Baculovirus infection, host immunity and pathogen competition in the cabbage looper, *Trichoplusia ni*

by

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Abstract

Competition between pathogens within a host could change the short term and evolutionary outcomes of an infection. These interactions include resource-based and immune-mediated competition. We understand little about the insect immune response to virus infection and how this alters the host for future co-infecting pathogens. I characterised several immune parameters of the cabbage looper, *Trichoplusia ni*, in response to challenge by a baculovirus, TnSNPV, and found that all parameters reduced with increasing virus dose. To measure the effect of competing pathogens on virus success, I exposed *T. ni* to TnSNPV followed by either the bacterium, *Bacillus thuringiensis*, or the fungus, *Beauvaria bassiana*. The fungus reduced the infection success of the baculovirus and the bacterium negatively impacted virus replication. This outcome has consequences for microbial control agents in insect pest management and adds to our fundamental understanding of the effect of mixed infections on pathogen and host populations.

Keywords: Baculovirus; co-infection; invertebrate immunity; competition; dose dependence; virus yield; host-pathogen interaction.
This thesis is dedicated to my loving and supportive family who have always encouraged me to follow my dreams; most especially to my grandpa, Gordon, who has inspired me throughout my life and continues to do so - thank you for believing in me.
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Chapter 1. Thesis Introduction

Competition is ubiquitous across living organisms and occurs between individuals of the same and different species when space, resources or mates are limited. The effects of competition are widespread and have been documented to affect fecundity and survivorship of individuals, as well as having population level impacts including altering population growth rate, carrying capacity and community structure (Begon et al 1996). Diversification within species is believed to be driven by intraspecific competition for resources by selecting for specialisation and niche expansion, as seen in *Drosophila melanogaster* where populations in treatments facing high competition adapted more rapidly to a given environment (Bolnick 2001). Interference competition takes place when organisms interact directly with each other, for example fighting over territories, whereas exploitation competition is indirect, with individuals depleting a resource that other individuals or species require (Begon et al. 1996). Whilst ecology largely focuses on interactions between macro-organisms, competitive interactions between micro-organisms within a host are often overlooked. Several studies have documented the impacts of single pathogens on host populations in various invertebrates, such as western tent caterpillars and a nucleopolyhedrovirus (Cory & Myers 2009), *Drosophila spp.* and sigma viruses (Longdon et al. 2011), *Daphnia magna* and a bacterial parasite (Decaestecker et al. 2007), and vertebrates, such as amphibians and Chytrid fungus (Berger et al. 1998), and humans with malaria (Enayati & Hemingway 2010). More recently, studies focusing on the pathogen community within a single host species have indicated a level of complexity previously underexplored. For example, Telfer et al. (2010) found that population level impacts in a field vole population were driven by interactions between the micro-parasite community that infected them. Colony collapse disorder in honeybees has also been attributed to the impact of multiple pathogens and parasites (Nazzi et al. 2012; Cornman et al. 2012). This emphasizes the need to study pathogens from a community perspective, using an individual host as an ecosystem in which the micro-organism community presides (Graham 2008). Principles
of community ecology can be applied to within-host interactions as they are in larger ecosystems (Pedersen & Fenton 2007); for example, looking for the pressures exerted on pathogens and parasites by ‘bottom-up’ effects of the host’s resources and ‘top-down’ impacts by the host immune response, as with a predator-prey interaction (Graham 2008). The ecology of various pathogens, most importantly viruses, and the interactions they have with their hosts and other micro-organisms is understudied. One of the main drawbacks is a lack of mechanistic understanding about the different ways that pathogens interact within a host and the variables that alter these interactions.

An increased understanding of the ways that pathogens interact within a host will inform decisions on infectious disease management (Balmer & Tanner 2011; Griffiths et al. 2011). For example, when a drug is applied against a specific pathogen, how does this affect the rest of the within-host community including pathogenic and commensal organisms? Additionally, many pathogens exist as multiple genotypes. The development of vaccines and new drugs to combat infectious disease often considers a host infected with a single, rather than multiple, species, and also rarely looks at the effect of the treatment on multiple genotypes. Drug applications may have differential effects on genotypes and could alter between-genotype interactions and thus the outcome of a drug treatment; one of many potential reasons why malaria is so hard to treat (Read & Taylor 2001). The evolution of drug resistance in malaria occurs faster in multiple strain infections due to resistant genotypes experiencing ‘competitive release’ as susceptible genotypes are wiped out, thus increasing access to host resources (Pollitt et al. 2014). Collecting observational data on the pathogens and parasites that are present in wild host populations is challenging, however conducting large scale, long term manipulative field studies is considerably more difficult, and almost impossible with viruses, that cannot be vaccinated against. Collaborations with theoretical ecologists have enabled lab studies or small scale field studies to be modelled at the population level (Rigaud et al. 2010). Models involving single host-parasite interactions are common but the field has been slow to recognise the more complex interactions that exist (Alizon & van Baalen 2008). However several mathematical models have tried to capture the complexity of co-infections, with a particular focus on intraspecific interactions between pathogen genotypes and the evolution of virulence, but empirical studies are required to support these models (Alizon et al. 2013).
1.1. Host-pathogen interactions

Pathogens are under selection pressure to both replicate within their host and to ensure transmission to a new host. The trade-off theory predicts that an intermediate level of virulence is optimal so that a pathogen can maximise fitness by delaying host mortality until adequate replication has occurred (Anderson & May 1981; Bull 1994; Alizon et al. 2009). Virulence has various definitions in the literature. For example, evolutionary biologists define it as the reduction in host fitness as a result of parasite infection (Mideo 2009), whereas insect pathologists prefer to say it is the disease-producing power of a pathogenic organism (Shapiro-Ilan et al. 2005), but definitions vary within, as well as between, fields (Thomas & Elkinton 2004). An intermediate level of virulence will therefore be one where the pathogen or parasite undergoes some within-host replication and therefore causes some harm, but not enough to result in quick host mortality. In Monarch butterflies, Danaus plexippus, and their protozoan parasite, Ophryocystis elektroscirrha, parasites that are too virulent kill their host prematurely resulting in lower transmission potential (de Roode et al. 2008). The trade-off depends on the mode of transmission of the infectious organism, for example, vector transmitted pathogens can be transmitted while their hosts are still alive, and incapacitated (Ewald 1995), whilst O. elektroscirrha, described above, is vertically transmitted by living adult hosts to their offspring, and therefore lower virulence is selected for in order to keep the host alive (de Roode et al. 2008). This trade-off may be different in obligate killers, such as the entomopathogens used in this thesis, because these pathogens and parasites require their host to die before they can be transmitted (Ebert & Weisser 1997). The terms of the trade-off model may vary in an obligate killing parasite (Cory & Franklin 2012), however, it is still supported theoretically and empirically in a Daphnia spp. host and its castrating bacterial parasite, Pasteuria ramosa. The theoretical model demonstrated that pathogens that kill their host too rapidly, that is, they are too virulent, undergo less replication within the host and have reduced overall fitness (Ebert & Weisser 1997). Experimentally, parasites with intermediate levels of virulence, or time to host death, exhibited maximum levels of within-host replication (Jensen et al. 2006). Our understanding of the trade-off model comes primarily from studies on single pathogen-host systems and it is unknown how within-host competition among pathogens could vary the balancing selection between virulence and transmission.
1.2. Insect pathogens in pest management

Biological control involves the release of natural enemies (predators, parasitoids or pathogens) to manage a target pest in order to reduce damage by that pest (Eilenberg et al. 2001), and is often aimed at maintaining damage below a certain economic threshold. Different application strategies are used in biological control. These include classical biological control, where the intention is that the agent will establish as a permanent addition to the biological community; inoculation biological control, where it is expected that the agent will cycle through some generations and provide future control, although this may not be long term; and inundative biological control, which requires multiple applications of an organism at high densities and does not expect the organism to multiply or reproduce to limit a pest in the future (Eilenberg et al. 2001). An inundative release strategy is most commonly used in microbial applications due to a lack of environmental persistence of applied pathogens in the field (Vega & Kaya 2012). Another factor, however, is a lack of understanding of the ecology of these microorganisms, resulting in them being applied inundatively, as though they were chemical pesticides. The bacterium *Bacillus thuringiensis* is the most widely applied microbial control agent in the commercial sector (Bailey et al. 2010), not including the use of the *Bt* toxin in transgenic plants. Other agents include entomopathogenic fungi, baculoviruses, and nematodes (Cory & Franklin 2012). The evolution of resistance to common chemical pesticides, as well as an increased focus on environmentally sustainable methods of pest management, has made microbial agents an increasingly popular option (Lord 2005).

One major issue with applying pathogens to pest populations is the way that the agent could interact with naturally occurring pathogens within the host. Target pests are highly likely to carry several pathogens prior to encountering the control agent (Bruck 2004; Meyling & Eilenberg 2006) and interactions between the already-present pathogens and the microbial agent might alter the outcome, causing an increase or decrease in expected pest mortality. If an inoculation strategy is being used, subsequent generations of the pathogen may evolve changes in virulence in response to competition thus altering its effect on the pest. Additionally, due to evolved resistance to microbial controls, such as *Bt*, resistance management is now a key focus in pest management.
(Janmaat & Myers 2003; Bates et al. 2005; Cervantes et al. 2011; Cory & Franklin 2012). Multiple control agents should be applied to a pest population in order to reduce the chances of resistance evolving by enforcing multiple or varied selection pressures on a population (Raymond et al. 2006; Raymond et al. 2007). However, when multiple pathogens are applied, our lack of understanding of the ways that they can interact within a host could result in unexpected outcomes.

The ways in which microbial control agents interact could differ depending on pathogen groups, environmental factors, and host condition including immune capabilities. A clear understanding of the host immune response to different pathogen groups is required in order to shed more light on the potential outcome of a given interaction.

