pest management strategy where natural enemies (in
this case, pathogens) are released to reduce pest damage to below economic levels (Eilenberg et al., 2001). Entomopathogens or microbial biocontrol agents tend to be used like chemical insecticides, where large numbers of the pathogen are sprayed onto high density pest populations, sometimes multiple times, rather than utilizing their natural capacity to recycle and be dispersed within insect populations (Vega and Kaya, 2012). This approach has implications on both the financial cost of using the microbial control agents but also the likelihood of natural selection for insect resistance. The sections below go into more details about the three main groups of entomopathogens with the focus of this thesis (the baculoviruses) being covered in more detail.

The bacterium *Bacillus thuringiensis* (Bt) is the most successful commercial insect pathogen used in insect pest management representing around 2% of the total insecticide market (Bailey et al., 2010; Bravo et al., 2011). Different isolates of Bt kill mostly larvae of different insect Orders, mainly Lepidoptera, Coleoptera, and Diptera through the disruption of the midgut tissue followed by septicemia (Raymond et al., 2010). Upon sporulation, Bt produces insecticidal crystal inclusions that are formed by insecticidal proteins (i.e. Cry or Cyt toxins) (Bravo et al., 2005). The Cry and Cyt toxins are secreted as water soluble proteins that insert into the host midgut membrane after undergoing conformational changes. In addition to Bt insecticide sprays, the Cry toxins have been included in genetically modified (GM) crops which provides a more effective way to target and control insect pests.

Entomopathogenic fungi are commonly found in insects (e.g. in Coleoptera, Lepidoptera, Orthoptera, Hemiptera, Hymenoptera) and are used as biocontrol agents; e.g. the fungus *Beauveria bassiana* is used to control many species of insect pests (Hajek and St. Legar, 1994; Shah and Pell, 2003). Entomopathogenic fungi infect hosts without the need for ingestion, unlike Bt and baculoviruses. The fungi produce conidia (infective spores) that attach to the cuticle of the host and then germinate and penetrate into the host (Roy et al., 2006). Once inside, the fungi exploit resources of their hosts and proliferate, eventually killing the host and produce more conidia for transmission (Inglis et al., 2001; Pell et al., 2001).

Insects can be infected by many groups of viruses and the main family used for pest management is the insect-specific baculoviruses. Baculoviruses are dsDNA viruses which have mainly been isolated from the Order Lepidoptera, but are also found in
Diptera and Hymenopteran sawflies (Jehle et al., 2006). There are two main types of baculovirus which are divided into four families: Alphabaculovirus, Deltabaculovirus, and Gammabaculovirus are types of nucleopolyhedrovirus (NPV) that infect the insect Orders Lepidoptera, Diptera and Hymenoptera respectively; while Betabaculovirus is a type of granulovirus (GV) only found in the Order Lepidoptera. In the NPVs, multiple genomes are encapsulated in virions in the transmission unit, which is called an occlusion body (OB). NPVs and GVs are distinguished by the different protein matrix (polyhedrin or granulin) that surrounds the occlusion derived virions (ODVs) (Herniou and Jehle 2007). NPVs are further divided by the number of nucleocapsids within a virion: single (SNPV) or multiple (MNPV) (Rohrmann, 2013) (Figure 1.1.). MNPVs contain multiple virions, which can be different variants, which contributing to the substantial (intra-specific) genetic variation in pathogenicity, productivity and speed of kill of baculoviruses (Hodgson et al., 2001; Cory et al., 2005).

Baculoviruses occlusion bodies (OBs) are the units which infect insect larvae. After the OBs are ingested and have entered the midgut, the alkaline pH and proteases release the virions which then pass through the peritrophic membrane of the midgut wall (Cory and Myers, 2003). The virions fuse with the midgut columnar cells’ plasma membranes and the nucleocapsids inside move in to initiate infection of the nucleus (Slack et al., 2008; Hoover et al., 2010). In Lepidoptera, the infected cells produce budded virus (BV) which spread within the host haemocoel via the tracheal cells (Volkman, 2007; Passarelli, 2011)). At the end of infection, the insect larval body usually disintegrates and millions of OBs are released into the surroundings (Hoover et al., 2011) (see Figure 1.2. for a visualized life cycle of baculovirus). The OBs can persist for a long time in the environment but can be degraded by UV light (Carruthers et al., 1988). Baculoviruses are exposed to two distinct selections: transmission to a new host and proliferation within the current host; therefore, genotypes that maximize transmission may not be as successful at within host infection (within versus between host dynamics) (DeFillippis and Villarreal, 2000). This can lead to a trade-off between transmission and virulence which could contribute to the maintenance of genetically diverse baculovirus populations in the wild (Hodgson et al., 2001; Cory et al., 2005; De Roode et al., 2008; Alizon et al., 2009).

Baculoviruses are used to control numerous insect species across a wide range of habitats (e.g. field, forestry, and greenhouse). The earliest example of a baculovirus
insecticide was in 1892 in Germany against the nun moth (*Lymantria monacha*) in pine forests (Huber, 1986). Since the first registered baculovirus insecticide (ElcarTM) in 1975 against the cotton bollworm (*Helicoverpa zea*), many baculovirus have been used in the field. Some of the most successful examples include *Anticarsia gemmatalis* MNPV against the velvet bean caterpillar in over 1 million hectares of soybean annually in Brazil (Moscardi, 1999) and over 100 tons of *Helicoverpa armigera* NPV insecticide is produced against the cotton bollworm in China (Sun et al., 2002).

Baculovirus insecticides have many advantages. Baculoviruses have a narrow host range (often they are species specific) meaning that they are harmless to non-target species (e.g. beneficial insects) (Gröner, 1990), can be used with chemical insecticides and other biological control agents (Hawtin and Possee, 1993), and can persist in environments if protected from UV (e.g. in soil) for years (Jaques, 1975). However, there are drawbacks of using baculovirus insecticides. Their narrow host range means that they cannot control multiple pest species on a single crop, can be rapidly inactivated by UV exposure, and have higher production costs and slower speeds of kill than chemical insecticides (Inceoglu, 2001). The slower speed of kill allows the infected larvae to feed and damage crop for several days until death. The use of baculoviruses is, therefore, limited in high value crops with a low damage threshold (Bonning and Nusawardani, 2007).

### 1.2. Insect resistance

If the insect populations are frequently exposed to pathogens (i.e. microbial insecticides treatments in field crops) they could be selected to become more resistant. Although resistance was not expected, there are several examples of resistance against microbial biocontrol agents and most of these are against Bt (both bacteria and toxins; GM plants expressing Bt toxins are not covered here). Bt has been used to manage a wide range of pests, particularly Lepidoptera, on row crops, in orchards, and in forestry in many countries around the world (Lacey et al., 2015). Lab studies can demonstrate the potential to develop resistance and many have shown the potential for insects treated with Bt to decrease their susceptibility (Ferré and Van Rie, 2002; Siegwart et al. 2015; Melo et al., 2016). The development of resistance in the field has been rarer; the first example was in the stored product pest, the Indian meal moth, *Plodia interpunctella* (McGaughey, 1985). This has been followed by widespread resistance (up to >200-fold)
in the diamondback moth, *Plutella xylostella* populations from the Philippines and subsequently worldwide (Kirsch and Schmutterer, 1988; Tabashnik, 1994); and in cabbage looper (*Trichoplusia ni*) populations in greenhouses in British Columbia (Janmaat and Myers, 2003). *Bacillus thuringiensis israelensis* (Bti) contains six different toxins and this is thought to delay the evolution of resistance in mosquito populations. While resistance to individual Bti toxins of up to 30-fold can be selected rapidly in the lab, the overall resistance to all six toxins is lower (2-fold) (Paris et al., 2011).

For baculoviruses, there are numerous examples where resistance has been selected in lab populations, demonstrating the potential for baculovirus resistance to develop. For example, fall armyworm (*Spodoptera frugiperda*) became 3X more resistant after 8 generations of selection to its NPV (Fuxa and Richter, 1989); the velvetbean caterpillar (*Anticarsia gemmatalis*) became up to 1000X more resistant to its NPV (Abot et al. 1996). There is only one example of baculovirus resistance in the field: codling moth, *Cydia pomonella*, populations became up to 100,000X more resistant to CpGV in Europe in early 2000s; a more detailed discussion of this can be found in chapter 2 (Eberle and Jehle, 2006; Asser-Kaiser et al., 2007; Berling et al., 2009; Schmitt et al., 2013). Resistance against fungal pathogens is less studied; currently there is little evidence of resistance against fungal pathogens (Dubovskiy et al., 2013).

It is important to note that Bt is different from other pathogens (i.e. baculoviruses) because Bt kills insects primarily with endotoxins, which act more similarly to chemical pesticides (i.e. disrupt vital processes) and there is evidence that indicates a role for gut microbiota in the killing mechanism of Bt which further complicates the matter (Caccia et al., 2016). One possible mechanism behind Bt resistance was found in *Heliothis virescens* (the tobacco budworm) where resistant caterpillars expressed lower levels of membrane-bound alkaline phosphatase (Jurat-Fuentes et al., 2011). Other resistance mechanisms include mutation in the ABC transporter that could prevent toxin binding to the midgut epithelium and modification of cadherin receptor that prevented the docking and recognition of the toxin (Zhang et al., 2005; Heckel, 2012).

There are multiple ways the host can evolve to be resistant (i.e. changes in behaviour, morphology, and immunity) (Hoover et al., 2000; Lee et al., 2004) and resistance against different types of pathogens is likely to be different since their methods of infection are different. The resistance mechanism behind pathogens such as
baculoviruses that hijack host cellular machinery for reproduction is likely to be different from pathogens such as Bt that produce harmful endotoxins upon sporulation. There are currently few studies on the genetic basis of resistance against baculoviruses. Possible resistance mechanisms against baculoviruses include modification of midgut receptors (similar to Bt) or the peritrophic membrane and changes in innate immunity. Resistance to CpGV in *C. pomonella* was related to early blocking of virus replication and was found to be sex-linked and incompletely dominant (Asser-Kaiser et al., 2011). The resistance is thought to be caused by a mutation in the CpGV *pe38* gene, which encodes a protein that is suggested to contribute to augmentation of apoptosis (Prikhod’ko and Miller, 1999). However, the function and the resistance mechanism against CpGV are still not fully understood (Jehle et al., 2017).

It is important to note that there are other factors that affect susceptibility to pathogens in an insect that will modulate genetically-based resistance. Susceptibility to baculoviruses and Bt decreases with age (both within and between instars) due to weaker immunity in younger larvae (Cory and Myers, 2003). One major form of defence in lepidopteran larvae is sloughing off baculovirus-infected midgut lining cells (Hoover et al., 2000); *T. ni* larvae were most susceptible to AcMNPV (*Autographa californica* multicapsid nucleopolyhedrovirus) after molting (Engelhard and Volkman, 1995) when it is possible that the gut has been reformed. Insect density can also affect their susceptibility to virus. African armyworm larvae (*Spodoptera exempta*) living in high density have reduced individual susceptibility to NPV compared to singly reared larvae possibly due to increased melanization and increased immune function (Reeson et al. 1998).