1.3. The invertebrate immune response

Ongoing studies demonstrate that the invertebrate immune response is complex (Loker et al. 2004), and that invertebrates can mount species-specific responses to diverse pathogens and parasites (Gillespie et al. 1997). To successfully enter a host and begin replicating, a pathogen must first overcome the host’s primary barriers – the cuticle or gut lining. The cuticle is the common mode of entry of many fungal pathogens, including *Beauvaria bassiana* studied in Chapter 3. Baculoviruses and many bacteria species are ingested and some hosts demonstrate significant resistance to these pathogens at the gut level. For example, gut enzymes of the silkworm, *Bombyx mori*, have antiviral activity (Nakazawa et al. 2004). The peritrophic membrane is a semi-permeable layer that lines the insect GI tract and its thickness has been shown to affect host resistance to virus (Wang & Granados 1998; Plymale et al. 2008).

Once these defences are breached by the pathogen, the host will attempt to defend itself via the production of immune molecules including antimicrobial peptides (AMPs) and enzymes, haemocytes, and molecular interference strategies. For non-viral pathogens, these responses are initiated by the host’s successful recognition of the pathogen using pattern recognition receptor (PRR) proteins that recognise and bind to molecules on the microbial surface membrane, known as pathogen-associated
molecular patterns (PAMPs) (Schmid-Hempel 2005). For Gram-positive bacteria, hosts use peptidoglycan recognition proteins (PGRP) whereas Gram-negative binding proteins (GNBP) are utilised for Gram-negative bacteria and fungi (Yoshida et al. 1996; Lemaitre & Hoffmann 2007; Ferrandon et al. 2007). These bind to peptidoglycans or lipopolysaccharides in the bacterial cell membranes, and β-(1-3)-glucans in the fungal cell walls (Ferrandon et al. 2007). Recognition of viral pathogens is not fully understood, however, RNA interference pathways are currently thought to be the primary mechanism involved in virus recognition (Kemp et al. 2013; Kingsolver et al. 2013).

Pathogen recognition triggers a response cascade that activates one or multiple immune pathways and as well as an immediate cellular response. The presence of a pathogen in the haemolymph will stimulate the differentiation of haemocytes into function specific cells. Functions include encapsulation of the foreign body, agglutination or clumping of the cells around it, and the transportation of humoral enzymes to the location of the pathogen (Strand 2008). The immune pathways that could be activated are Toll, IMD, JAK-STAT and RNA interference. Both the Toll and the IMD pathway produce certain AMPs but the Toll pathway is more commonly associated with defence against Gram-positive bacteria and fungi, and IMD against Gram-negative bacteria (Tanzi & Ip 2005). Both pathways have been implicated in defence against virus; for example mosquitoes infected with Dengue virus show an up-regulation of both Toll and IMD transcription factors (Xi et al. 2008), and the Toll pathway is required for inhibition of Drosophila X virus in Drosophila melanogaster (Zambon et al. 2005). However, results between insect groups as well as between different viruses vary, indicating that there may be species-specific differences in immune responses. The JAK-STAT pathway mediates the cellular immune response (Marmaras & Lampropoulou 2009) and has become a recent focus for potential antiviral defence (Kingsolver et al. 2013).

Two mechanisms of defence used against viruses are RNA interference (RNAi) and cell apoptosis. RNAi is now well characterised in insects in defence against RNA viruses (Gammon & Mello 2015). The presence of viral double stranded RNA (dsRNA) in a cell generates the production of small interfering RNAs (siRNAs) which then bind to sequence specific sites on the viral RNA and degrade it (Terenius et al. 2011; Gammon & Mello 2015). The use of RNAi as a host defence mechanism against DNA viruses is
less well known however there do appear to be some similar mechanisms, as shown in *Helicoverpa armigera*, the cotton bollworm, infected with a baculovirus, HaSNPV (Jayachandran et al. 2012). DNA viruses may induce a different pathway of RNA interference by the host in the synthesis of microRNAs (miRNA) rather than siRNAs (Kollipoulou & Swevers 2014), as shown recently in the silkworm, *Bombyx mori*, and its obligate baculovirus, BmNPV. Hosts that were blocked from producing miRNA had higher virus titres indicating a functional role in defence (Singh et al. 2012). Furthermore, this baculovirus produces a miRNA that interferes directly with the transport of host miRNAs from the nucleus into the cytoplasm (Singh et al. 2012), signifying that host manipulation at the molecular level is a popular offense in these viruses (Gammon & Mello 2015). Cell apoptosis, or programmed cell death, is a conserved process across the animal kingdom and can reduce the ability of a virus to replicate and spread from one cell to another (Cooper & Mitchell-Foster 2011). Several virus sequences contain anti-apoptotic genes indicating long term coevolution of this defence mechanism between insect hosts and their viruses (Clem 2005).

It is becoming clear that there are substantial connections and cross-talk between the different defence mechanisms (Hoffmann & Reichhart 2002; Behura et al. 2011; Kingsolver et al. 2013) and, as a result, it is challenging to elucidate the importance of each across different host-virus interactions. Studies are rarely conducted in multiply-infected hosts and therefore interactions via the host’s immune response are not considered. Additionally, experiments are frequently conducted on cells *in vitro* rather than on whole organisms, or apply methods where pathogens are injected, which could alter the natural activation sequence of a host’s defence. For example, if certain defences are triggered by pathogen presence in the gut this could alter the sequence and activation of immune responses in a way that is missed when pathogens bypass this route. In addition to the host response to pathogens, there may also be competitive interactions between pathogens within a host.

### 1.4. Within-host competition between pathogens

Three mechanisms for within-host competition have been outlined in the literature and recently reviewed by Mideo (2009). Some pathogens are capable of
releasing a toxic compound that can inhibit a competitor. This interference competition is common in bacteria that synthesize bacteriocins, which can interfere with cell functions including enzyme inhibition, DNA transcription, and degradation of cell membranes (Riley & Wertz 2002). Bacteriocins have evolved to target closely related strains in order to directly inhibit a competitor and their presence can therefore be mediated by relatedness of the pathogens to each other (Massey et al. 2004; Buckling & Brockhurst 2008).

Resource-based or exploitation competition occurs when pathogens compete over the host’s resources, much like resource-based competition in community ecology (Pedersen & Fenton 2007; Graham 2008). If resources are limited, competition between the pathogens is thought to be high and therefore a pathogen that can quickly dominate the available resource is at an advantage, resulting in selection for increased virulence (Mideo 2009). This has been demonstrated in rodent malaria parasites, *Plasmodium chabaudi*, where more virulent parasites were more successful competitors, both in terms of within-host replication and between host transmission (de Roode et al. 2005; Bell et al. 2006). Resource-based competition will be reduced when there is niche separation between pathogens meaning that they exploit different regions or tissues in the host body. For example, closely related trematode parasites that co-infect will occupy either the lens or vitreous body of their fish host’s eye (Karvonen et al. 2006), and strains of a nucleopolyhedrovirus may partition the resources of their noctuid host, *Panolis flammea*, during a co-infection (Hodgson et al. 2004). However, because they are occupying the same host, co-infecting pathogens can still be impacted via reduced quality of host resources. These are also required by the host for immune responses. This is where host-pathogen interactions differ from a standard predator-prey dynamic as depletion of the resources by the pathogen, or ‘prey’, will also deplete the resources available to the host immune response, or ‘predator’ (Pedersen & Fenton 2007; Cressler et al. 2014).

Immune-mediated competition occurs when the host immune response is activated by the presence of a pathogen or parasite, altering the state of the host encountered by subsequent or co-infecting pathogens. For example, in multiple strain infections of the rodent malaria, avirulent clones were competitively suppressed;
however when placed in an immune deficient host they were released from this competitive pressure and had equal fitness to the more virulent clone (Barclay et al. 2008). A further study did not, however, show strong evidence for an interaction between single or mixed infections and host CD4+ T cell depletion and concluded that resource-based competition was the more likely driver of the result (Barclay et al. 2008). These conflicting interpretations illustrate the difficulties associated with teasing apart different types of competition and their outcomes. Invertebrate host immunity can also play a key role in mediating a pathogen interaction. *Aedes aegypti* and *Anopheles gambiae* mosquitoes that were injected with bacteria prior to feeding on hosts infected with *Plasmodium spp.*, had reduced parasite growth and oocyst presence in the midgut (Lowenberger et al. 1999). Similarly, in a bumblebee host previously challenged with heat-killed bacteria, the outcome of a dual strain infection of *Crithidia bombi* was different compared to unchallenged hosts; both strains were more successful in a host that was immune challenged (Ulrich & Schmid-Hempel 2012). This may have been due to the cost to the host of mounting an immune response (Moret & Schmid-Hempel 2000), which could manifest itself in terms of which immune pathways are activated as well as the region in the host body where a pathogen establishes (Ulrich & Schmid-Hempel 2012).

### 1.4.1. Priority effects

Immune-mediated competition may be most affected by the order in which the competing pathogens enter the host, known as priority effects. Prior residence of a pathogen in a host may facilitate entry of a second pathogen due to either a general weakening of host immune capabilities, or a cost of mounting an initial response, as shown with both cellular and humoral mechanisms (Siva-Jothy et al. 2005; Valtonen et al. 2010). Therefore, an invertebrate host that can tailor its immune response to specific pathogens may have to trade-off resources between different immune components or pathways (Cotter et al. 2004; Sadd & Schmid-Hempel 2009; Ulrich & Schmid-Hempel 2012). This trade-off may weaken the host response and cause increased host mortality, or synergism between the co-infecting pathogens. This hypothesis is not well supported by empirical evidence however, which more often reports reduced host mortality with sequential infections compared to simultaneous ones. For example, sequential infection
in a *Daphnia spp.* host by a protozoan and a fungal pathogen (Lohr et al. 2010), and a separate study in the same host using multiple strains of the bacteria *Pasteuria ramosa* (Ben-Ami et al. 2008), reduced host mortality. Desert locusts infected with two fungal entomopathogens also showed reduced mortality when infections were sequential (Thomas et al. 2003). These results do not support the facilitation hypothesis. Rather, they could be explained by an immune priming mechanism demonstrated in several invertebrates whereby initial challenge with a sub-lethal dose of a pathogen confers increased resistance to subsequent infections (Sadd & Schmid-Hempel 2006; Tidbury et al. 2011; McTaggart et al. 2012). Bumblebee hosts exhibited species-specific priming to bacterial pathogens for several weeks (Sadd & Schmid-Hempel 2009), and *Daphnia magna* had species-specific, but not strain-specific, priming to their obligate bacterial pathogen, *Pasteuria ramosa* (McTaggart et al. 2012). Interestingly, although not a lot is known about the lepidopteran immune response to virus, there is some evidence for immune priming in a noctuid, *Plodia interpunctella*, to a DNA virus (Tidbury et al. 2011), but this is not supported in *Drosophila melanogaster* with Drosophila C Virus, an RNA virus (Longdon et al. 2013). Whilst these studies report host immune priming to the same pathogen, rather than across pathogen groups, the effect that each pathogen has on the immune response must be noted and a general up-regulation may result in a host better prepared for infection by any pathogen, thus reducing overall host mortality.

### 1.5. Study system

#### 1.5.1. Insect Host

*Trichoplusia ni* (Hübner) (Lepidoptera: Noctuidae) is a common greenhouse and field crop pest from California to British Columbia (Franklin et al. 2010), and occurs worldwide including in Asia and Europe (Capinera 2008). Insects migrate north from California, where they overwinter, or can survive the winter in greenhouses in BC (Cervantes et al. 2011). They are generalist herbivores and will consume crops ranging from Brassicas, such as broccoli and cabbage, to greenhouse vegetable crops such as tomatoes and peppers. Resistance of *T. ni* to *Bt* (Janmaat & Myers 2003) has encouraged the development of other biological control products, such as baculoviruses.
These are particularly important for organic growers who have a limited selection of products to choose from. Increasing our understanding of how potential microbial control agents affect their hosts has important applications in the management of this pest; however they are additionally a useful model system in which to study ecological immunology and the ecology of DNA viruses.

1.5.2. Pathogen – Baculovirus

The family *Baculoviridae* are common DNA viruses of several insect orders. Although they are most common and diverse in Lepidoptera, they have also been identified in Diptera and Hymenoptera and show clear phylogenetic branching that matches their host species, indicating long term coevolution (Herniou et al. 2004). *Trichoplusia ni* Single Nucleopolyhedrovirus (TnSNPV) falls in the Alphabaculovirus clade, along with other lepidopteran baculoviruses (Jehle et al. 2006). As well as single nucleocapsid baculoviruses, this clade includes multiple nucleocapsid baculoviruses, such as the extensively-studied AcMNPV. Alphabaculoviruses exist in two phenotypic forms - occlusion bodies (OBs), the transmission form of an Alphabaculovirus, and budded virus, the within-host replicating form. OBs range in size from 0.4-3 µm with a cuboid structure. OBs need to survive outside of the host until a new host consumes them, therefore they are environmentally hardy but can be broken down by UV; a key limitation in the use of these viruses in microbial control (Griego et al. 1985). OBs are consumed by the host and release occlusion-derived virions in the insect gut which, in AcMNPV, penetrate the midgut cells of *T. ni* around 24 hours post infection (Keddie et al. 1989; Engelhard et al. 1994). After this, virions transform into the budded virus phenotype which bud through the cell membranes of infected cells and begin infecting the haemocoel via the tracheal cells (Engelhard et al. 1994; Jehle et al. 2006; Passarelli 2011). Infection with a baculovirus can affect insect development, particularly by preventing the moulting of the larval host into the next instar (Cory et al. 2004). In *T. ni*, infection will last 4-7 days, although this is temperature dependent, during which time the virus undergoes rapid replication. At death, the host cadaver will lyse, spilling millions of OBs into the environment for transmission to a new host.
1.5.3. Competing pathogens – *Bacillus thuringiensis kurstaki* and *Beauvaria bassiana*

*Bacillus thuringiensis* is a gram-positive bacteria with several strains, each capable of infecting the larval stages of multiple insect orders (Bravo et al. 2011). Each strain expresses a unique combination of Cry toxins, which cause midgut damage in the host allowing the bacterial spores to enter the haemolymph and replicate (Bravo et al. 2011). This insecticidal activity of *Bt* toxins is foremost in its success as the most widely applied microbial control agent, as well as the incorporation of the toxins into transgenic crops (Vachon et al. 2012). In a natural infection (that is, one containing both toxins and bacteria), the host dies either due to irreparable gut damage or septicaemia as a result of spore proliferation in the haemocoel (Raymond et al. 2010). *Bt* is a fast acting pathogen, often killing *T. ni* hosts within 3-4 days of exposure.

*Beauvaria bassiana* is a generalist fungal entomopathogen and a widely applied microbial control agent. Globally, over 50 registered products use *B. bassiana* as the active agent (Faria & Wraight 2007). The strain used here, GHA, is the active agent in the commercial product BotaniGard™, and is most commonly applied on greenhouse crops to control aphids, whiteflies and thrips but has been demonstrated to be effective against *T. ni* (Ericsson et al. 2010). Infection begins with conidia adhering to the host cuticle. Enzymes produced by the conidia then break down the various layers of the insect cuticle to enable entry and proliferation within the haemocoel (Hajek & St. Leger 1994). The host dies 5-7 days later and the fungus sporulates within 48 hours after host death.

1.6. Thesis aims

This thesis explores the effect of within-host competition on a baculovirus, TnSNPV, in the insect host, *T. ni*, and the potential role of the host immune response in mediating interactions between baculovirus and other pathogens. In chapter 2, I study the dose dependent and temporal host immune response to baculovirus exposure by measuring cellular and humoral components of immunity. I additionally challenge virus-exposed hosts with bacteria and use quantitative PCR to measure antimicrobial peptide
activity to assess the effect the virus has on the host immune response to subsequent infecting pathogens. Chapter 3 investigates sequential co-infections of baculovirus followed by either the bacterium, *Bt*, or the fungus, *B. bassiana*, with each pathogen being applied at two doses. I measure host mortality to investigate the efficacy of these combinations for pest management. Virus yield and production efficiency are also measured to gain insight into how virus replication is affected by the presence of another pathogen. Understanding the ways that a baculovirus can affect its host directly, in terms of host immune capabilities, as well as the ways it interacts with other entomopathogens within the host, will contribute substantially to microbial control strategies and, more broadly, to our ecological knowledge of the interactions between viruses and other pathogen groups.

References


Chapter 2.  Dose dependent, temporal changes in immune response to baculovirus exposure and implications for competing pathogens

2.1. Introduction

Living organisms continuously encounter pathogens against which they must defend themselves. Defence can act through behavioural avoidance of pathogens (Ouedraogo et al. 2004; Parker et al. 2010), in the form of a physical barrier, such as the gut lining or cuticle (Barnes & Siva-Jothy 2000; Dubovskiy et al. 2013), or an active immune response following the invasion of the pathogen into the host (Tzou et al. 2002; Schmid-Hempel 2005; Strand 2008). Pathogens are highly diverse; a host is often infected with multiple genotypes of a species (Lagru et al. 2007), as well as a community of different pathogens (Johnson & Hoverman 2012). Therefore, a host must be capable of multiple different responses depending on the types of pathogens that they encounter (Gillespie et al. 1997; Wang et al. 2010), as well as have the ability to detect them accurately upon infection (Loker et al. 2004). Despite the lack of an adaptive immune response (Hoffmann et al. 1996), invertebrates demonstrate a remarkably broad range of immune abilities, from the encapsulation of large organisms such as parasitoid wasp eggs or larvae (Smilanich et al. 2009), to the production of many specialised antimicrobial peptides in response to invasion by bacteria and fungi (Lowenberger et al. 1995; Lowenberger 2001; Lai & Gallo 2009; Tassanakajon et al. 2015).

Many aspects of the host response to these parasite and pathogen groups have been extensively characterized, including cellular and humoral responses (Lavine & Strand 2002), particularly immune gene expression (for example, Eleftherianos et al. 2007; Riddell et al. 2011). However, the invertebrate immune response to virus infection is less well-understood than anti-bacterial responses. Recent advances in molecular technologies have opened the field to investigating the effects of virus infection on gene
activity in primary host organisms (Gatehouse et al. 2009; Choi et al. 2012) as well as insects that act as vectors of these viruses (Barón et al. 2010; Ocampo et al. 2013). There is evidence for the suppression of immune function in *Aedes aegypti* cells infected with Dengue virus (Sim & Dimopoulos 2010), and for the down-regulation of lepidopteran host immune gene expression following baculovirus infection (Salem et al. 2011; Jakubowska et al. 2013). Antiviral defence strategies include RNA interference (RNAi) and apoptosis. RNAi is well documented in *Drosophila spp.* against RNA viruses (Kemp et al. 2013) but evidence also comes from a lepidopteran host infected with a DNA virus (Jayachandran et al. 2012). Apoptosis of infected cells is another widespread method of host defence against various viruses (Clem 2005; Cooper & Mitchell-Foster 2011). The presence of anti-apoptotic gene sequences in baculoviruses suggest an ancient history of host-pathogen coevolution and confirms the importance of this mechanism in host defence (Clem 2007; Thiem 2009).

Comprehensive information on the insect response to virus infection is limited to a small number of host and pathogen species, primarily *Drosophila spp.*, with a strong focus on RNA viruses (Breitenbach et al. 2011). However, evidence suggests that Diptera may have some unique characteristics, particularly in cellular immunity (Ribeiro & Brehélin 2006). For example plasmatocytes found in Diptera appear very different to those present in Lepidoptera (Ribeiro & Brehélin 2006), warranting caution when comparing across insect orders. Additionally, differences exist within Lepidoptera (Strand et al. 2006). This highlights the need to investigate immune responses in a wider range of invertebrate taxa (Kingsolver et al. 2013) and to a more diverse suite of pathogens, particularly different viruses (Huszar & Imler 2008). Additionally, it is likely that invertebrates are highly diverse in their responses to pathogen infection (Loker et al. 2004; Cooper & Mitchell-Foster 2011) and clarifying this diversity will be an important component of future research.