Insect susceptibility to pathogens is also affected by what they eat. Plant phytochemicals have been shown to affect host mortality due to baculoviruses (Farrar and Ridgway, 2000; Raymond et al., 2002; Cory and Hoover, 2006). *H. virescens* larvae inoculated with AcMNPV that fed on cotton were 2.5-fold less susceptible than those that fed on lettuce or artificial diet; this was likely due to higher levels of foliar peroxidase on cotton which damage the midgut lining and result in infected cell sloughing early on (Hoover et al., 2000). *Spodoptera littoralis* (the African cotton leafworm) larvae infected with NPV is able to reduce mortality by eating a high protein to low carbohydrate diet; protein in the diet is likely important in immunity and/or larval development (Lee et al., 2004). The larvae could possess the ability to self-medicate by increasing protein intake.
after infection when given a choice (Lozano, 1998; Lee at al., 2004). Larval nutrition also affects susceptibility to other pathogens. The greater wax moth (Galleria mellonella) larvae starved for seven days displayed increased susceptibility to the yeast Candida albicans. Their hemolymph displayed reduced expression of many antimicrobial peptides and immune proteins (Banville et al., 2012).

1.3. Costs of immunity and resistance

Evolved resistance often involves maintaining immunological resistance mechanisms which can have energetic costs and redirect resources that would have been used for other life-history traits (Schmid-Hempel, 2005; Libert et al., 2006). Immune responses reduce the total resources available for normal body functions, which affects the individual’s survival and fitness. In one study, researchers induced immune responses in four different species of insects and found increased metabolic cost of immunity and trade-offs between different immune responses: phenoloxidase and antimicrobial lysozyme levels (Ardia et al., 2012). Another study, in the field cricket (Teleogryllus oceanicus), induced an antibacterial immune response and the challenged individuals displayed slower growth and reduced sperm viability (Simmons, 2011). These costs could explain the rapid reversal of resistance often found in resistant populations. Further details on the cost of immunity and resistance will be covered in Chapter 2.

1.4. Diverse pathogen interactions

Natural pathogen populations are diverse and mixed infections (intra-specific) are common (Read and Taylor, 2001; Clavijo et al., 2010, Votýpka et al., 2010; Ulrich et al., 2011). Insects are hosts to diverse pathogen communities which often have high intra-specific diversity (Cory et al., 2005; Tanada and Kaya, 2012). When pathogens with different genotypes share the same host, there are different ways they could interact that could affect overall virulence (degree of damage experienced by the host). The fitness of individual genotypes in mixed infections could be better, worse, or the same as in a single infection depending on whether those genotypes co-operate (e.g. suppressing the host immune system) or if they compete, either directly or indirectly (Zhan and McDonald, 2013; Redman et al., 2016). Within-host competition between pathogen
genotypes is likely in mixed infections because individual genotypes will compete for host resources (if limited) (Mideo, 2009). Mixed pathogen competition for the shared host resource can select for more aggressive exploitation strategies which may lead to premature host death and subsequently, pathogen death; this short-sighted evolution process is often referred to as the ‘tragedy of the commons’ (Berngruber et al., 2015).

One of the leading hypotheses to explain the evolution of lower virulence assumes a trade-off between pathogen virulence and transmission. There is a balance between the extent and rate at which the host is exploited and the risk of killing the host too quickly which will curtail transmission (Thomas et al., 2003; Alizon et al., 2013). For example, virulence is less in co-evolving lines of the obligate-killer microsporidian pathogen *Nosema whitei* to the red flour beetle (*Tribolium castaneum*) compared to non-co-evolving lines (Bérénos et al., 2009). However, the trade-off is more complicated in diverse pathogen infections (Alizon et al., 2009). In mixed infections, pathogens that exploit the host resources too slowly will be outcompeted. Even if pathogen replication is stopped when the host dies prematurely due to excessive exploitation, individuals that exploited host resources less will do disproportionately more poorly in terms of producing transmission stages (Ebert, 1998); although this depends on if the pathogen is able to transmit at this point. Where individuals are less related in mixed infections, virulence is predicted to be higher: e.g. faster speed of kill was favoured in competition between strains of the entomopathogenic fungus *Metarhizium anisopliae* in *G. mellonella* compared to single strains (Staves and Knell, 2010). Multiple Bt toxins in mixed infections result in synergism (more toxic than combined toxicity of individual toxins) (Sharma et al., 2010). Mixed baculovirus infections are more pathogenic than single strains (Hodgson et al., 2004; Simon et al., 2005). However, there are likely to be differences between within host and between host transmissions; the strains that are most successful in within host competition are not necessarily those which are most successfully transmitted.

Alternatively, virulence could be reduced in mixed infections due to competitive inhibition through production of antagonistic compounds, which is both energetically costly and can decrease virulence (Chao et al., 2000; Garbutt et al., 2011). Different pathogen genotypes can interfere with each other through either direct attacks or chemical/mechanical exclusion (Gold et al., 2009). Some viruses interfere with conspecifics by actively synthesizing molecules which prevent cell entry (Hart and Cloyd,
In mixed strain infections of the pathogenic bacterium *Pseudomonas aeruginosa* in *G. mellonella*, virulence is reduced compared to some single infections due to competition favouring non-contributing, cheater strains (does not contribute to infection but focus on multiplying) whose presence reduces overall growth (Harrison et al., 2006). Additional suppression methods include bacteriocin production in *P. aeruginosa* which minimized overall growth rate and virulence (Inglis et al., 2009). Diverse pathogen infections could also lower the severity of infection by limiting the development of extremely virulent genotypes (Smith et al., 1999). Additionally, there could be ‘parasitic’ genotypes (their genome is defective and requires another genotype to be present to replicate or be transmitted) in mixed infections that could affect the overall virulence. For example, parasitic genotypes in the beet armyworm (*Spodoptera exigua*) NPV reduces the overall pathogenicity of the entire population and can make up 30% of viral progeny (Muñoz and Caballero, 2000).

**1.5. Diverse pathogen resistance**

The selection imposed by diverse pathogens on the host could be directional or stabilizing. In directional selection, beneficial alleles (e.g. resistant host genotypes) will increase in frequency in a host population until fixation (Woolhouse et al., 2002). Less diverse pathogen infections would likely result in directional selection for increased host resistance; diverse pathogen infection will likely stabilize host population diversity by preventing the fixation of resistant genotypes to one pathogen. It is less likely for resistance to evolve against a diverse pathogen population (MacDonald and Linde, 2002). This assumes individuals carrying genes resistant to all genotypes in the pathogen population are rare. For example, the frequency of resistant alleles against Bt (Cry1Ac toxin) in field populations of *H. virescens* was estimated to be 0.0015 (Gould et al., 1997; Shelton et al., 2007; Tabashnik et al., 2009). Mounting immune responses against diverse infections can be costly to the host resource (Boots and Begon, 1993; Ferguson et al., 2003; Ulrich and Schmid-Hempel, 2012). Thus it is unlikely that resistance against multiple strains of pathogens will evolve at the same rate as against a single strain. Alternatively, mixed pathogen may be ineffective in reducing resistance rate due to more intense selection pressure that can result from increased virulence (Tabashnik, 1989). As well, if the host evolves general immunity to all variants of the pathogen then the outcome may differ.
Only one study has investigated the impact of strain diversity on the evolution of resistance in entomopathogens. In a multi-generational selection experiment, the impact of strain diversity on the rate of evolution of resistance of the diamondback moth (*Plutella xylostella*) was compared using single and mixed-strain Bt (containing both different strain isolates and Bt biopesticide mixtures). Cages of *P. xylostella* were inoculated with different levels of Bt diversity and after five generations of selection, the rate of evolution of resistance was not significantly different (Raymond et al., 2013); this study will be discussed in greater detail in chapter 2.

### 1.6. Baculovirus diversity

Baculoviruses are highly diverse in both genotype and phenotype. For example, 24 distinct PfNPV genotypes were isolated from a single pine beauty moth (*Panolis flammea*) larva and had significant variation in both pathogenicity and speed of kill (Cory et al., 2005). The presence of parasitic genotypes in the NPV population also supports the idea that mixed infections are common in many cases (Munoz and Caballero, 2000). One way this diversity is generated in the NPV population is through recombination, which is common in cells infected with multiple genotypes; e.g. the homologous recombination rate between related AcMNPV genotypes is up to 41% in cell culture (Hajos et al., 2000) and a similar result is seen *in vivo* in *T. ni* as well (Bull et al., 2003).

Baculoviruses also vary in phenotype; e.g. NPV genotypes from a single *P. flammea* larva differed in LD50 by 7-fold and had significant differences in speed of kill and virus yield (Hodgson et al., 2001); genotypes of TnSNPV differed in LD50 by over 6-fold (Harrison et al., 2012). Phenotypic diversity can be maintained through trade-offs and differential selection. One common trade-off is between OB production and infection duration; slower speed of kill results in higher virus yield; e.g. AcMNPV in the cabbage moth (*Mamestra brassicae*) (Hernandez-Crespo et al. 2001). Some genotypes perform better under certain conditions (e.g. in different host species or in different environmental conditions); this could result in differential selection. For example, when mixed AcMNPV was passaged 20 times in *P. xylostella*, the genotypic mixture and the nucleocapsid structure was significantly altered. Additionally, the passaged AcMNPV mix became 15 times more infective towards its adapted host, *P. xylostella* (Kolodny-Hirsch & van Beek 1997).
1.7. Pathogen – The baculovirus: AcMNPV

*Autographa californica multicapsid nucleopolyhedrovirus* (AcMNPV) is a genetically diverse Alphabaculovirus. AcMNPV has a broad host range and is capable of infecting at least 30 lepidopteran species (Goulson, 2003; Clem and Passarelli, 2013; Chateigner et al., 2015). AcMNPV ODVs contain multiple nucleocapsids which can differ in genotype; multiple genotypes as well as ‘parasitic’ genotypes (natural recombinant strain that was incapable of producing OBs on its own) can be co-occluded (Bull et al., 2001; Bull et al., 2003).

The infection pathway of AcMNPV, like other baculoviruses, begins after a host larva ingests OBs which contain the ODVs protected within a protein matrix. The OBs dissolve in the alkaline environment in the host midgut releasing the ODVs which in turn penetrate the peritrophic membrane and initiate primary infections in the midgut epithelial cells, which typically occurs within 24 hours (Slack et al., 2008; Hoover et al., 2010). Infected midgut cells produce budded virus (BV) which spreads within the host in the secondary infection of haemocoel via tracheal cells (Volkman, 2007; Passarelli, 2011). Eventually, if sufficient numbers of OBs are produced, the host is slowly killed by first preventing moulting to the next instar followed by tissue lysis and cadaver liquefaction; this process depends on the dosage and presence of viral egt gene (Hoover et al., 2011). In AcMNPV, infection usually takes around 4-10 days to kill the host, depending on temperature, and releases millions of OBs into the surroundings ready for transmission to the next host.

1.8. Host – The Cabbage Looper

The cabbage looper (*Trichoplusia ni* (Hübner)) belongs to the Order Lepidoptera and the moth Family Noctuidae. The species name ‘ni’ is derived from the lowercase Greek letter ‘v’ (ni) which resembles the adult moth forewing marking. *T. ni* is found on all continents except Antarctica. It is a generalist herbivore that feeds on many plants including many within the family *Brassicaceae*, in addition to other important crops (e.g. tomato, cucumber, bell peppers). The insect is multivoltine with a life-cycle of around four weeks consisting of a five-instar larval stage followed by pupation and adult moth (Figure 1.3.). The larvae can be cannibalistic when reared at high densities. The adult males are slightly larger than the females and the adults can mate with multiple partners.
The females usually lay between 200-500 eggs. Due to the voracious appetite of larval stage, *T. ni* is a major pest both in field and greenhouse in British Columbia (they either migrate from California or overwinter in local greenhouses) (Cervantes et al., 2011). The commercial development of a baculovirus-based microbial insecticide in Canada provides farmers with another option to control *T. ni* populations that can become resistant to Bt and other insecticides. Loopex (website: http://www.andermattbiocontrol.com/sites/products/bio-insecticides/baculovirus/loopex.html) is a commercial AcMNPV insecticide used to control *T. ni* on vegetables. Understanding the resistance potential of *T. ni* against this baculovirus and how to mitigate increases in resistance are important, not only for management of *T. ni* but also as a model for other Lepidopteran species and the evolution of resistance of insects against baculoviruses.