In addition to apoptosis and RNAi, immune pathways linked to antiviral responses include Toll, IMD and JAK-STAT. However, the roles of these pathways differ in response to different viruses, and between host species and regions of the host body (Huang et al. 2013). Two of these pathways, Toll and IMD, were first identified in the insect immune response to bacteria and fungi. Although it was initially believed that
immune pathways acted independently of each other (Hultmark 2003), it is now accepted that there is cross-talk between them (Hoffmann & Reichhart 2002; Behura et al. 2011; Kingsolver et al. 2013). The Toll, IMD, and JAK-STAT pathways are all activated in Dengue virus defence in the mosquito, *Aedes aegypti* (Xi et al. 2008) indicating that there are general responses that activate multiple immune pathways when a host is infected with bacteria and viruses (Zambon et al. 2005; Costa et al. 2009). However, these results are incomplete because they target gene level responses only and do not account for downstream components such as haemocyte presence or enzyme activity. Comprehensive studies that investigate multiple levels of the host response, i.e. that measure gene expression and cellular parameters are challenging but necessary. Mounting a cellular or humoral immune response is often considered energetically costly (Siva-Jothy et al. 2005; Valtonen et al. 2010) and trade-offs can occur both between different life history traits as well as among or within immune pathways (Cotter et al. 2004). Measuring these end point molecules is important for deciphering how and why trade-offs occur between different aspects or functions of immunity. The existence of within immune system trade-offs is supported by evidence from multiple insect systems (Siva-Jothy et al. 2001; Cotter et al. 2004; Ulrich & Schmid-Hempel 2012).

The host response may differ depending on the mode of entry of a pathogen, and could have a significant effect in the early stages of infection. A pathogen that enters the host by penetrating the gut, such as a baculovirus, needs to overcome the physical barrier of the gut prior to entering the host haemolymph (Keddie et al. 1989). For example, the peritrophic membrane of the GI tract is an important factor determining baculovirus infection in *Trichoplusia ni* (Wang & Granados 1998; Plymale et al. 2008).

Haemocytes have known functions in defence against Gram-positive and Gram-negative bacteria (Tzou et al. 2002; Costa et al. 2005; Wang et al. 2010), and fungi (Lee et al. 2005). Broadly, when the haemoceol is invaded by a foreign body, it is located by granulocytes and phagocytosed whilst a larger object is encapsulated by plasmatocytes (Lavine & Strand 2002; Strand 2008). Oenocytoid cells transport humoral compounds to the scene and, on bursting, the pro-phenoloxidase cascade is activated resulting in melanisation (Shrestha & Kim 2008). Despite a wealth of studies into functions of these
cells it remains unclear which, if any, are involved in anti-virus defence (for example, Trudeau et al. 2001). The baculovirus AcMNPV can infect all types of haemocytes in multiple lepidopteran species (Wang et al. 2010), although whether this is initiated by the host, as attempted phagocytosis, or by the virus, is unclear.

FAD-glucose dehydrogenase (GLD) is used in the production of free radicals and reactive oxygen species, an important component of humoral defence (Lovallo & Cox-Foster 1999; Lee et al. 2005). Phenoloxidase (PO) has multiple roles in insect physiology; it is involved in the melanisation of foreign objects and also in pigmentation, and is a good indicator of organism health (González-Santoyo & Córdoba-Aguilar 2012). In the emerging field of eco-immunology, PO is commonly used as a basic measure of immunocompetence in a host (Siva-Jothy et al. 2001), although it is not clear whether it functions in defence against all pathogen groups. PO plays a significant role in insect defence against invading pathogens due to its role in several stages of the biochemical production of melanin, which is used to eliminate larger parasites (Cerenius & Soderhall 2004). For example, PO is crucial for the melanisation of parasitoids (Smilanich et al. 2009) and the pro-phenoloxidase cascade is activated in response to wounding (Ardia et al. 2012). Some studies support a role in bacterial defence (Korner & Schmid-Hempel 2004; Cerenius & Soderhall 2004; Cerenius et al. 2008) but results vary and are species-specific (González-Santoyo & Córdoba-Aguilar 2012; Haine et al. 2008). Recent studies in the Indian meal moth and western tent caterpillar concluded that PO may not be involved in defence against baculoviruses (Saejeng et al. 2010; Myers et al. 2011). By altering host physiology, however, baculoviruses may alter the pro-phenoloxidase cascade and other important immune processes. Any change in PO activity due to virus exposure could leave the host vulnerable to, or alternatively, better prepared for, challenge by subsequent pathogens or parasites.

Antimicrobial peptides (AMPs) are essential components of defence across the tree of life (Tassanakajon et al. 2015). Cecropins were first identified in a lepidopteran (Hultmark et al. 1980) and, along with other AMPs, play a significant role in defence against Gram-positive and Gram-negative bacteria (Lowenberger et al. 1995; Hu et al. 2013), as well as some fungi (Ekengren & Hultmark 1999). While they appear to target different prokaryotic and eukaryotic pathogen groups (Lowenberger et al. 1996), the role
of AMPs in antiviral activity is unclear. In terms of function against viruses, more is known about mammalian AMPs which can deactivate some enveloped viruses (Lai & Gallo 2009). There is evidence from a lepidopteran cell line, Sf9, that infectivity of the baculovirus AcMNPV can be reduced by the activity of an AMP, gloverin (Moreno-Habel et al. 2012), however whole organism studies in lepidopterans on AMP functions in virus defence are limited.

Baculoviruses are naturally occurring DNA viruses of insects, primarily found in Lepidoptera (Cory & Myers 2003; Clem & Passarelli 2013). These obligate killers are responsible for large scale epizootics in several insect species (Cory & Myers 2003), making them an excellent candidate for the biological control of lepidopteran crop pests (Moscardi 1999). Baculovirus infection affects host development by preventing moulting, a strategy that increases host resources available to the virus and therefore virus fitness (O’Reilly & Miller 1991). AcMNPV prevents moulting in T. ni via the activity of the egt gene that interferes with ecdysis (O’ Reilly & Miller 1989). However, lower virus doses are slower acting so some insects will moult successfully into the next instar before succumbing to the virus (Fig. 2.1). This implies that a threshold level needs to be reached in order for moulting to be prevented (Cory et al. 2004). Virus dose has been studied extensively in terms of its effect on insect host mortality, particularly concerning the application of viruses in pest management (for example Burden et al. 2000; van Beek et al. 2000; Mascarin et al. 2010). However, less work has focused on measures of the host immune response to different virus doses. Linking the magnitude of the immune response to a range of pathogen doses will better enable eco-immunologists to clarify the costs of defence incurred to the host (Graham et al. 2011).
Figure 2.1  The timing of development in unexposed *Trichoplusia ni* and *T. ni* infected with a low or high dose of TnSNPV.

Here, I investigated temporal and dose-related changes in immune response to a baculovirus, *T. ni* Single Nucleopolyhedrovirus (TnSNPV), in the cabbage looper, *Trichoplusia ni*. I aimed to determine whether there was a dose dependent immune response to baculovirus infection, and to explore the temporal dynamics of this response. In the first experiment, I measured the presence of different haemocyte cells and the activity of two humoral enzymes in response to five virus doses and three time points post exposure. In experiment two, I assessed whether baculovirus exposure affected other immune responses such as the response to a subsequent bacterial challenge. I therefore measured the levels of four antimicrobial peptides in insects exposed to virus and then challenged with bacteria.

2.2. Methods

2.2.1. Organisms

Cabbage looper (*Trichoplusia ni* Hübner) eggs were collected from a long term laboratory colony established in 2001 from a greenhouse population (Janmaat & Myers 2003; Shikano & Cory 2014). Larvae were raised on an artificial wheat-germ based diet in groups of 20 from egg hatch until the 3rd instar and were then culled to 13 larvae until pupation. Up to 200 adult moths were then mass mated and eggs collected. The laboratory colony was maintained at 24°C with a 16:8 light:dark cycle.
2.2.2. Virus preparation

*T. ni* SNPV occlusion bodies (OBs) were originally isolated from an infected *T. ni* larva collected from a greenhouse in the Fraser Valley, British Columbia in 2000 (Janmaat & Myers 2003). The virus was amplified in *T. ni* hosts and semi-purified by multiple rounds of centrifugation to remove debris. The concentration was estimated by counting the OBs using a compound microscope at 400X magnification and an improved Neubauer haemocytometer (Hausser Scientific). Doses were then prepared by serial dilution. Blue food colour preparation (Club House) was added at a 1% concentration to visualize the virus suspension in the larval gut.

2.2.3. Experiment 1 – Investigating the temporal and dose response to TnSNPV

Initially, 1200 neonates were maintained individually at 23°C in bio-assay trays (128 cells, Bio-serv, Frenchtown, NJ) containing ample diet. After 7 days, 600 moulting 3rd instars were randomly selected, weighed and placed individually in 12-well tissue culture plates (Becton Dickinson Labware, Franklin Lakes, NJ). Once larvae begin moulting they stop feeding and slough their guts. Collecting them at this time ensured that all larvae were unfed 4th instars at the time of pathogen exposure. This minimised variation in infection due to the presence of food in the gut. Individuals were randomly assigned to one of four virus dose treatments or a distilled water control, with a minimum of 90 larvae per treatment. This resulted in n=30 larvae per time point per dose. The dose treatments were 10; 100; 1,000; 10,000 occlusion bodies, administered in a 1 µl droplet, and were selected to give a wide range of infection levels.

**Infection protocol**

A droplet feeding technique was modified from Hughes & Wood (1981). A 1µ droplet of blue coloured virus suspension was placed directly in front of the larvae in the 12-well plate and was usually ingested within a minute. Individuals that did not immediately drink the droplet, or walked in it, were discarded. Uptake could be confirmed by the blue colouring in the insect’s gut. This dosing technique ensured both accurate dosage and timing of exposure to the pathogen.
Exposures were evenly staggered over 8 hours to ensure that the timing of haemolymph collections was accurate and doses were spread throughout the day to control for any time of day effects. For example, the order of exposure was replicate 1 for control dose; replicate 1 for dose 1; replicate 1 for dose 2; etc. Haemolymph collections were taken at 18, 42, and 90 hours post virus-exposure with 30 replicates for each treatment (including controls) at each time point. A further 30 insects per treatment were maintained on diet to record infection status.