1.9. Thesis aims

This thesis aims to explore the role of pathogen diversity on the evolution of resistance in an insect using AcMNPV and *T. ni* as a model system. I first examine differences in virulence and genotype in a mixed AcMNPV population by isolating virus variants and comparing them both molecularly using Restriction Fragment Length Polymorphism (RFLP) gel electrophoresis and virulence bioassays. In a multi-generational selection experiment, replicated insect populations are challenged by a different level of virus diversity over five generations. Subsets of individuals from each treatment are taken and exposed to a dose-mortality assay to estimate the change in their resistance levels. Life-history traits are measured and compared for all treatments to investigate any cost of resistance. Experimental evolution studies are useful tools for examining the role of different mechanisms on the outcome of selection (Kassen, 2002). Understanding the ways that pathogen diversity can affect the evolution of resistance in its host will contribute substantially to the theory of pathogen-host interaction and evolution, disease ecology, and pest management strategies.
1.10. References


Sharma, P., Nain, V., Lakhanpal, S., & Kumar, P. A. (2010). Synergistic activity between Bacillus thuringiensis Cry1Ab and Cry1Ac toxins against maize stem borer (Chilo partellus Swinhoe). Letters in Applied Microbiology, 51, 42–47.


1.11. Figures

Figure 1.1. Illustration of the three baculovirus groups. Based on OB (occlusion body) morphology, baculoviruses were originally divided into: Nucleopolyhedrovirus (NPVs) and the Granulovirus (GVs). NPVs occlusion bodies are also called polyhedra and GV occlusion bodies are called granules. SNPVs contained a single nucleocapsid within an enveloped virion while MNPVs contained multiple nucleocapsids.
Figure 1.2  Baculovirus life cycle. 1. Insect ingests occlusion bodies (OBs) on foliage. 2. After ingestion of the OBs, the occlusion derived virions (ODVs) are released in the midgut and infect epithelial cells. 3. Virions migrate to the host cell nucleus where replication of viral genes initiate. 4. Nucleocapsids assemble and then bud through the cell membrane acquiring an envelope. 5. Budded virion (BV) spread infection throughout the insect in secondary infections. 6. Virions accumulate in the nucleus and are occluded into OBs until the larva disintegrates. 7. The OBs are released into the environment and the cycle repeats after a new host ingests the OBs.

Figure 1.3  The life cycle of *T. ni*: consists of larval instars followed by a pupal and adult stage. At 25°C, the average generation time is 28 days.
Chapter 2.

The role of pathogen diversity on the evolution of resistance

2.1. Introduction

Microbial control agent usage can select for resistance in the insect pest population; however, it is less known whether the genetic diversity of those agents makes a difference to either the speed at which resistance evolves or the level of resistance. The use of multiple Bt (*Bacillus thuringiensis*) toxins in genetically modified crops is one approach that has been successfully adopted to reduce resistance evolving (Cui et al., 2011). Genetically diverse entomopathogenic agents could be applied to a pest population in order to reduce the likelihood of resistance evolving through more varied selection on the host population (Raymond et al., 2007). However, the impact that pathogen diversity could have on host resistance is not clear and could have unanticipated consequences as a result of the effect of pathogen diversity on other traits, such as virulence (Read and Taylor, 2001). Understanding the circumstances under which resistance is more likely to evolve and the factors that modulate the rate of evolution are crucial if we want to extend the life of these important pest control products and develop appropriate application and resistance mitigation strategies. In addition, understanding the consequences of both pathogen and host diversity is of fundamental importance to disease ecology and evolution and how hosts and pathogens interact (Cory et al., 2005; Jabbour et al., 2011; Cory and Franklin, 2012).

The main groups of pathogens (micro-parasites) that have been developed as microbial insecticides are bacteria, fungi, and baculoviruses. Most examples of microbial resistance in the field are against Bt sprays (includes both spores and toxins). Resistance to *Bt kurstaki* (effective for Lepidoptera) in field populations has been limited to the diamondback moth (*Plutella xylostella*) populations from the Philippines and subsequently worldwide (Kirsch and Schmutterer, 1988; Tabashnik, 1994) and the cabbage looper (*Trichoplusia ni*) populations in greenhouses in British Columbia (Janmaat and Myers 2003). It should be noted that the selection for resistance to Bt
sprays and Bt crops is different since the endotoxins are continuously present in genetically modified crops and the insects are exposed continuously to the same concentration regardless of host population density.

For baculoviruses, there are numerous examples where increased resistance has been selected in lab populations, demonstrating the potential for baculovirus resistance to develop (Fuxa and Richter, 1989; Abot et al. 1996). There is only one example of baculovirus resistance in the field: codling moth *Cydia pomonella* populations became up to 100,000X more resistant to *C. pomonella* granulovirus (CpGV) in Europe in the early 2000s. Resistance evolved quickly in many locations due to repeated application of CpGV within a season over many years and the CpGV having low genetic diversity. Due to the structure of CpGV, GVs are less diverse than NPVs (e.g. SNPVs and MNPVs have multiple ODVs in the OB, MNPV ODVs contains multiple nucleocapsids). At least three resistance mechanisms have been found and will be discussed in detail in the discussion (Eberle and Jehle, 2006; Asser-Kaiser et al., 2007; Berling et al., 2009; Schmitt et al., 2013; Sauer et al., 2017b).

There are multiple ways the host can evolve to be resistant to pathogens (i.e. behavioural avoidance, changes in morphology and binding sites, and immune protection). Resistance against different types of pathogens is likely to vary since their methods of infection are different. For example, resistance against entomopathogenic fungi, which enter through the cuticle, is likely to be different to orally ingested pathogens. The resistance mechanism against pathogens, such as baculoviruses, that hijack host cellular machinery for reproduction is likely to be different from pathogens such as Bt that produce harmful endotoxins.

Invertebrates, including insects, lack the adaptive immune system present in vertebrates which allows for immunological memory, as well as high levels of specificity in its defense mechanism (Schmid-Hempel, 2005; Little et al., 2005). However, recent research provides evidence of some elements of immunological memory and specificity in invertebrates (Rodrigues et al., 2010; Netea et al., 2011). There are several ways the insect immune system can defend itself from pathogens. The physical barriers are the first line of defense against pathogens: e.g. the peritrophic membrane against baculoviruses and the cuticle against entomopathogenic fungi (Hoover et al. 2010; Ortiz-Urquiza and Keyhani 2013). Insect saliva and digestive juice often contain proteins or
enzymes that could inactivate ingested pathogens (Eichenseer et al., 2010; Ponnuvel et al. 2012). Once through the physical barriers, the pathogens can be recognized and trigger humoral and cellular immune responses (Lemaitre and Hoffmann, 2007), resulting in the production of reactive oxygen species and antimicrobial peptides and encapsulation, phagocytosis, and melanization (Tsakas and Marmaras, 2010).

Evolved resistance often involves costs of maintaining resistance mechanisms (Schmid-Hempel, 2005; Libert et al., 2006). Additionally, there could be genetic trade-offs between traits, such as within the insect immune system; for example, in *Spodoptera littoralis* (the African cotton leafworm), antibacterial activity exhibits a significant negative genetic correlation with haemocyte density (Cotter et al., 2004). Immune responses reduce the resources available for normal body functions, which can affect an individual’s fitness (Moret and Schmid-Hempel, 2000; Koella and Boëte, 2002). Frequent selection and bottlenecks as a result of high pathogen-induced mortality can rapidly increase, or even fix, the frequency of resistance alleles (Bourguet et al., 2003; Zichová et al., 2013). However, this does not always happen, partly due to the strong trade-off costs between resistance and other life-history traits (Schmid-Hempel, 2005; Paris et al., 2011). In some circumstances, the evolved resistance (against baculoviruses and Bt) is reversed within several generations (Fuxa and Richter, 1989; Gassmann et al., 2009) and this is mainly because of the trade-offs such as with other life history traits mentioned above. However, resistance reversal does not always occur and resistance can be persistent (Gassmann et al., 2009; Undorf-Spahn et al., 2012).

Insect pathogen populations often have high intra-specific diversity and this is observed for all pathogen groups (i.e. fungi, bacteria, and baculoviruses) (Cory and Myers 2003; Cory and Franklin, 2012; Tanada and Kaya, 2012). For example, 24 genotypes of NPV were isolated from an individual pine beauty moth (*Panolis flammea*) larva; comparison of 7 of the variants demonstrated significant differences in pathogenicity and speed of kill (Cory et al., 2005). In baculoviruses, there are many potential mechanisms (e.g. host resource partitioning, virulence trade-off, and variation in host immunity) that could maintain diversity (Hodgson et al., 2004). For example, host variation in susceptibility helps maintain phenotypic variation in the baculovirus that infects *Lymantria dispar* (the gypsy moth) (Fleming-Davies et al., 2015). In fungi, there is considerable intraspecific diversity among isolates of the fungi *Beauveria* spp (Wang et
al., 2005) and in bacteria, Bt is highly diverse in its endotoxins which vary in pathogenicity and for different hosts (reviewed in de Maagd et al., 2003). However, in commercial microbial control agents, pathogen diversity is often reduced due to the production process selecting for certain traits and the pathogen diversity is not often monitored.

Mixed infections can have other consequences. When pathogens with different genotypes share the same host, there are different ways they could interact that could affect the overall virulence (degree of damage experienced by the host) (Mideo, 2009). A leading hypothesis to explain the evolution of virulence assumes a trade-off between the level of pathogen virulence and transmission rate, which will mediate the severity of the infection (Alizon et al., 2009). There is a balance between the extent to which the host is exploited and the likelihood of transmission; if the host killed too quickly, then the opportunity for transmission is also reduced (Thomas et al., 2003; Alizon et al., 2013). This theory has been mainly applied to vectored pathogens where the host needs to be alive for the pathogen to be transmitted, but could also apply to obligate killers like many entomopathogens, which are transmitted directly (Blaser and Schmid-Hempel, 2005; Redman et al., 2016). However, the trade-off is more complicated in diverse pathogen infections because there are likely to be differences between within host competition and between host transmission success; what is most successful in within host competition is not necessarily the most successful at transmission to new hosts (Alizon et al., 2009). This has not been well studied in insect pathogens.

Diverse infections can be more virulent (Read and Taylor, 2001), although the virulence of mixed infections is often strain dependent and few studies have looked at the impact of increasing pathogen diversity on virulence. In the entomopathogenic fungus, *Metarhizium anisopliae*, intraspecific competition favoured more virulent (mortality) strains in the wax moth *Galleria mellonella* (Staves and Knell, 2010). In baculoviruses, mixed infections of two variants of PfNPV in *Panolis flammea* (the pine beauty moth) caused higher mortality and produced higher yield than single-genotype infections, although this was modulated by nutrition (Hodgson et al., 2004). Similarly, some mixtures containing parasitic SfNPV variants in *Spodoptera frugiperda* (the fall armyworm) resulted in higher virulence. Variant identity is important, virulence can go up or down in mixtures with different parasitic variants (Lopez-Ferber et al., 2003; Simon et al., 2005; Simon et al., 2006). These parasitic genotypes lack certain essential genes.
and require co-infection with complete infectious genotypes. However, parasitic genotypes can also reduce pathogenicity: for example, mixed SeMNPV infections in *Spodoptera exigua* (the beet armyworm) containing genotypes with defective genomes can reduce the pathogenicity of the viral population by up to 3.6X. The co-infection is persistent and the parasitic genotypes constituted up to 30% of the viral progeny (Muñoz and Caballero, 2000).