Once the droplet had been consumed, larvae were given a block of artificial diet and were kept individually at 23°C as described above. Caterpillars destined for the immune measurements were maintained in 12-well plates and fed diet ad libitum. Those designated for infection were moved into 1 oz plastic soufflé cups (Solo Cup Company, Lake Forest, IL) 24 hrs after exposure and were monitored and given fresh diet daily until death or pupation.

**Haemolymph measurements**

At each collection time, larval weight was recorded and haemolymph was collected by pricking the base of the middle proleg and extracting haemolymph using a pipette (Finnpipette F1, Thermo Scientific, Wilmington, DE, USA). This wounding caused substantial damage to larvae and they were swiftly disposed of once the sample was collected. Measurements taken at each time point are from individual larvae and are therefore independent.

**Haemocyte counts**

Two µl of haemolymph were gently mixed with 11µl 1xPhosphate Buffered Saline (PBS; pH7.4) and pipetted into an improved Neubauer haemocytometer. Haemocytes were counted immediately using a compound microscope at 200X magnification. Three types of haemocytes were selected as they were the most numerous and easily identified. Granulocytes were small, round cells, plasmatocytes were also small with extensions spreading outwards from the cell, and oenocytoids were very large with a darker pigment inside the cell.
Humoral enzyme activity measures

Six µl of haemolymph were diluted in 60µl 1xPBS and stored at -20°C for 24 hrs before being moved to -80°C to minimise cell damage due to rapid freezing thus preserving enzymes in an inactive form until activity could be measured. To measure PO activity, 50µl of the mixture were added to 150µl of dopamine hydrochloride solution (11.3mM in 1xPBS) in a 96-well tissue culture plate (Becton Dickinson Labware, Franklin Lakes, NJ). The absorbance at 492nm was measured every 45 seconds for 40 mins using a microplate reader (SpectraMax M2e, Molecular Devices, Sunnyvale, CA). Measuring the slope of the absorbance over time ($V_{\text{max}}$) gave the PO activity of each sample. Glucose dehydrogenase (GLD) activity was measured by taking 15µl of the remaining sample and modifying methods from Lovallo & Cox-Foster (1999). The sample was mixed with 200µl of 2, 6-Dichlorophenolindophenol (DCIP) reagent (48 µM DCIP, 76 mM β-D-glucose, 0.1 M Tris-HCl, pH7.0) in a 96-well plate. The absorbance at 600 nm was measured every 45 seconds for 40 mins where change in absorbance indicated the activity of GLD.

At 18 hrs post exposure, only 5µl of haemolymph per larvae could reliably be collected as the larvae were small. Therefore the PO and GLD measures for this time category are the combined samples from two caterpillars in the same dose treatment (26 larvae = 13 replicates). At 42 and 90 hours post exposure, 8µl haemolymph per larvae was more easily extracted so these replicates are from an individual larva.

2.2.4. Experiment 2 – Does TnSNPV exposure alter the host’s ability to respond to a bacterial challenge?

Early 4th instar larvae were exposed as described above to one of two treatments – baculovirus at a dose of 100 OBs or control (dH2O). Thirty hours later, larvae were pierced with either a needle dipped into a suspension of live *Micrococcus luteus* (Gram-positive) and *Escherichia coli* (Gram-negative), a sterile needle (‘wounded’), or unpierced (control). This resulted in six treatments – control; control plus wounding; control plus bacteria; virus alone; virus plus wounding; virus plus bacteria. The insects were pierced with a 25 gauge needle one third of the way down the body on the ventral side. Eight hours after bacterial treatment, larvae were placed in 1.5 ml eppendorfs on
ice and were stored at -80°C within 10 minutes. The time was chosen to mirror the middle time point from experiment 1 but could not be exactly the same due to logistical issues; therefore measurements are taken at 38 hours post virus exposure in experiment 2 compared to 42 hours post virus exposure in experiment 1.

**RNA extraction & quantitative PCR of antimicrobial peptides**

RNA was extracted from four individual whole larvae per treatment using a standard Trizol (TRI Reagent, Sigma-Aldrich, Oakville, ON, Canada) protocol. Following initial grinding, addition of chloroform, and centrifuging, the aqueous phase was transferred into a new tube with 500µl Isopropanol and was centrifuged (12,000g at 4°C for 8 minutes) to form a pellet. This was washed twice with 1 ml ethanol before being dissolved in 25µl DEPC treated water. RNA was quantified using a Nanodrop 2000C (Thermo Scientific, Wilmington, DE, USA) and diluted appropriately to 5000 ng in 12 µl DEPC treated water (417ng/µl). cDNA synthesis was done using reverse transcription (6 µl LVRT buffer (Promega, Madison, WI, USA), 5 µl dNTPs (Thermo Scientific, Wilmington, DE, USA), 3 µl 0.1M DTT (Invitrogen, Carlsbad, CA, USA), 1 µl MgDT (IDT, Coralville, IA, USA), 1 µl protector RNase inhibitor (Roche Diagnostics, Laval, QC, Canada), 1µl MMLV reverse transcriptase (Promega, Madison, WI, USA) with 12 µl dissolved RNA) with a cycle of 37°C for 90 mins; followed by 95°C for 5 mins. cDNA was then diluted with 170µl H2O.

Quantitative polymerase chain reaction (qPCR) was carried out using a Rotor-gene 3000 (Corbett Research, Concorde, NSW, Australia). Each reaction contained 3µl nuclease free water, 6µl PerfeCTa SYBR green supermix (Quanta Biosciences, Gaithersburg, MD, USA), 1µl each of forward and reverse primers (50 ng each), and 1µl of the cDNA sample. Two technical replicates were run per sample, and the mean of these was used as the raw expression (C_T) value for that AMP. The activity of the housekeeping gene, EIF (Eukaryotic initiating factor), and 4 antimicrobial peptides – HDD (“Hyphantria differentially displayed” - Woon Shin et al. 1998), cecropin, defensin, and gloverin – were measured for each sample. The thermo cycle conditions were as follows: 95°C for 2 mins, 35 repeats of 95°C for 10 secs (denaturing), 60°C for 10 secs (annealing), 72°C for 30 secs (extending). Melt curves for each sample were analysed for a single peak to ensure primer specificity (Taylor et al. 2010).
2.2.5. Statistical analysis

Host mortality to virus was analysed using a generalized linear model with a binomial distribution and a logit link function. Dose was transformed to ‘log_{10}(virus dose)’ and was classified as continuous. The full model consisted of dose as a main effect and weight at exposure as a covariate. Only treatments that received virus (i.e. not the controls) were included in mortality analysis. General linear models were used to analyse immune cell count and enzyme activity. Five models were run for the different response variables. Haemocyte counts – granulocytes, plasmatocytes, and oenocytoids – were square root transformed, phenoloxidase activity was log_{10} transformed, and glucose dehydrogenase activity was squared, to ensure the data were normally distributed. Larval weight at sampling was included as a covariate and the main effects were virus dose (continuous, log_{10}(dose + 1); including control treatment) and time (ordinal, 3 levels). This therefore gave a full model of ‘response ~ dose + time + dose*time + weight’. Non-significant interactions were removed first followed by non-significant main effects until the most parsimonious result was reached. Full models are reported in Appendix A.

qPCR data were processed using the ΔΔC_{T} method according to Table 1 in Livak & Schmittgen (2001). Data were log_{10} transformed for display. All analyses were done using JMP® (Version 10.0, SAS Institute Inc, Cary, NC) and figures were made in R (Version 3.1.0, The R Foundation for Statistical Computing).

2.3. Results

2.3.1. Experiment 1 – Temporal and dose dependent responses to TnSNPV

Mortality to virus

As expected, virus-induced mortality increased with increasing virus dose ($\chi^2_1=42.60$, p<0.001, Table A1, Fig. 2.2). Mortality due to virus was not detected in the control treatment and background mortality was minimal. Weight at exposure was included as a covariate and was not significant ($\chi^2_1=1.74$, p=0.1866, Table A1).
**Moulting & development**

A larva’s ability to moult into the next (fifth) instar was clearly affected by dose (Fig. 2.3). The likelihood of moulting decreased with increasing virus dose. For this reason, immune measurements collected at 90 hours were done on a mixture of fifth instars and infected fourth instars. Previous time points consisted of all fourth instars.

**Immunity measures**

**Cellular immunity**

Of the three types of haemocyte that were counted, granulocytes were the most numerous, making up roughly 65% of cells seen, and oenocytoids the least, at just 5% of total haemocytes (Fig. 2.4). Therefore, the pattern seen when all haemocytes are combined is very similar to that seen when granulocytes are considered alone.

The number of granulocytes varied over time and was affected by virus dose (Table 2.1). At 18 hours post exposure, virus dose had very little effect on the number of granulocytes present; however, by 42 hours post virus challenge there was a significant reduction in granulocytes as virus dose increased. This trend continued at 90 hours post exposure. A very similar pattern was observed in the plasmatocyte cells with small differences across virus dose at 18 hours but significant reductions with increasing virus dose at 42 and 90 hours post exposure.

The 42 hour time point marked an interesting stage in the development of the larvae. All larvae were still fourth instars, however, those insects whose development had not been stalled by the virus were preparing to moult into the fifth instar (Fig. 2.1 & 2.3). Here, there is a peak in granulocytes and plasmatocytes in the control and lower dose groups (Fig. 2.4), a potential indication that moulting is about to occur (McNeil et al. 2010). In contrast, all haemocyte types remained low in the high virus dose treatments, mirroring levels seen in fourth instar larvae at previous time points.
Figure 2.2  Proportion of *T. ni* larvae killed following exposure to one of four doses of TnSNPV or a control. Each treatment consisted of up to 30 replicates.

Figure 2.3  The development stage of *T. ni* larvae at each TnSNPV dose recorded at 90 hours post exposure, shown as a percentage. These individuals were used to collect 90 hour post exposure immune measurements. Larvae were either infected fourth instars (4 inf, red); fourth instars not yet displaying signs of infection (4, peach); fourth instars that had failed to moult and died, and were therefore excluded from the study (failed, light grey); or fifth instars (5, dark grey).
The presence of oenocytoid cells increased over time however, unlike the other two cell types, counts were highest at 90 hours post exposure, when control or low dose larvae had moulted into the fifth instar. Further, as with the other types of haemocytes, oenocytoids decreased as virus dose increased, with this difference becoming more pronounced through time (Table 2.1). Heavier or fifth instar larvae had higher numbers of oenocytoids whereas larval weight did not have a significant effect on granulocytes or plasmatocytes (Table A2).