Virulence could also be reduced in mixed infections due to competitive inhibition through production of antagonistic compounds which is both energetically costly and can decrease virulence (Chao et al., 2000). Different bacteria genotypes can interfere with each other through either direct attacks or chemical/mechanical exclusion (Gold et al., 2009). Diverse pathogen infections could also lower the severity of infection by limiting the development of extremely virulent genotypes (Smith et al., 1999). Selection in mixed Bt infections resulted in improved suppression of competitors and decreased virulence (Garbutt et al., 2011). Moreover, mixed infections were less virulent than single-strain infections and had decreased pathogen growth both *in vivo* and *in vitro*.

It is theorized that it is less likely for resistance to evolve against a diverse pathogen population because mounting immune response against diverse infections can be costly to the host (MacDonald and Linde, 2002; Ulrich and Schmid-Hempel, 2012). Although this depends on how generalized the response to infection is. For example, the mosquito *Anopheles stephensi* infected by mixed genotypes of the parasite *Plasmodium chabaudi* experienced reduced bloodmeal size which in turn reduced fecundity (Ferguson et al., 2003). Additionally, individuals carrying resistant alleles for multiple strains are often extremely rare (e.g. the frequency of resistant alleles again Bt in the Lepidoptera *Heliothis virescens* was estimated to be 0.0015) (Gould et al., 1997; Shelton et al., 2007; Tabashnik et al., 2009). Thus it is unlikely that resistance against multiple strains of pathogens will evolve at the same rate as against a single strain. Alternatively, mixed pathogens may be ineffective in reducing resistance rate due to more intense selection pressure that can result from increased virulence (Tabashnik, 1989).

There has been little research on the impact of pathogen diversity on the evolution of resistance in terms of pest management. In practice, there is a tendency to use single virus clones or isolates of unknown diversity. However, it is important to understand the impact of diversity. Additionally, if the microbial insecticide is produced
using large scale in vitro techniques, such as cell culture, selection would favour genotypes that are not necessarily as effective in vivo. Only one study has addressed this question in insect pathogens. In a multi-generational selection experiment, the impact of strain diversity on the rate of evolution of resistance of the diamondback moth Plutella xylostella was compared using single and mixed isolates (but not clones) of Bt. The rate of evolution of resistance (i.e. susceptibility to Bt) was not significantly different (Raymond et al., 2013). It is important to note that Bt is different from other pathogens because Bt kills insects primarily with endotoxins, which act more similarly to chemical insecticides (e.g. paralyzing digestive tract), thus this example might not be a good predictor for other insect-pathogen systems.

This study aims to explore the role of pathogen diversity on the evolution of resistance in an insect using Autographa californica multicapsid nucleopolyhedrovirus (AcMNPV) and the cabbage looper, Trichoplusia ni (Hübner), as a model system. AcMNPV is a baculovirus which is genetically highly variable (Erlandson, 2009; Chateigner et al., 2015) and has a relatively broad host range, being capable of infecting more than 30 Lepidopteran species (Goulson, 2003; Clem and Passarelli, 2013). AcMNPV occlusion derived virus (ODVs: present in a polyhedrin matrix and responsible for the primary infection) can contain multiple nucleocapsids which can differ in genotype and phenotype (Bull et al., 2001; Bull et al., 2003; Erlandson, 2009; Harrison et al., 2012; Ikeda et al., 2015) this often results in mixed infections within the host. However, AcMNPV diversity has been often examined using insect cell culture lines which selects only from budded virion infections and may not reflect genotypic variation in the original wild-type isolates (Cory et al., 2005). T. ni belongs to the Order Lepidoptera and Family Noctuidae. It is a multivoltine generalist herbivore that feeds on many plants including many within the family Brassicaceae, in addition to other important crops (e.g. tomato, cucumber, bell peppers). Due to the voracious appetite of larval stage, T. ni is a major pest both in field and greenhouse in British Columbia and on most continents (Cervantes et al., 2011). The commercial development of Loopex (AcMNPV microbial insecticide; website: http://www.andermattbiocontrol.com/sites/products/bio-insecticides/baculovirus/loopex.html) in Canada provides growers with another option to control T. ni populations that have been shown to develop resistance against Bt (Janmaat and Myers, 2003), and also to reduce the use of chemical controls. Understanding the resistance potential of T. ni against baculovirus and how to mitigate
the rapid development of resistance are important, not only for management of *T. ni* but also as a model for other Lepidopteran species and the evolution of resistance of insects against baculoviruses.

Experimental evolution studies are useful tools to examine the plausibility of different mechanisms on the outcome of selection (Kassen, 2002). Here I summarize the methods I used to achieve the study goal above. I first isolated AcMNPV variants and compared them at the DNA level using Restriction Fragment Length Polymorphism (RFLP) gel electrophoresis and virulence bioassays. I then created a colony of genetically diverse *T. ni* in order to maximize the possibility of resistance alleles being present (King and Lively, 2012). In a multi-generational selection experiment, replicated insect populations were challenged with different levels of virus diversity over five generations. Subsets of individuals from each treatment were taken and exposed to a dose-mortality assay to estimate the change in their resistance levels. Life-history traits were measured and compared to investigate any costs of fighting infection.

### 2.2. Methods

#### 2.2.1. Preparation of the insect colony

In order to increase genetic diversity, a colony of the cabbage looper *Trichoplusia ni* (Hübner, 1803) was produced by combining individuals from three sources: insects collected locally in September 2014 from a broccoli field (Delta, British Columbia, Canada), a colony imported from a commercial supplier (Benzon Research Inc., Carlisle, Pennsylvania, USA), and an existing lab colony originally collected from a commercial tomato greenhouse in Delta in 2001 (Janmaat and Myers, 2003). All colonies were maintained on the same wheat-based artificial diet (Shikano et al. 2015) at 25±1 °C and L16:D8 for several generations before mixing. A total of 100 breeding pairs from the three cultures (Table 2.1.) were mated singly for the first generation and then en masse in the following generations. The new mixed colony was maintained at 250 individuals (assumed roughly equal sex ratio) and reared for eight generations under the same conditions prior to the selection experiment to ensure sufficient genetic homogeneity and reduce the effect of linkage disequilibrium.
2.2.2. Isolation and characterization of virus variants

2.2.2.1. In vivo dilution cloning

The wild-type AcMNPV mixture contains many genotypes and was first isolated in 1964 from a single field-collected caterpillar of *Autographa californica* (the alfalfa looper) by Crumb’s methods (Crumb, 1956; Vail, 1971). The AcMNPV isolate used in the experiment was obtained from E. Herniou (IRBI, Tours, France) who subjected it to deep sequencing to assess the genetic diversity (Chateigner et al., 2015). This isolate was originally obtained from P. Vail by J. Cory and is the closest to the original AcMNPV isolate that is available. It has been amplified in *T. ni*, and although the number of amplification cycles is unknown, it is likely to be less than 10. Baculovirus genotypes (variant/clone refer to baculoviruses that are thought to be genetically identical (i.e. a single genotype) and here I use the terms interchangeably) are usually isolated for molecular studies using cell culture (Pijlman et al. 2001; Pijlman et al., 2003). However, *in vitro* (i.e. tissue culture) cloning tends to select for genotypes that grow well in cells, potentially losing some of the natural diversity within the population. In addition genes that are important for infecting insects can be rapidly lost during this process (Pijlman et al. 2001). Dilution cloning *in vivo* avoids the problems associated with cell culture studies, although any genotypes that are not capable of oral infection will be lost during this process (Muñoz and Caballero, 2000; Serrano et al., 2013). *In vivo* dilution cloning is a process where insects (larvae) are challenged with decreasingly low concentrations of virus OBs (occlusion bodies). This assumes that the probability of infection follows a Poisson distribution, thus the virus is diluted low enough such that infection is ideally initiated by only one OB (van Beek et al. 1988; Zwart et al., 2008). This allows single clones of virus to be isolated from an original mixture. However, because of the multiply-enveloped morphology of NPVs, a true clone cannot be guaranteed. To try and avoid retaining mixed genotypes, several rounds of *in vivo* cloning are performed.

Variants were isolated from the AcMNPV isolate using three rounds of *in vivo* dilution cloning. Neonate *T. ni* were reared individually on artificial diet in wells of a 128-well bioassay tray (Frontier Agricultural Sciences, BAC128) until the early fourth instar. They were then transferred to a 24 well plate (VWR Standard Line Multiwell Cell Culture Plates) and starved for 24 h prior to inoculation with virus using the diet plug method (Shikano et al. 2015). Insects were inoculated with 5-10 OBs/3μL virus suspension
pipetted onto a 1 x 4 mm diet plug. The doses were chosen after a preliminary dose-
mortality assay with 10, 100, 1000 OBs/3 μL found that around 10 OBs should result in
<10% mortality. A total of 542 fourth instar larvae were inoculated in the first round (218
for 5 OBs dose and 324 for 10 OBs dose). After 24h, individuals that had eaten their diet
plug were transferred into individual 1 oz. cups (Solo P100N) with diet. Insects were
maintained in the same conditions as the colonies described above, and monitored daily
until death or pupation. The two subsequent rounds of cloning were carried out at 1
OB/larva with 40 insects for each of 29 clones isolated in round one. One cadaver was
kept for the next round for each ‘clone’, usually the first cadaver with a sufficient virus
yield. Virus-killed cadavers were placed into 1.5 mL microcentrifuge tubes (VWR) and
were kept at -20°C until characterization.

2.2.2.2. Enumeration of occlusion bodies

The concentration of virus OBs was estimated using a haemocytometer (Hausser
Scientific Bright-Line 3100). The virus was released (see section 2.2.2.4.) and
suspended using a vortex mixer and then serially diluted down to 1 in 1000 or 1 in 100
(depending on concentration, assessed visually) with distilled water and counted using a
phase-contrast microscope (Nikon Eclipse 50i) at 400x magnification; ten counts were
made for each sample/cadaver.

2.2.2.3. Initial Mortality Screening

Putative clones isolated through the three rounds of dilution cloning were
compared in two ways to screen for differences. Firstly, 29 clones were assayed for
differences in virulence (mortality) at a single virus dose. Early 4th instar T. ni larvae were
challenged with 100 OBs in 3μL and monitored as described above. The assay was
repeated three times, two of which were carried out at the same time.