**Enzyme activity – Phenoloxidase and glucose dehydrogenase**

Phenoloxidase activity decreased as virus dose increased (Table 2.1). The most noticeable effect of dose on PO levels is seen in the highest dose treatment at 90 hours post exposure (Fig. 2.4e) where activity was considerably higher across all doses, including in control larvae. These data are supported by the oenocytoid cell counts in Figure 2.4d. However, unlike the oenocytoids cells, phenoloxidase activity was not affected by larval weight (Table A2).

Glucose dehydrogenase activity changed between time points and across doses (Table 2.1, Fig 2.4f). Activity was highest at 18 hours and decreased over time. As with PO, GLD activity reduced across doses. GLD activity was affected by weight with increasing weight resulting in a slight reduction in activity (Table 2.1).
Table 2.1  Statistical output from linear models for measurements of all haemocyte cells, and two immune enzymes, taken at three time points following exposure of *T. ni* larvae to one of four doses of TnSNPV, or a control. Output from minimal models is displayed with non-significant terms or covariates removed from the model. Maximal model output is in Table A1 in Appendix A.

<table>
<thead>
<tr>
<th>Response</th>
<th>Predictor Variable</th>
<th>Degrees of Freedom</th>
<th>Sum of Squares</th>
<th>F ratio</th>
<th>Prob &gt; F</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total Haemocytes</td>
<td>Dose</td>
<td>1</td>
<td>2.70</td>
<td>0.50</td>
<td>0.4799</td>
</tr>
<tr>
<td></td>
<td>Time</td>
<td>2</td>
<td>661.86</td>
<td>61.32</td>
<td>&lt;0.0001  ***</td>
</tr>
<tr>
<td></td>
<td>Dose*Time</td>
<td>2</td>
<td>273.70</td>
<td>25.36</td>
<td>&lt;0.0001  ***</td>
</tr>
<tr>
<td>Granulocytes</td>
<td>Dose</td>
<td>1</td>
<td>3.00</td>
<td>0.58</td>
<td>0.4459</td>
</tr>
<tr>
<td></td>
<td>Time</td>
<td>2</td>
<td>565.46</td>
<td>54.84</td>
<td>&lt;.0001   ***</td>
</tr>
<tr>
<td></td>
<td>Dose*Time</td>
<td>2</td>
<td>246.45</td>
<td>23.90</td>
<td>&lt;.0001   ***</td>
</tr>
<tr>
<td>Plasmatocytes</td>
<td>Dose</td>
<td>1</td>
<td>0.058</td>
<td>0.06</td>
<td>0.801</td>
</tr>
<tr>
<td></td>
<td>Time</td>
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<td>150.57</td>
<td>83.22</td>
<td>&lt;.0001   ***</td>
</tr>
<tr>
<td></td>
<td>Dose*Time</td>
<td>2</td>
<td>31.89</td>
<td>17.63</td>
<td>&lt;.0001   ***</td>
</tr>
<tr>
<td>Oenocytoids</td>
<td>Dose</td>
<td>1</td>
<td>9.24</td>
<td>9.26</td>
<td>0.0025   **</td>
</tr>
<tr>
<td></td>
<td>Weight at collection</td>
<td>1</td>
<td>12.25</td>
<td>12.28</td>
<td>0.0005   **</td>
</tr>
<tr>
<td></td>
<td>Time</td>
<td>2</td>
<td>22.75</td>
<td>11.40</td>
<td>&lt;.0001   ***</td>
</tr>
<tr>
<td>Phenoloxidase</td>
<td>Time</td>
<td>2</td>
<td>17.42</td>
<td>49.78</td>
<td>&lt;.0001   ***</td>
</tr>
<tr>
<td></td>
<td>Dose</td>
<td>1</td>
<td>5.83</td>
<td>33.33</td>
<td>&lt;.0001   ***</td>
</tr>
<tr>
<td>Glucose Dehydrogenase</td>
<td>Dose</td>
<td>1</td>
<td>12.86</td>
<td>5.68</td>
<td>0.0178   *</td>
</tr>
<tr>
<td></td>
<td>Weight at collection</td>
<td>1</td>
<td>10.58</td>
<td>4.67</td>
<td>0.0315   *</td>
</tr>
<tr>
<td></td>
<td>Time</td>
<td>2</td>
<td>51.30</td>
<td>11.33</td>
<td>&lt;.0001   ***</td>
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</tbody>
</table>
Figure 2.4  Mean immune measures +/- standard error of *T. ni* larvae across three times post-exposure to one of four doses of TnSNPV or a control treatment. Transformed data are shown as indicated on axes labels. Counts were taken at three times post challenge depicted by point and line type where circles and solid line = 18 hours, triangles and dashed line = 42 hours, and squares and dotted line = 90 hours. Lines shown are for the fitted model (Table 2.1).


2.3.2. Experiment 2 – The effect of TnSNPV exposure on the antibacterial response of T. ni

This experiment had two aims - primarily, to determine if previous exposure to baculovirus altered the host antibacterial response and secondly, to clarify which, if any, of the AMPs could be implicated in direct defence against baculovirus. Gene expression for gloverin was hugely upregulated when the host was exposed to bacteria, and previous exposure to baculovirus did not affect the magnitude of this response (Table 2.2, Fig. 2.5). Gloverin was increased in response to wounding but this did not differ between control and virus treatments, and was not nearly as high as treatments with bacteria (Table A3, Fig. A1). Exposure to baculovirus alone slightly increased gloverin expression compared to the control treatment (Table 2.2, Fig. 2.5).

For cecropin, hosts exposed to virus alone showed similar levels of expression compared to controls (Table 2.2, Fig. 2.5). There was a significant difference between bacteria only and virus plus bacteria treatments, with the latter treatment having half the degree of expression. HDD expression was upregulated in larvae exposed to virus alone compared to a control, showing five times increased expression (Table 2.2, Fig. 2.5) and did not differ significantly between bacteria only and virus plus bacteria treatments (Table 2.2, Fig. 2.5). Defensin expression was increased in virus-only exposed groups compared to controls, but there was no difference between bacteria only and virus plus bacteria treatments (Table 2.2/Fig. 2.5). Expression levels for all treatment groups, including control and wounding treatments, are shown in Figure A1 and Table A2 of Appendix A.
Table 2.2  Fold change in *T. ni* antimicrobial peptide expression in insects exposed to virus (compared to control insects) and insects challenged with bacteria following virus exposure (relative to insects only challenged with bacteria). ΔΔC_t values (top line) and associated ranges based on standard deviations (below in brackets) were calculated according to Livak & Schmittgen (2001).

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Antimicrobial Peptide</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Gloverin</td>
</tr>
<tr>
<td>Control (no pathogen or wounding)</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>(0.1-7.3)</td>
</tr>
<tr>
<td>Virus alone</td>
<td>3.4</td>
</tr>
<tr>
<td></td>
<td>(0.7-16.6)</td>
</tr>
<tr>
<td>Bacteria alone (positive control)</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>(0.0-25.6)</td>
</tr>
<tr>
<td>Virus plus bacteria</td>
<td>1.8</td>
</tr>
<tr>
<td></td>
<td>(0.0-80.1)</td>
</tr>
</tbody>
</table>
Figure 2.5  \( \log_{10} \) transformed fold change in expression of the four AMPs in relation to a designated control group set at zero. Plot a displays data for expression in treatments where *T. ni* larvae were exposed to TnSNPV only, relative to a control that received no pathogen or challenge. Plot b shows data for expression of AMPs in treatments that received TnSNPV plus a bacterial challenge, relative to a positive control treatment where larvae were challenged with bacteria only. Bars that fall below the zero line indicate reduced gene expression relative to the control for that plot, whereas bars above zero show increased gene expression.

2.4. Discussion

Three of the four AMPs measured had increased expression in insects that were exposed to virus versus control insects. However, increasing baculovirus dose negatively affected the presence of haemocytes and the activity of the two immune enzymes measured. For the immune enzymes, PO and GLD, this reduced activity occurred as a direct and immediate effect of increasing dose across all time points, a result mirrored by the oenocytoïd cells. However, the granulocyte and plasmatocyte cells exhibited a dose response only after 18 hours post exposure.

Additionally, there were significant changes in all parameters over time. This highlights the importance of taking measurements at multiple times post-exposure so as to gain a temporal perspective on disease progression within the host at different doses.
Changes to host physiology or immunity that are related to infection may only become apparent sometime after the pathogen has established indicating that a single time point may not be a reliable measure. This may be particularly interesting in intracellular pathogens, such as viruses, that need to hijack the host’s genetic machinery in order to replicate. Many baculoviruses express genes capable of altering host physiology and development prior to replication (Thiem 2009). For example, the egt gene in some baculoviruses demonstrates the potential consequences for host development by preventing host moulting into subsequent instars (O’Reilly & Miller 1989; O’Reilly & Miller 1991). The presence of the egt gene increases virus replication within the host (Cory et al. 2004) however arresting host development may also be a tactic to reduce the host’s defence capabilities, as resistance increases with each instar (Engelhard & Volkman 1995; Cory & Myers 2003). The effect of the egt gene on host immune capabilities has not been investigated; it is possible that by arresting host development, the baculovirus prevents the host from mounting a stronger immune response in the next instar. This could be in addition, or alternative, to suppressing host immune gene activity and would result in immune parameters remaining at a level indicative of larval stage at the point of infection. Understanding these dose dynamics is therefore important as responses may differ depending on the infectious dose and the speed at which infection progresses inside the host.

Saejeng et al. (2011) present evidence for a systemic and dose dependent immune response to a granulovirus (another type of baculovirus) in the Indian meal moth *Plodia interpunctella*. However this is based solely on measures of mortality and life history costs and lacks an actual immune measure; therefore the role of immunity is not conclusively demonstrated. Nystrand & Dowling (2014) simplified the interaction by using a non-replicating pathogen mimic in a *Drosophila* host and found that the immune response did not show clear dose dependency.

The most curious finding here was the overwhelming downward trend with increasing virus dose that was observed in the number of immune cells and the activity of the enzymes. Possible explanations for this are discussed below. Firstly, the response within the haemolymph may be localised to a certain region of concern for the host, or traded off with an unmeasured response; secondly, it may be a consequence of illness
within the host, rather than a component of defence; and thirdly, the baculovirus may be causing host immune output to be reduced.

2.4.1. Localisation of immune response to gut region

TnSNPV needs to be ingested to initiate infection. Since entry of the virus is limited to the gut, this should be an important area for host defence against baculovirus infection (Nakazawa et al. 2004). Host plant has an important effect in mediating the outcome of infection (Cory & Hoover 2006) and more specifically, this can occur at the gut level rather than systemically (Hoover et al. 2000). Routine sloughing of mid-gut cells is also a mechanism to void infected cells from the body (Keddie et al. 1989). These effects are frequently not considered in studies that inject budded virus into organisms, or work on cells in vitro.

Additionally, an immune response within the gut cannot be ruled out. Activation of an immune response within the gut has been documented in other insects including *Anopheles gambiae* (Dimopoulos et al. 1997), *Stomoxys calcitrans* (Lehane et al. 1997; Boulanger et al. 2002), *Drosophila melanogaster* (Tzou et al. 2000) and *Spodoptera littoralis* infected with the baculovirus, AcMNPV (Rivkin et al. 2006). All studies report increased antimicrobial peptide activity either within the gut or generated by the epithelial cells of the gut surface membrane. Although tested on bacteria rather than viruses, it is possible that a similar mechanism exists in host defence against viruses, considering that they use the same mode of entry.

Whilst defence mechanisms may occur within the gut itself, the host may also localise its response to the gut region in order to tackle budded virions emerging from the gut lining into adjacent tracheal cells (Keddie et al. 1989; Passarelli 2011). I extracted haemolymph from the proleg of exposed insects. Therefore, if the response within the haemolymph is localised to the gut, it may result in a reduced response elsewhere in the body. This explanation is not supported, however, by two of the three haemocyte types not showing a dose dependent reduction until the 42 hour time point. Although exact timing is not known for TnSNPV infection, Engelhard et al. (1994) found that AcMNPV was present in tracheal cells and haemocytes of *T. ni* by 36 hours post
infection (h p.i.). This indicates that the virus crosses the midgut barrier around 24 h p.i., and then becomes systemic (Passarelli 2011), although different doses could alter this timeline. For the two later time points measured here, it is unlikely that the gut is still a specific area of concern suggesting that a localised response is not the cause of this result.

2.4.2. Trade-off with an unmeasured immune response

Life history studies often focus on trade-offs between immunity and other life history traits such as growth or reproduction (for example, Freitak et al. 2007). There is also evidence that a host may trade-off between different aspects of the immune response in order to defend itself against pathogen challenge (Ulrich & Schmid-Hempel 2012; Ardia et al. 2012). This may help to explain why some host-pathogen systems do not show evidence of trade-offs between life history traits (Labbé et al. 2010). In order to support a within immune system trade-off, an increase and a corresponding decrease in a functional, and effective, immune factor, would need to be shown. Across several parameters measured here, I did not measure an up-regulation in response to virus exposure. The clear reduction in haemocytes, PO and GLD in response to virus dose may be indicative of a trade-off occurring elsewhere, however further investigation is required before any firm conclusions can be drawn.

2.4.3. Physiological symptom of illness

Lower doses of baculovirus take longer to arrest host development, and can allow a host to moult into a subsequent instar before succumbing to infection. Alternatively, higher doses result in very few larvae moulting into the 5th instar (Fig. 2.1 & 2.3). It is possible that the changes in larval development indirectly halt or restrict further production of immune components by limiting the host physiologically.

For granulocyte and plasmatocyte cells, the counts seen at 18 h p.i. are mirrored by those in the highest dose at the two later time points, supporting the idea that immune activity is stunted from the point of infection onwards. Furthermore, there is no dose effect on these two cell types at 18 h p.i., with the dose effect only appearing later once
infection has established and disease is progressing. The dynamics seen in these cells may therefore be a physiological symptom of illness in the host rather than a deliberate response by either host or pathogen.

This explanation does not suffice, however, for PO, GLD and oenocytoids, which show a dose effect at 18 h p.i. and across all time points. It is interesting that oenocytoids are not affected by host development, or lack thereof, in the same way as the other haemocytes. The increase in cell numbers seen at 90 hours post exposure (Table 2.1; Fig. 2.4d), including in the control treatment, indicates that this cell type may be utilised more by larvae towards the end of their development and to a far lesser extent in earlier instars. Oenocytoids synthesise PO (Shrestha & Kim 2008) and therefore we would expect a similar trend between cell number and enzyme activity as seen in Fig. 2.4. My data support recent findings in two other species of Lepidoptera that PO is probably not involved in defence against virus (Saejeng et al. 2010; Myers et al. 2011). In *Plodia interpunctella* hosts infected with a granulovirus, phenotypically resistant individuals do not show increased levels of enzyme activity and individuals with higher PO levels are not less likely to become infected (Saejeng et al. 2010). These measurements were only taken three days after exposure but here I demonstrate that the reduction in PO activity is present earlier, at 18 hours post exposure.

### 2.4.4. Baculovirus altering host immune ability

Some baculovirus species are known to infect haemocytes (Keddie et al. 1989; Barrett et al. 1998) and these may initiate apoptosis to eliminate the virus. If haemocytes are utilised in host defence against baculovirus, this will weaken the host defences further because the cells required for defence are already infected by the pathogen; an effective strategy by the virus to overcome host defences.

All viruses have the ability to directly interfere with host gene expression. For example, in *Bombyx mori*, BmNPV can interfere with host production of anti-viral miRNAs and thus evade the host response (Singh et al. 2012). Other areas of focus include inhibiting host cell apoptosis (Gatehouse et al. 2009) and host development via hormone disruption, such as the *egt* gene (O’ Reilly & Miller 1989; Choi et al. 2012).
AcMNPV, the most commonly studied baculovirus, can alter host immune gene expression (Salem et al. 2011; Jakubowska et al. 2013) and this may explain the reduction I find in cellular components of immunity with TnSNPV exposure. The targeting of host defences may have broader consequences than just preventing host attack against baculovirus; it could affect multiple aspects of immunity, including those that act against different pathogens.

We tested this in experiment 2 by challenging virus-exposed hosts with live bacteria and measuring the expression of four antimicrobial peptides (AMPs). By using a bacterial cocktail I aimed to activate all anti-microbial immune pathways to see if baculovirus infection was affecting AMP activity.

Contrary to the overall reduction seen in experiment one, three of the four AMPs measured did not have reduced expression in hosts that were exposed to virus followed by bacteria. Cecropin was the exception to this with a down-regulation when the host was exposed to virus prior to bacterial challenge. In Heliothis virescens larvae challenged with Helicoverpa zea SNPV, cecropin was upregulated (Breitenbach et al. 2011) but a study in Spodoptera frugiperda cells infected with AcMNPV recorded a down-regulation (Salem et al. 2011). The down-regulation seen in cecropin expression warrants further investigation of this AMP. HDD had significantly increased expression in insects exposed to virus alone compared to the control treatment. The function of this AMP is not known, although it is immune-related (Woon Shin et al. 1998; Freitak et al. 2007). This result encourages future investigation into this protein, as this up-regulation may indicate a role in anti-viral activity. Although gloverin had an increase in expression in virus-exposed T. ni, this was not in line with the magnitude of the response by gloverin seen in bacterial challenged treatments. Gloverin is present in both the fat body and haemocytes of Trichoplusia ni (Lundstrom et al. 2002) and, as supported by my data, it is highly expressed when hosts are exposed to bacteria. I can additionally conclude that gloverin expression in response to a bacterial challenge is not affected by baculovirus exposure in T. ni, but there are no solid conclusions across different systems. For example, gloverin was shown to reduce infectivity of AcMPNV budded virus in Sf9 cells, probably by perforating the viral membrane (Moreno-Habel et al. 2012). Results from in vitro studies should be treated with caution, however, as they may have different
outcomes from in vivo experiments. In Spodoptera frugiperda larvae, gloverin was strongly down-regulated at six hours post infection with AcMNPV (Choi et al. 2012) whereas in H. armigera with the same virus there is an up-regulation of gloverin at three hours post infection (Wang et al. 2010). Defensin was recently shown to be up-regulated in Aedes aegypti following a blood meal containing dengue virus (Wasinpiyamongkol et al. 2014). By challenging virus infected hosts with LPS, Furukawa et al. (2007) demonstrated that BmNPV infection does not alter antimicrobial activity in the silkworm, Bombyx mori. My data support this conclusion, however family and pathogen specific differences caution against generalisations. The potential for family specific differences is supported by work on a lepidopteran specific AMP, hemolin. This was implicated in baculovirus defence in a saturnid, the Chinese oak silk moth, Antheraea pernyi (Hirai et al. 2004), but is not supported in Noctuidae (Terenius et al. 2009).

While the structure and molecular function of AMPs have extensively studied, there is a need for an ecological approach in understanding how these proteins interact with the rest of the immune response and whether there are specific costs associated with increased AMP expression. Additionally much of what we do know about their function in Lepidoptera comes from in vitro studies and it will be important to link these with the whole organism in future. The range of study organisms, viruses, and experimental design reported in the literature makes it difficult to draw common conclusions. However, there is clearly a case for continued investigation into the involvement of AMPs in virus defence as well as the effects that virus infection may have on immune responses against other pathogens.