2.2.2.4. Viral DNA Extraction

Individual virus-killed caterpillars were macerated in an equal volume (topped up
to 1 mL with distilled water) using an electric tissue homogenizer (VWR Fixed Speed
Vortex Mixer) in order to release the viral OBs from the body tissue. DNA was extracted
using 75 μL of undiluted OBs in 0.6mL microcentrifuge tubes. Fifteen μL 0.5M EDTA
(Bioshop, Burlington, Ontario, Canada) and 1.5 μL Proteinase K (Qiagen) were added to
the viral suspensions, and left to incubate in a water bath (Thermo Scientific, Lindberg
Blue M) at 37°C for 90 min with occasional inversion of the tubes. Then 15µL sodium carbonate (1M NaCO₃; Caledon Laboratories Ltd., Georgetown, Ontario, Canada) was added to the samples and incubated for another 90 min at 37 °C. After incubation, 10 µL 10% SDS (Calbiochem, Japan) was added and the sample was then incubated for an additional 15 min in the water bath at 37 °C. The samples were centrifuged at 17949 rcf (g) (Eppendorf Centrifuge 5417R) for 30 sec to pellet unwanted insect debris. The resulting supernatant was removed and placed in a new tube for phenol-chloroform extraction of DNA. An equal amount of phenol/chloroform (1:1) (Sigma-Aldrich) was added to each sample and mixed for 5 min by inverting the tubes. The samples were then centrifuged at 17959 rcf (g) for 10 min. The aqueous top layers were then removed to new tubes, and the organic layer was discarded. This was repeated and then chloroform/isoamyl alcohol (24:1) (Sigma-Aldrich) was added to remove any excess phenol. The final resulting top aqueous layer was removed and transferred to dialysis chambers. A mini dialysis chamber was made using a heated sharp knife to cut off the top section and lid of a plastic 1.5 mL microcentrifuge tube (Smith and Crook, 1988). The DNA was pipetted into the upturned lids with a single layer of dialysis tubing (that had been boiled for at least 1h) and placed into 4 L of dialysis buffer (1% TE buffer in Milli-Q water) in a cold room at 4 °C for at least 24 h with constant stirring and three changes of buffer.

2.2.2.5. DNA analysis

Each cadaver yielded approximately 100-130 µL DNA. Restriction endonuclease digestion was carried out on 20 µL (each sample was standardized to 3.3 µg) purified DNA, 25 µL Milli-Q water, 5 µL of the appropriate buffer, and 1 µL (5U) of restriction endonucleases (Either HindIII or EcoRV (Eco32I) (Thermo Scientific). The DNA was digested for at least 2 h at 37 °C. Afterwards, 10 µL of 6 x DNA loading dye (Thermo Scientific R0611; Bromophenol Blue, Xylene Cyanol FF) was added to the DNA samples and Quick-Load® 1 kb Extend DNA Ladder (0.5-48.5 kb; New England Biolabs Inc. N3239S) at 6:1 ratio. The agarose gel consisted of 0.8% molecular grade agar and 1 x TBE (90mM TRIS Borate (Invitrogen), 2mM EDTA at pH 8.0). DNA was stained using 10000x Biotium GelRed Nucleic Acid Stain. The gel was run at 35V for 24 h (Thermo Scientific Owl Horizontal Gel Electrophoresis System A2). An UViPro Gold GAS7400 Gel Documentation System, running UviPro software, was used to capture and analyze the gel image.
2.2.3. Effect of diversity on virulence

A dose-mortality assay was carried out to determine the differences in virulence between the virus clones to determine the doses to be used in the selection experiment. The isolates were selected based on differences in RFLP profile and those that had a range of virulence levels according to the initial mortality assay. The mixtures were chosen based roughly on their individual virulence levels from the initial assay. The assay used the same method of infection and rearing as described in the in vivo dilution cloning section above; 24 early 4\textsuperscript{th} instar individuals were inoculated at each dose (1000, 600, 300, 100, 30 OBs/3μL) for each treatment and 24 individuals for the control (3μL of water). The virus treatments were: the parent wild-type mixture (given a nominal diversity of 20), an 8-variant mixture (contains the eight variants examined using RFLP profiles and had a range of virulence based on initial assay: URFJVIKQ), and three 4-variant mixtures ('vifj'='Low', ‘urfj’='Medium’, ‘urkq’='High’) of varying levels of virulence. All mixtures contained equal proportions of each variant. The assay was repeated three times.

2.2.4. Selection experiment

A multi-generational experimental evolution protocol was used to select for resistance in the T. ni population. Three different diversity levels were used to select for resistance: single clones, mixtures of four clones and the parent wild type isolate. There were eight virus treatments: 4 single clones (‘u’, ‘r’, ‘f’, and ‘j’), 3 four-clone mixtures representing different levels of virulence (‘vifj’='Low', ‘urfj’='Medium’, ‘urkq’='High’) and the original mixed variant wild type isolate, plus an unexposed, water only, control. The 8-variant mixture was not used due to logistical constraints (not enough insects and supplies available).

For each virus/diversity treatment, 168 early 4\textsuperscript{th} instar larvae were weighed and starved for 24 h prior to challenge with an LD70 dose via the ‘diet plug’ method already described. LD70 is the dose of virus used where 70% of challenged hosts die; 70% was selected to ensure a high enough selection pressure while not so high so as to drive resistance genotypes rapidly to fixation. The LD70 varied among treatments, thus virus mortality was equal across treatments but virus dose varied. Insects were monitored twice daily until pupation or death.
Pupal mass of the survivors was measured (within 3 days of pupation) and sex was recorded. The next generation was produced by haphazardly selecting 15 pairs from each treatment and mating each pair singly in a 16 oz. Solo cup. Mating adults were fed with 10% sugar water; black construction paper lined the interior of the 16 oz. cup to allow for oviposition and easier egg counting. All eggs laid for the 72h period after the initiation of oviposition were counted (eggs start to hatch after 3 days thus making egg counting less accurate); peak oviposition usually occurs on the 2\textsuperscript{nd} and 3\textsuperscript{rd} day. Neonates from all pairs contributed approximately evenly to the next generation. Initially 256 neonates (haphazardly chosen) were set up individually, and 168 of these were used for selection. This allowed the selected individuals to be as consistent in size and age (early 4\textsuperscript{th} instar) as possible.

The selection experiment ran for five generations and each treatment was replicated five times giving a total of approximately 36,000 insects. The selection dose remained constant for all generations. All individuals were kept in 24h darkness and maintained at 25.7-26.3 °C.

2.2.5. Changes in resistance

In order to monitor changes in host resistance to virus over time, a dose-response assay was carried out on every generation of the selected line and compared with the unselected controls. The selected lines were reared through for one generation without selection prior to assay to account for possible parental influences on virus resistance, such as immune priming (Tidbury et al., 2011). In each treatment, 50 neonates were haphazardly selected from each replicate and reared through and 15 males and 15 females were singly-mated to form the generation tested in the resistance assay. Each mating pair contributed roughly equally to the next generation. Each treatment line was compared with the virus clone or mixture of clones that it was selected against, paired with unselected controls. Each assay consisted of 5 treatments of 24 early 4\textsuperscript{th} instar larvae each, evenly spread over four doses (1000, 450, 150, 50, OB/3μL) and an untreated control (fed 3μL water instead; otherwise treated the same as treatments individuals). Larval weight at inoculation was also recorded. Mortality, speed of kill and time to pupation were recorded. All other conditions were kept the same as for the selection experiment. The assay had five replicates. A total of 44,400 individuals were assayed.
2.2.6. Statistical analysis

Dose-mortality assays were analysed using a generalized linear model (GLM) using binomial distribution and a logit link function to compare virus mortality among clones or mixed clone (diversity) treatments. The wild type virus was given a nominal diversity of 20 and analysed as ordinal. The number ‘20’ was chosen because I believe there is at least 20 genotypes within the wt AcMNPV isolate. Wild-type isolate of *S. exempta* NPV contain at least 17 genotypes (Redman et al., 2010); 24 *P. flammea* NPV genotypes were isolated from a single larva (Cory et al., 2005). Ultra Deep Sequencing of the same wt AcMNPV isolate showed it is highly diverse (Chateigner et al., 2015). Additionally, ‘20’ is analysed as ordinal, thus its numeric value does not impact the analysis.

Corrections for over-dispersion were included where appropriate. Generation and log_{10} virus dose were included as continuous variables (except in the initial diversity-virulence assay, where virus dose was not transformed). All terms and their interactions were included initially in the models and removed if not significant. Contrasts tests (the contrasts function in JMP using the GLM model) were used to compare between pairs or groups of clones and between individual clones and diversity treatments, as well as for comparisons within generations. LD70s and LD50s were estimated according to individually fitted inverse prediction functions based on GLM, with each treatment analyzed separately with combined replicates.

The background mortality (virus death not caused by baculovirus) in the selection line (< 4.2%) and the resistance assay (< 3.8%) were low. However, in the resistance assay, the background mortality had reached around 10% in a few replicates; therefore, mean log_{10} larval weight was included in the resistance assay as a co-variate instead of log_{10} larval weight as in the selection experiment. The total was adjusted for mortality according to: \( T_{adj} = T_d \times (1-(V_c+U_c)/T_c) \) (Olson, 2014). \( T_{adj} \) is the adjusted dose-specific total number assayed, \( T_d \) is the dose-specific total assayed, \( V_c \) is the number killed by virus in the untreated controls, \( U_c \) is the number dead by unknown causes for the untreated control dose, and \( T_c \) is the total number assayed in the controls. The resistance assay analysis was first split by selected and unselected lines and analyzed separately. Then the differences in mean proportionate mortality change between selected and unselected lines (unselected subtracted from selected for each replicate)
was compared using a standard least squares model with the virus treatment or diversity level and generation as the main factors. The LD50 was estimated separately for each treatment and generation. The resistance ratio was calculated by dividing the LD50 of the selected lines by the unselected lines in the 5th generation.

In the main selection line; pupal weight and egg count were checked for normality using the Shapiro-Wilk W test; egg count was squared-root transformed and pupal weight was log10-transformed. A standard least squares model and Tukey-Kramer HSD test was used to compare the pupal weight and egg count between the treatments groups. All analyses were carried out using JMP 12.

2.3. Results

2.3.1. Initial Mortality Screening

At the initial concentrations used for cloning, 5 and 10 OBs per larva; 10/218 and 19/324 insects died of virus infection respectively. The two subsequent rounds of cloning were carried out for each of the 29 clones (all 29 from the two doses in the first round were carried through), and the mortality was less than 10% in each round. All of the 29 in vivo-derived clones went through the three rounds of dilution cloning and were compared by virulence screening using a single dose bioassay. There was clearly a difference in virus mortality between the clones ($\chi^2 = 115.07$, $P < 0.0001$, df$_{29,58}$ with repeat included in the model) with average mortality ranging from 8% to 52% in response to a challenge of 100 OBs. The wild-type displayed higher virulence than the single isolates combined and caused 66.6% mortality (wt vs all the clones: $\chi^2 = 193.06$, $P <0.0001$) (Figure 2.2.). After three rounds of cloning, some but not all of the 29 clones were compared using RFLP (due to time/material constraints) until eight with distinct RFLP profiles were identified (Figure 2.3.). These clones were used in the dose-mortality assay and selection experiment.

2.3.2. Effect of diversity on virulence

I selected eight variants that differed in RFLP signature and induced mortality levels at the single dose and subjected them to a full bioassay, in comparison with different mixtures (levels of diversity) and the wild type virus. The three 4-variant
mixtures combinations were based on the virulence levels (high, medium, low). The four individual variants used in the selection experiment and variant combinations differed in their dose-response (virus variants, $F_{12,148} = 167.07$, $P<0.0001$, scale parameter 1.67; virus*dose, $F_{12,148} = 40.6$, $P<0.0001$; log dose, $F_{1,148} = 411.43$, $P<0.0001$; there was no difference between bioassay repeats, $F_{2,146} = 3.7$, $P=0.157$) (Figure 2.4.) (The response of all eight variants are shown in Appendix A Figure A1). I used the data from the bioassay to estimate individual LD70 doses for each clone, mixture and the wild type virus for the selection experiment (Table 2.2.).

Virus diversity affected the dose-response such that mortality increased with the number of variants at any dose (virus diversity, $F_{3,169} = 29.03$, $P<0.0001$, scale parameter 2.8; log dose, $F_{1,169} = 229.76$, $P<0.0001$; virus diversity*log dose $F_{3,164} = 0.734$, $P=0.865$) (Figure 2.5.). Challenge with the wild-type virus resulted in greater mortality than any combination of variants (wt vs. variant diversities 1, 4 & 8, $\chi^2=13.53$, $p=0.0002$). Single and 4-variant mixtures ($\chi^2=2.15$, $p=0.14$) and 4-variant and 8-variant mixtures ($\chi^2=2.18$, $p=0.14$) do not differ in mortality; but single and 8-variant mixtures did differ significantly in mortality ($\chi^2=5.97$, $p=0.015$).

2.3.3. Selection experiment

2.3.3.1. Virus mortality

I selected for resistance with different levels of virus diversity (1, 4 and wild type) over five generations at a consistent LD70 dose. In general, virus mortality went down over time in a non-linear manner. In terms of diversity, mortality from the single variants decreased more than the more diverse treatments which reached a plateau earlier (diversity, $\chi^2=493.5$, $p<0.0001$, scale parameter 1.0; diversity*generation, $\chi^2=184.0$, $p<0.0001$; generation, $\chi^2=1272.8$, $p<0.0001$; generation*generation, $\chi^2=47.2$, $p<0.0001$). Generation*generation*diversity was not significant ($\chi^2=1.4$, $p=0.50$). Virus mortality was affected by $\log_{10}$(larval weight) ($\chi^2=76.9$, $p<0.0001$); heavier larvae are more likely to survive. There was a difference between replicates ($\chi^2=10.5$, $P=0.032$) (Figure 2.6a.). Contrasts on diversity in the 5th generation shows that mortality in single isolates is different from the wt ($\chi^2=246.3$, $p<0.0001$) and 4-isolates mixtures ($\chi^2=264.5$, $p<0.0001$); 4-isolates mixtures are different from the wt ($\chi^2=18.1$, $p<0.0001$).
Individual virus variants and virus mixtures followed a similar pattern. Proportionate virus mortality decreased over time for all virus treatments and at different rates in a non-linear manner (virus variants, \( F_{7,31654} = 284.9, p<0.0001 \), scale parameter 1.0; variant*generation, \( F_{7,31654} = 194.3, p<0.0001 \); generation, \( F_{1,31654} = 1369.4, p<0.0001 \); generation*generation, \( F_{1,31654} = 46.7, p<0.0001 \); generation*generation*variant, \( F_{7,31654} = 36.9, p<0.0001 \)). Virus mortality was affected by \( \log_{10}(\text{larval weight}) \) (\( F_{1,31654} = 77.9, p<0.0001 \)). However, larval weight at virus challenge did not differ among treatments (\( F_{7,31682} = 2.00, p = 0.051 \)). There was a difference between replicates, \( F_{4,31654} = 10.7, P = 0.031 \).

In order to confirm that the selection dose produced an equal effect, we compared virus mortality in the first generation of selection. There was no difference in mortality between the virus treatments in generation 1 (\( \chi^2=12.7, p=0.079 \)); nor was there any difference between replicates (\( \chi^2=7.9, p=0.096 \)). However, by the fifth and final generation, the virus treatments differed significantly in mortality from each other (\( \chi^2=355.9, p<0.0001 \)); again there was no difference between replicates (\( \chi^2=8.8, p=0.067 \)) (Figure 2.6b.).

### 2.3.3.2. Potential costs

The pupal weight of the survivors of viral challenge differed depending on virus diversity (\( F_{3,16630}=422.45, p<0.0001 \)); single variant lines had heavier pupae than 4-variant mixtures and the wt and the uninoculated lines had the heaviest pupae (Figure 2.7a.). Pupal weight also decreased over time although the rate differed with diversity level (generations: \( F_{1,16630}=1.24, p=0.27 \); diversity*generation, \( F_{3,16630}=55.62, p<0.0001 \)) primarily because the control pupae did not change over time. As expected, males had heavier pupae than females (LSmean females: 0.2211g SE=0.0009; males: 0.2389g SE=0.0009) (\( F_{1,16630}=851.97, p<0.0001 \)) and male weight decreased at a faster rate than the female (sex*generation, \( F_{1,16630}=5.09, p=0.024 \)). However, both sexes responded in the same way to changes in virus diversity over time (diversity*sex: \( F_{3,16611}=0.20, p=0.90 \); diversity*sex*generation \( F_{3,16614} = 0.93, p = 0.42 \)). Changes were consistent across replicates (\( F_{4,16617} = 0.89, p = 0.47 \)).

Changes in pupal weight could also be the result of increased selection pressure as mortality decreased more slowly with diversity, in addition to differing among variants. When average mortality per replicate/generation is added as a co-variate, it has no
influence on pupal weight ($F_{1,16630}=0.22$, $p=0.64$). This suggests that selection related to mortality is not important and the decrease in pupal weight is likely to be a cost of more diverse infections and/or of evolved resistance. This conclusion is also supported by the decrease in pupal weight over time.

Female fecundity increased with pupal weight but the relationship was weak ($F_{1,6749}=269.9$, $p<0.0001$, $R^2 = 0.074$). Fecundity was only different between unselected (0 diversity) and selected individuals (diversity, $F_{3,3365}=7.60$, $p<0.0001$). The number of eggs laid decreased over time (generation, $F_{1,3365}=31.46$, $p<0.0001$) but was unaffected by virus diversity (diversity*generation, $F_{3,3365}=1.88$, $p=0.13$). The uninoculated lines laid more eggs than the inoculated lines ($F_{1,3365} = 12.7$, $p = 0.0004$) (Figure 2.7b). As with pupal weight, when mortality is added as a co-variate, egg count was not significantly affected ($F_{1,3365}=3.52$, $p=0.061$); this also suggests that mortality level is not important and the decrease in egg count is primarily a cost of surviving virus infections.

2.3.4. Change in resistance

2.3.4.1. Comparison between variants and variant mixtures

I looked at the change in resistance at each generation in the selected lines by comparing the difference in the dose-response against the unselected control. Each line was only compared with the virus that it had been selected against and the control line was also tested with the same virus. Initially the selected and unselected lines were analysed separately. Virus mortality in the unselected lines did not change over time (generation: $\chi^2=0.50$, $p=0.48$) and there was no difference between replicates ($\chi^2=0.015$, $p=0.90$). The only factors affecting virus mortality in the unselected lines was virus treatment ($\chi^2=1109.0$, $p<0.0001$) and dose ($\chi^2=2872.4$, $p<0.0001$). As demonstrated above (section 2.3.2.) virus treatments had different dose-responses (dose*virus, $\chi^2=389.5$, $p<0.0001$); as expected, the virus mortality is also affected by the mean (log) larval weight ($\chi^2=140.0$, $p<0.0001$). The dose-mortality graphs for all treatments and generations can be found in Appendix D Figure D2. When using a standard LS model to compare the larval weight between dead/survived, larvae that weighed less were more likely to die ($F_{1,96345}=290.2$, $p<0.0001$).

In the selected lines, as before, mortality was significantly influenced by virus treatment and dose (virus treatment: $\chi^2=1771.5$, $p<0.0001$; log$_{10}$dose, $\chi^2=1985.7$, $p<0.0001$).
However, virus mortality decreased over time (generation) in a non-linear manner and started to level out in later generations (generation*generation, $\chi^2=13.4, p=0.0003$; generation, $\chi^2=448.0, p<0.0001$), but not at the same rate for different virus treatments (generation*virus $\chi^2=114.4, p<0.0001$; generation*generation*virus is not significant ($\chi^2=7.4, p=0.39$). Virus mortality was also affected by the mean (log) larval weight ($\chi^2=126.4, p<0.0001$). There was no difference between replicates ($\chi^2=1.4, p=0.23$).

I also estimated LD50s for the resistance assay using the model above with the inverse prediction function for each individual treatment and replicate. This comparison is not as accurate as the main mortality analysis described above as there was a significant dose*virus interaction (i.e. the dose-responses were not parallel). However, it allows the results to be more easily summarized and visualized. In addition, it allows the estimation of resistance ratios (RR) to illustrate the changes in resistance between different treatments. The LD50 is unchanged in the unselected lines while the LD50 in the selected line increased for all treatments ('r' and 'u' increased considerably) (Figure 2.8.). There is a lot of variation in the amount of resistance achieved amongst the single isolates; when comparing the RR between treatments, some single isolate (i.e. ‘r’) became over 285-fold (dividing RR of ‘r’ by wt: 690/2.42=285) more resistant than the wt, others (i.e. ‘j’) is only 1.2-fold more resistant (see Appendix C Table C2. for full details).

### 2.3.4.2. Comparison between levels of diversity

I also looked at the change in resistance at each generation between different levels of virus diversity. As expected, the result in the unselected lines is similar for diversity as for the analysis on variants/variant-mixtures (in section 2.3.4.1.). Virus mortality did not change over time: generations ($\chi^2=0.40, p=0.53$) or between replicates ($\chi^2=0.012, p=0.91$). The only factors affecting virus mortality were the diversity of the virus treatment ($\chi^2=379.6, p<0.0001$) and dose ($\chi^2=1207.8, p<0.0001$); different diversity levels had different dose-responses (dose*diversity, $\chi^2=31.2, p<0.0001$). Viral death is affected by mean (log_{10}) larval weight ($\chi^2=123.1, p<0.0001$).

Looking at the effect of viral diversity in the selected lines, virus mortality was significantly affected by diversity level and dose (diversity, $\chi^2=1238.0, p<0.0001$; dose, $\chi^2=729.4, p<0.0001$; dose*diversity, $\chi^2=17.8, p=0.0001$). Virus mortality decreased over time (generation) in a non-linear manner and started to level out in later generations.
(generation\*generation, $\chi^2=12.5$, $p=0.0004$; generation, $\chi^2=447.4$, $p<0.0001$), but not at the same rate for different diversity levels (generation\*diversity, $\chi^2=87.3$, $p<0.0001$) (Figure 2.9). Again, viral death is affected by mean (log) larval weight ($\chi^2=129.0$, $p<0.0001$). There was no difference between replicate ($\chi^2=1.3$, $p=0.25$).

**2.3.4.3. Mortality differences between selected and unselected lines**

Although the previous analyses (Section 2.3.4.1. and 2.3.4.2.) indicated differences between virus and diversity treatments, the data are confounded by the impact that diversity has on virulence. Therefore to examine the impact of virus treatment and diversity on the evolution of resistance I looked at the difference in mortality between paired selection and unselected treatments by subtracting the control mortality from the selected line.

When analyzed using individual virus treatments, the difference in virus mortality between the selected and unselected lines changed in a non-linear fashion over time and starts to level out in later generations (generation\*generation, $F_{1,799}=6.0$, $p=0.015$). The difference increased with generation ($F_{1,799}=197.5$, $p<0.0001$), but not at the same rate for different virus treatments: generation\*virus ($F_{7,799}=8.5$, $p<0.0001$) (Figure 2.10a.). The difference in mortality is dependent on the virus treatment ($F_{7,799}=32.7$, $p<0.0001$). The difference is not dependent on the mean (log) larval weight ($F_{1,799}=0.63$, $p=0.43$). The result was consistent between replicates ($F_{4,799}=0.74$, $p=0.57$).

When analyzed using levels of diversity, the conclusion is the same. The difference in virus mortality between the selected and unselected lines changed in a non-linear fashion over time and starts to level out in later generations (generation\*generation, $F_{1,799}=5.4$, $p=0.021$). The difference increased with generation ($F_{1,799}=93.3$, $p<0.0001$), but not at the same rate for different diversity levels: generation\*diversity ($F_{2,799}=14.0$, $p<0.0001$) (Figure 2.10b.). The difference in mortality is dependent on the diversity level ($F_{2,799}=68.6$, $p<0.0001$). The difference is not dependent on the (log) larval weight ($F_{1,799}=0.19$, $p=0.67$). The result was consistent between replicates ($F_{4,799}=0.51$, $p=0.73$).
2.4. Discussion

In this experiment I investigated the role of pathogen diversity on the evolution of resistance. I predicted that host populations subjected to lower pathogen diversity would have a higher rate of evolution of resistance. I found that host populations subjected to selection with single variants had a higher rate of resistance development compared to more diverse treatments. There was considerable variation in the response to individual genotypes and the relative resistance ratio of insects exposed to some of the single variants was more than 200-fold greater than that for the wild-type virus. As expected, I found that the wild-type isolate was more virulent than less diverse isolates on average; although variant ‘r’ was not different in virulence to the wild-type. Higher virulence (mortality) might be expected to exert stronger selection for resistance; however, this was not the case. It is possible that the choice of selection level was too low, but more likely, the results support the hypothesis that resistance to diverse infections is more costly or less likely due to the rarity of resistant alleles, higher costs and possible trade-offs of disease resistance (Ferguson et al., 2003; Ulrich and Schmid-Hempel, 2012). The initial frequency of individuals that are relatively more resistant to a single virus variant in the population is likely to be low and the frequency of individuals resistant to multiple virus variants is likely even lower. This assumes that resistance to some virus variants is based on different resistance mechanisms (e.g. as shown in CpGV covered below). In that case, despite the higher selection pressure due to increased virulence in diverse virus infections, the rate of resistance is low simply due to the rarity of individuals with resistant genotypes. However, two of the single isolates developed significant levels of resistance within a few generations, suggesting that the initial frequency of resistant individuals may not be as low as predicted. Additionally, although the change in resistance is relative, when it reaches a certain level, it would not be economically viable (or even possible) to spray such a high concentration of OBs in the field; at that point, the insects are effectively totally resistant.

Two of the single variants, ‘u’ and ‘r’, showed much higher levels of resistance than ‘f’, ‘j’, and more diverse treatments, suggesting that multiple mechanisms might be involved (at least one mechanism for u and r and another for f and j). Although AcMNPV is genetically diverse (Chateigner et al., 2015), the different genotypes do not necessarily differ in virulence. It has been theorized that perhaps resistance to a single
isolate is common but with multiple pathogen mixtures, it is more difficult for host population to achieve a high level of resistance quickly (Alizon et al., 2009; Alizon et al., 2013). In spite of the higher level of selection imposed by the more virulent mixture, the ability of the host to evolve resistance appears to be limited due to difficulty of fighting off multiple infections. The interaction and the role different isolate play in a pathogen mixture may account for these results (Ben-Ami et al., 2008; Luijckx et al., 2011); however, additional research is required.

The mechanism of baculovirus resistance is not well understood. In baculoviruses, there is one known example of resistance in the field: the resistance of *C. pomonella* to CpGV. The resistance to the first strain of CpGV is thought to be dominantly inherited in a monogenic sex chromosome-linked mode that quickly spread across Europe within a few years (Asser-Kaiser et al., 2007; Asser-Kaiser et al., 2011). Resistance to the first strain was overcome, in a practical sense, with a new resistance-breaking variant that had a different resistance mechanism (Eberle et al., 2008; Berling et al., 2009; Graillot et al., 2014). However, very recently, some populations were able to break resistance again and achieve cross resistance to the second strain of CpGV with dominant autosomal inheritance (Sauer et al., 2017a); and a third resistance mechanism has also been identified (Sauer et al., 2017b). The identity of the specific resistance mechanism against CpGV is unknown, although it has been narrowed down to exclude peritrophic membrane modification, midgut receptor modification, and innate immune response changes (Asser-Kaiser et al., 2011). However, the resistance mechanism and inheritance is different between the strains; this suggests there are likely to be multiple resistance mechanisms to CpGV. NPV resistance mechanisms are also poorly understood but appear to be linked to the expression of two proteases (caspase-1 and serine protease) in the silkworm *Bombyx mori* resistance to BmNPV (Qin et al., 2012). Increased melanization is linked to the African armyworm (*Spodoptera exempta*) resistance to its NPV (Reeson et al., 1998). It is impossible to say what the likely resistance mechanisms are in my study, but any of the following changes could explain the varying degree of changes in resistance; for example, modification of midgut receptor and/or peritrophic membrane, increased sloughing of infected cells, or increased melanization in the midgut (McNeil et al., 2010). Due to the short period of time of my experiment, it is unlikely for novel resistance mechanism to evolve independently; the resistance mechanism likely already existed in some form in the population. There is
likely variation in the level that different defence mechanisms are present in the population (e.g. genetically-based variation in how quickly infected midgut cells are sloughed (Hoover et al., 2000)) and the selection process simply increased the proportion of individuals in the population expressing that trait. However, since the resistance level for some single isolates became over 600X higher, there could be a more 'all or nothing' mechanism (e.g. modification of midgut receptor). More research is needed in this area to narrow down the specific mechanisms for AcMNPV and other baculoviruses.

In this study, we observed reduced pupal weight and female fecundity in individuals under selection, particularly in more diverse virus treatments. Pupal weight and fecundity (at least over the first three days) decreased over time in the selected populations, while it was unchanged in the unselected populations. However it is also possible that the pattern and timing of oviposition could also have been changed under selection and further work is needed to discover whether temporal changes are taking place. It has been shown in previous studies that surviving pathogen challenge can have life-history costs (e.g. reduced fecundity and pupal weight as found in this study) (Abot et al., 1996; Lee et al., 2006; Povey et al., 2009). My result is similar to other studies: e.g. the Indian meal moth (Plodia interpunctella) subjected to its GV experienced reduced growth rate and fecundity (Sait et al., 1994); in the western tent caterpillars, Malacosoma californicum pluviale, reduced fecundity of moths could be explained by the costs of resistance to virus or as a sublethal effect surviving virus challenge (Myers, 2000; Cory and Myers, 2009). However, it should be noted that the cost is not necessarily of genetically-based resistance. Costs could be a result of fighting infection or some other sublethal effect and evolved resistance to some baculoviruses has been shown to have little cost, at least under the conditions studied, for example, T ni. against TnSNPV (Milks et al., 1998; Milks et al., 2002). There is a trend of increasing costs to the host with higher virus diversity in my experiment; this cost could be a sub-lethal effect of surviving the viral challenge or a cost of evolved resistance as the measurements were carried out on the generation that was challenged with virus. Since my data show that virus mortality does not significantly influence pupal weight; the decrease in pupal weight could be a cost of evolving resistance to more diverse infections.
Resistance to baculoviruses has not been found widely in the field possibly due to limited usage (and therefore selection), only used on less damage sensitive crops (therefore sprayed less often), compared to microbial pathogens such as Bt. This does not mean that resistance against baculovirus is less likely to develop because it has in one of the most widely used baculovirus insecticide (CpGV, mentioned in detail above) (Sauer et al., 2017a). During baculovirus insecticide production, genetic diversity/composition is often not monitored or considered, and in some cases, limited genetic diversity can make a product easier to register commercially. In these cases, it could result in pest populations being selected by less diverse or single virus variants which this study has found to be easier for resistance to evolve and thus make the insecticide less effective in the long term. Effective resistance management would depend on reducing the fitness of initially resistant individuals, using genetically diverse insecticide application, discovery of new ‘resistance breaking’ strains (e.g. in CpGV) (Sauer et al., 2017a), or co-evolution with the host. For example, Brazilian farmers spray AgNPV and collect the dead velvetbean caterpillar (Anticarsia gemmatalis) cadavers in the field every season allowing for co-evolution (Moscardi, 1999). In Bt, increased fitness cost in resistant individuals provides a counter selection against Bt resistance, especially in periods of Bt absence in the environment, resulting in subsequent resistance decline (Tabashnik, 1994; Janmaat and Myers, 2003). Perhaps the increasing fitness cost of resistance through the use of more diverse AcMNPV infections can achieve a similar result of reducing the rate of resistance development by providing this counter selection pressure. This strategy is, however, untested in baculoviruses since it is unknown whether AcMNPV resistance is comparable to models of Bt resistance such as the ‘single major gene’ model (Heckel et al., 1999; Carriere et al., 2010). If AcMNPV resistance follows the ‘single major gene’ model of Bt, the relative fitness of resistant to susceptible insects to AcMNPV could determine the rate of resistance evolving. High frequency and intensity (% mortality) of selection could rapidly increase resistance, however, inflicting a higher cost of resistance (i.e. reduced pupal weight with increased pathogen diversity) might prevent fixation of resistant individuals in the population and provide the opportunity for resistance to decrease or be lost in subsequent generations without selection, or slow down resistance through the use of untreated refuges (Sanchis and Bourguet, 2008; Huang et al., 2011).
There have been few studies on the impact of pathogen diversity on the evolution of host resistance in insects. In the one previous selection study using *P. xylostella*, Bt mixtures did not reduce the rate of evolution of resistance (Raymond et al., 2013). This is perhaps not surprising as Bt and baculoviruses infect and kill their host in very different ways and likely have very different resistance mechanisms. Additionally, Bt often have multiple toxins and strains often share the same or closely related toxins and cross-resistance can occur (Tabashnik, 1989; Raymond et al., 2013). Therefore, combining Bt strains did not necessarily increase the infection diversity greatly. There were also several key differences between my study and the Bt study: the Bt isolates used were not clones, just different commercial products and isolates from different locations which are likely to contain diverse toxins, in addition to different bacterial genotypes. I used higher number of individuals in the selection experiment (7200 vs 108 individuals in the 1st generation of selection) which increased the gene pool of potential resistant individuals. I also individually reared the larvae and each mating pair to ensure genetic mixing and prevent any horizontal transmission or any effect of density (while they were mated en masse in Raymond et al., 2013). The exposure time of my experiment was also shorter (24 h vs. 1 week). Rearing and inoculating in groups can alter disease resistance in Lepidoptera (either through direct interactions among insects or changes in diet quality or availability), and result in variable exposure to the pathogen (Reeson et al., 1998; Wilson et al., 2001). Additionally, when reared in groups, some species of caterpillar, including *T. ni*, can be cannibalistic, particularly if competing for food. These factors will likely complicate the effect of virus selection. Individually mated pairs ensure higher genetic diversity in the subsequent generation because in *T. ni*, males can mate with multiple females and thus potentially resulting in some males contributing disproportionately into the gene pool of the next generation. The virus concentrations in my experiment were originally set to not be high enough to rapidly select for resistance and to allow for observation of how different levels of diversity may affect the rate of resistance.

This study is the one of the first to show that challenge with diverse pathogen populations slows down the rate of resistance evolving. In summary, host populations subjected to selection with single virus variants had a higher rate of evolution of resistance compared to more diverse pathogen treatments. The impact of virus diversity on resistance development should not be overlooked in the development of virus
insecticides and potential resistance management, because significant levels of resistance to single isolates can develop rapidly. Once resistance reaches a certain level, it would not be economically viable for that baculovirus to be used for pest management because the virus density required to achieve sufficient levels of insect mortality would be too high. More research is needed to uncover the specific mechanisms behind the differences in evolved resistance, which could allow rapid screening of virus populations. Similar studies need to be carried out in other baculovirus systems, and on other insect pathogens, to examine whether this is a widespread phenomenon. The development of appropriate application and resistance mitigation strategies for microbial insecticides is crucial for their continued usage and development. In addition, this and future research on the maintenance and consequences of pathogen diversity will provide further insights on the evolution of disease resistance and pathogen-host interactions.
2.5. References:


Olson, G. L. (2014). Transgenerational effects of food quantity and quality on disease resistance in the western tent caterpillar, Malacosoma californicum pluviale (MSc dissertation, SFU Science: Biological Sciences Department).


van Beek, N. A., Wood, H. A., & Hughes, P. R. (1988). The number of nucleocapsids of enveloped *Autographa californica* nuclear polyhedrosis virus particles affects the survival time of neonate *Trichoplusia ni* larvae. *Journal of Invertebrate Pathology, 52*(1), 185-186.


2.6. Tables and Figures

Table 2.1. The origin of individuals in the original mixed *T. ni* population. Individuals were sourced from three cultures: wild, lab, and imported, and combined in different pair combinations. Approximately half of each colony type was male/female (the sex ratio was roughly equal) in all pair combinations.

<table>
<thead>
<tr>
<th>Number of pairs</th>
<th>Colony origins</th>
</tr>
</thead>
<tbody>
<tr>
<td>33</td>
<td>Wild/Import</td>
</tr>
<tr>
<td>33</td>
<td>Wild/Lab</td>
</tr>
<tr>
<td>17</td>
<td>Wild/Wild</td>
</tr>
<tr>
<td>11</td>
<td>Import/Lab</td>
</tr>
<tr>
<td>3</td>
<td>Import/Import</td>
</tr>
<tr>
<td>3</td>
<td>Lab/Lab</td>
</tr>
</tbody>
</table>
Table 2.2  LD70 (OBs) +/- 95% CI of four single AcMNPV isolates (f,j,r,u), three 4-isolate mixtures (L,M,H), and wild-type (wt) used in the selection experiment in early 4th instar T. ni.

<table>
<thead>
<tr>
<th>virus</th>
<th>LD70</th>
<th>Lower 95% CI</th>
<th>Upper 95% CI</th>
</tr>
</thead>
<tbody>
<tr>
<td>f</td>
<td>867.8</td>
<td>694.7</td>
<td>1271.2</td>
</tr>
<tr>
<td>j</td>
<td>765.6</td>
<td>641.2</td>
<td>1009.4</td>
</tr>
<tr>
<td>u</td>
<td>651.0</td>
<td>444.8</td>
<td>1237.7</td>
</tr>
<tr>
<td>r</td>
<td>316.1</td>
<td>262.5</td>
<td>365.3</td>
</tr>
<tr>
<td>L</td>
<td>623.1</td>
<td>610.8</td>
<td>636.0</td>
</tr>
<tr>
<td>M</td>
<td>633.7</td>
<td>480.2</td>
<td>934.4</td>
</tr>
<tr>
<td>H</td>
<td>408.2</td>
<td>301.5</td>
<td>543.1</td>
</tr>
<tr>
<td>wt</td>
<td>276.2</td>
<td>52.5</td>
<td>437.4</td>
</tr>
</tbody>
</table>
Figure 2.1  Diagrammatic summary of the *T. ni* – AcMNPV selection experiment and virus resistance assay. Pt I: Selection experiment; *T. ni* population infected with AcMNPV at LD70 with one of eight treatments: four single AcMNPV isolates (f,j,r,u), three 4-isolates mixtures (Low, Medium, High), and wild-type (wt) for five generations. Pt II: Change in resistance assay; compared selected vs. unselected line after skipping selection for one generation. Both experiments had five replicates.
Figure 2.2  Comparison of virus mortality in early 4\textsuperscript{th} instar \textit{T. ni} at 100 OBs between 29 AcMNPV isolates and the mixed wild-type (wt) (proportion mortality +SE: N= 3 repeats with 24 insects per clone in each repeat). The 8 clones used in the selection experiment (red) and the wild-type (black) are highlighted.
Figure 2.3  Gel electrophoresis of 8 AcMNPV isolates and the wildtype (Wt) isolate using (a) EcoRV and (b) HindIII enzymes with a size ladder (M; 0.5-48.5 kb). Gels were run at 35V for 24h with 0.8% agar; 2.9 micrograms of DNA were loaded in each lane. Banding differences between clones are identified with white dots.
Figure 2.4  Dose-response of early 4\textsuperscript{th} instar \textit{T. ni} to four single AcMNPV isolates (f,j,r,u), three 4-isolate mixture (L,M,H), and the wild-type (wt). Virus mortality is given in logits (log(p/1-p)). ‘p’ denotes proportion mortality due to virus. Lines are from the final statistical model and jittered mean experimental points are in the same corresponding colour. N=3 repeats, with 22 larvae per treatment per replicate on average.
Figure 2.5  
AcMNPV-induced mortality (shown as logits) in early 4th instar T. ni at different levels of virus diversity: 1 (isolates: f,j,r,u,v,i,k,q), 4 (mixes: L,M,H), 8 (all 8 single isolates), 20 (wt) with modeled lines and jittered mean experimental points in corresponding colour. Virus mortality is given in logits (log(p/(1-p))). ‘p’ denotes proportion mortality due to virus. N=3 repeats.
Figure 2.6  AcMNPV mortality in early 4\textsuperscript{th} instar \textit{T. ni} after challenge with an LD70 dose over five generations. Lines from the final statistical model are shown and mean virus mortality for each replicate in each treatment is represented with a jittered data point in the corresponding colour (N=5). 

a) Comparison between the three levels of diversity: single isolates (1), 4-isolates mixtures (4), and wild-type (20). 
b) Comparison between the four single isolates (solid line), three 4-isolates mixtures (dashed lines), and wild-type (dotted line).
Figure 2.7  The impact of virus (AcMNPV) diversity on a) T. ni pupal weight (back-transformed LS-mean +/- SE) and b) eggs laid in the first three days of oviposition (+/- SE) averaged for all generations based on Tukey-Kramer HSD test. Replicate lines were subjected to varying levels of AcMNPV diversity (1, 4, 20) and control (0) over five generations. Significant differences (p<0.05) are shown by letters. N= 16631 for pupal weight and N=3375 for egg count.
Figure 2.8  Comparison of log$_{10}$(LD50) of early 4$^{th}$ instar T. ni to AcMNPV between a) unselected and b) selected lines across generations with +SE for all treatments: single (u,r,f,j), 4-isolates mixtures (L,M,H), and wild-type (wt).
Figure 2.9  Comparison of mortality due to AcMNPV in early 4th instar T. ni between selected and unselected lines across generations. Modeled lines and jittered data points are shown for all levels of diversity: singles (green), 4-isolates mixtures (red), and wild-type (black).
Figure 2.10 The difference in percent AcMNPV mortality in early 4th instar T. ni between selected and unselected lines across generations with model line and data points for a) all treatments: single (u,r,f,j), 4-isolates mixtures (l,m,h), and wild-type (wt); b) shows average difference for diversity levels: single (1), 4-isolates mixtures (4), and wt (20). N=5 repeats.
Appendix A:

Virulence comparison between the 8 single AcMNPV isolates

Figure A1. Virulence comparison between the 8 single AcMNPV isolates used in the selection experiment and resistance assay. Log(dose) and logit (log(p/1-p)) probability of virus mortality in early 4th instar T. ni comparison between the 8 single AcMNPV isolates (f,j,r,u,i,k,q) with modeled lines and jittered mean experimental points with corresponding colour. 'p' denotes proportion mortality due to virus. Analysis is same as the ones with mixtures; N=3 repeats.
Appendix B:

Virulence (log(LD50)) comparison

Table B1. Virulence (log(LD50)) comparison between the eight treatments used in the selection and resistance assay. Modeled log(dose) log(LD50) (OBs) in early 4th instar T. ni of four single AcMNPV isolates (f,j,r,u), three 4-isolates mixtures (L,M,H), and wild-type (wt) along with 95% CI. – denotes negative LD50 number outside the range. N=3 repeats.

<table>
<thead>
<tr>
<th>virus</th>
<th>LD50</th>
<th>Lower 95% CI</th>
<th>Upper 95% CI</th>
</tr>
</thead>
<tbody>
<tr>
<td>u</td>
<td>2.72</td>
<td>2.43</td>
<td>3.39</td>
</tr>
<tr>
<td>r</td>
<td>1.99</td>
<td>0.60</td>
<td>2.29</td>
</tr>
<tr>
<td>f</td>
<td>2.74</td>
<td>2.66</td>
<td>2.84</td>
</tr>
<tr>
<td>j</td>
<td>2.53</td>
<td>2.34</td>
<td>2.72</td>
</tr>
<tr>
<td>L</td>
<td>2.23</td>
<td>1.65</td>
<td>2.62</td>
</tr>
<tr>
<td>M</td>
<td>0.86</td>
<td>-</td>
<td>1.47</td>
</tr>
<tr>
<td>H</td>
<td>1.51</td>
<td>-</td>
<td>2.15</td>
</tr>
<tr>
<td>wt</td>
<td>1.95</td>
<td>-</td>
<td>2.28</td>
</tr>
</tbody>
</table>
Appendix C:

Change in resistance

Table C1. Change in resistance (resistance ratio (RR)) is compared between the eight treatments after selection. Modeled log(dose) back-transformed LD50 (OBs) in early 4th instar T. ni of four single AcMNPV isolates (f,j,r,u), three 4-isolates mixtures (L,M,H), and wild-type (wt) in the 5th generation for the selected and unselected lines; and RR—LD50 of selected insects ÷ LD50 of unselected. N=5 repeats.

<table>
<thead>
<tr>
<th>virus</th>
<th>LD50 Selected</th>
<th>LD50 Unselected</th>
<th>RR</th>
</tr>
</thead>
<tbody>
<tr>
<td>u</td>
<td>34698.92</td>
<td>68.58</td>
<td>505.98</td>
</tr>
<tr>
<td>r</td>
<td>17087.17</td>
<td>24.78</td>
<td>689.61</td>
</tr>
<tr>
<td>f</td>
<td>2417.42</td>
<td>450.83</td>
<td>5.36</td>
</tr>
<tr>
<td>j</td>
<td>1261.96</td>
<td>422.97</td>
<td>2.98</td>
</tr>
<tr>
<td>L</td>
<td>588.46</td>
<td>341.62</td>
<td>1.72</td>
</tr>
<tr>
<td>M</td>
<td>641.67</td>
<td>166.73</td>
<td>3.85</td>
</tr>
<tr>
<td>H</td>
<td>180.10</td>
<td>54.44</td>
<td>3.31</td>
</tr>
<tr>
<td>wt</td>
<td>121.22</td>
<td>50.03</td>
<td>2.42</td>
</tr>
</tbody>
</table>
Appendix D:

Dose response over time

![Graphs showing dose response over time for different generations with various treatments.]
Figure D1. The dose response of 4\textsuperscript{th} instar *T. ni* larvae to AcMNPV in a) selected lines and b) unselected lines through five generations. The actual proportion of virus mortality at four doses (50, 150, 450, and 1000 OBs) for each of the eight treatments: single (u,r,f,j), 4-isolates mixtures (L,M,H), and wild-type (wt). N=5 repeats.