2.4.5. Conclusion

Here, I demonstrate a significant reduction in cellular parameters and immune enzymes in a lepidopteran following baculovirus exposure. Both virus dose and time post-exposure affected immune parameters. Although it remains unclear if these molecules are directly involved in defence against virus, they may be important indicators of infection. My second experiment showed some support for the involvement of AMPs in virus defence, but limited support for the down-regulation of AMPs by the virus. However, one of the four AMPs, cecropin, did show reduced expression in
bacteria-challenged hosts previously exposed to baculovirus. This does not fully support a hypothesis for widespread manipulation of host immune genes by TnSNPV, as shown previously with AcMNPV in *T. ni* larvae and in *S. frugiperda* cells (Salem et al. 2011; Jakubowska et al. 2013). If, however, haemocytes and humoral enzymes are not used in viral defence, the adjusted levels we see here may still alter the host for subsequent co-infecting pathogens. Anything that may alter host quality or defence capabilities will have knock on effects for the pathogen community that infects it. As we discover more about invertebrate immune responses, it is important to study them from a pathogen community perspective rather than a single pathogen-host interaction. Pathogen diversity has significant effects on population cycles but we know very little about the competitive mechanisms underlying these processes (Johnson & Hoverman 2012).

Additionally, more needs to be known about DNA viruses, their diversity, and the various ways that they can influence their hosts. Whilst many host-pathogen interactions have been studied from an evolutionary ecology perspective, baculovirus ecology remains poorly understood. Here I demonstrate the effect of baculovirus exposure on commonly measured immune parameters. By comparing these with what we know about the host response to other pathogens, we gain a broader view of how baculoviruses affect their hosts and the trade-offs that may be involved. Increasing understanding of the host response to DNA viruses, and applying this to the existing host-pathogen evolutionary ecology framework, will be a key component of future research. Little is understood about the costs related to dose responses and how this might affect selection on the evolution of host resistance. Expanding our perspective on these processes will enable advances in the application of baculoviruses as biological control agents of insect pests, particularly in understanding responses that may affect co-infecting pathogens.

References


Chapter 3. The effects of competing pathogens on baculovirus infection and within-host replication

3.1. Introduction

Competition for limited resources occurs frequently both within and between species. This is especially true for pathogens and parasites that are bound to their host for survival and replication and therefore cannot easily escape a competitor. Multiple infections are common in nature (Knowles et al. 2013; Johnson & Hoverman 2012) indicating that competitive interactions are likely to occur within a host. A pathogen that cannot efficiently replicate and be transmitted to a new host will be outcompeted and suffer reduced fitness. Additionally, pathogens may attack each other directly via the synthesis of toxic compounds (Riley & Wertz 2002; Gardner et al. 2004) and can encounter defences from the host’s immune system (Cox 2001; Ulrich & Schmid-Hempel 2012). Despite significant research into host-pathogen interactions, our understanding of co-infections and multiple infections is still in its infancy. However, theoretical and empirical evidence suggests that within-host interactions between pathogens and parasites can have important consequences, such as regulating host populations (Telfer et al. 2010; Lello et al. 2008), and in the evolution of virulence (de Roode et al. 2005; Garbutt et al. 2011; Alizon et al. 2013).

Insects are infected and killed by a diverse range of parasitic organisms including bacteria, fungi, parasitoids, nematodes and viruses (Hawkins et al. 1997). Microbial control is the use of insect pathogens in pest management (Tanada 1959), and licensed agents comprise organisms from all these groups (Cory & Franklin 2012). In the utilisation of pathogens, parasites, and parasitoids as biological control agents of insect pests, it is essential that we understand the ways that within-host interactions can alter host mortality as this could affect the efficacy of a control product. Additionally, the long term effects of competition, such as changes in virulence, should be expected (Cory &
Franklin 2012; Jabbour et al. 2011). Many insect pests have evolved resistance to synthetic chemical pesticides and, more recently, to some microbial control agents. Resistance to the bacterial insecticide, *Bacillus thuringiensis* (*Bt*), and also to its toxins, particularly those expressed in transgenic plants (Tabashnik et al. 2008), has evolved in several species in the field (Janmaat & Myers 2003, Cory & Franklin 2012). Some Lepidoptera are not highly susceptible to the available *Bt* strains or require different natural enemies for different life stages. This diversity has encouraged the development and use of several different microbial products, which could be applied in combination and therefore interact with each other. In addition, pathogens are being applied to insect populations that may naturally carry several covert or chronic infections, such as DNA and RNA viruses (Jakubowska et al. 2014; Virto et al. 2014), which might interact with the applied pathogen and could alter the expected management outcome (Thomas et al. 2003). For public health, the potential effects of drug or vaccine treatments on the within-host pathogen community, including gut microbiota (Raymond et al. 2008), are underappreciated. Using a model system to study co-infections could improve management strategies of infectious disease in wildlife, agriculture and humans (Balmer & Tanner 2011; Griffiths et al. 2011).

**Effect of pathogen interactions on the host**

A host that is required to fight off multiple pathogens at once may suffer costs, leading to trade-offs with other life history traits. Whilst trade-offs are often considered between life history traits, they also can occur within a trait, such as different aspects of host immunity (Siva-Jothy et al. 2001; Cotter et al. 2004; Ulrich & Schmid-Hempel 2012). If two pathogens stimulate a similar defence, for example, they activate the same immune pathway or infect hosts via the same route of entry, this may allow the host to fight the two invaders with the same tools. Alternatively, two different pathogens could force the host to trade-off between different immune responses resulting in an overall weakening of defence. These scenarios have been discussed and studied in vertebrate hosts (reviewed in Viney et al. 2005 and Bradley & Jackson 2008) but studies in invertebrates are rare. Additionally, most pathogen and parasite groups can be removed from hosts, for example the use of anthelminthic drugs to remove nematodes (Knowles et al. 2013) but viruses cannot be removed or vaccinated against making it difficult to study the effect of virus presence or absence on the within-host microbial community.
**Long term effect on pathogens**

Pathogens are under selection pressure to trade-off optimally between virulence and transmission – if too virulent its host will die whilst the pathogen is still replicating, resulting in reduced transmission potential and lower fitness (Anderson & May 1982; Bull 1994; de Roode et al. 2008; Alizon et al. 2009) – although the trade-off shifts for obligate killers, such as the entomopathogens used here (Ebert & Weisser 1997). This trade-off may be simple in a single infection; however the presence of another pathogen could result in a considerable shift in the optimal strategy. This is because the pathogen has to fight not only the host’s defences but also has to compete with another organism that is potentially exploiting the same resources or space.

Multiple theoretical models have demonstrated the effects that competition could have on pathogen infection success, replication and virulence (Alizon et al. 2013). In an empirical study, mixed genotype infections of *Schistosoma mansoni* in a snail host resulted in increased host mortality and decreased host reproduction compared to hosts infected with a single genotype (Davies et al. 2002). Different types of competition, detailed below, may result in different outcomes in terms of virulence evolution (Bashey et al. 2012), host mortality, and pathogen replication.

**Types of competition**

Three types of within-host competition - interference, resource-based, and immune-mediated - have been outlined previously in the literature and are reviewed by Mideo (2009). These can be applied to both intra- and interspecific interactions. Interference competition is most common between bacteria (Garbutt et al. 2011) and will not be considered here. I will focus on the latter two modes with more attention on immune-mediated competition and how this might affect a baculovirus in an insect host.

Resource-based competition is a bottom-up pressure where the pathogens compete directly for the resources that a host provides (Graham 2008). The rodent malaria *Plasmodium chabaudi* provides a well-known example, as different genotypes compete over red blood cells, a limited host resource (Mideo 2009; Bell et al. 2006). In this system, more virulent (defined here as ‘parasite-induced morbidity and mortality’) strains, are more successful at replicating within the host, possibly due to outcompeting
other strains by utilizing the host’s resources faster (Bell et al. 2006). An additional study presents evidence that within-host competitive success could also predict the success of *P. chabaudi* genotypes to be transmitted between hosts, indicating a long term advantage (de Roode et al. 2005). It is important to note that this outcome may be unique to vector-borne parasites that can afford to be more virulent as they do not rely on direct transmission from an infected host to a healthy one. Obligate killers, such as the pathogens used in the current study, could be affected differently by resource-based competition within the host due to differences in the terms of the trade-off model between these directly transmitted pathogens. Furthermore, different types of competition are not mutually exclusive and one of the main challenges facing this field is identifying which mechanism is most significant in a given interaction, as this could change the outcome (Mideo 2009).

Competing pathogens will encounter the host immune response, which could be considered as a predator as it limits population growth and could therefore be considered top-down control (Graham 2008; Fenton & Perkins 2010). This can result in immune-mediated competition. Vertebrates exhibit an array of highly specific immune responses (Litman et al. 2010), whereas invertebrate immunity is not adaptive but still surprisingly complex, and can be pathogen specific (Loker et al. 2004). For example, several immune genes of the cotton bollworm, *Helicoverpa armigera*, showed differential expression when the insect was exposed to Gram-positive and Gram-negative bacteria, fungi or baculovirus (Wang et al. 2010). In addition, there is substantial cross-talk between pathways making it difficult to distinguish whether specific pathways are utilised against certain groups (Hoffmann & Reichhart 2002). Invertebrate defence strategies against viruses include RNA interference (Jayachandran et al. 2012; Kemp et al. 2013; Kolliopoulou & Swevers 2014) and cell apoptosis (Clem 2005). Several systems have documented evidence of immune priming in invertebrates including *Bombus terrestris* with bacteria (Sadd & Schmid-Hempel 2006), *Daphnia magna* with bacteria (McTaggart et al. 2012), and a lepidopteran, *Plodia interpunctella* with a baculovirus (Tidbury et al. 2011). Hosts therefore respond differently depending on the pathogens that are present within them. For example, Ulrich & Schmid-Hempel (2012) found that an initial challenge with a bacterial elicitor in a bumblebee host induces a change in host immunity that alters the competitive interaction between genotypes of a trypanosome parasite,
*Critithidia bombi*. Not only did the challenge increase levels of overall infection by the trypanosome population, it also resulted in strains that are usually weaker, and outcompeted by conspecifics, being more successful. This was believed to be due to an increase in ‘immune space’ available to the trypanosomes as a result of the costs and immune changes incurred from responding to the bacterial challenge. Evidence suggests that a baculovirus may suppress the lepidopteran immune response (Jakubowska et al. 2013), and this could therefore alter the defence that a second pathogen could encounter. There may also be differences in the ability of the host immune response to recognise diverse pathogens (Sadd & Schmid-Hempel 2009), as well as if and how a pathogen recognises a competitor.

**Baculoviruses and competition**

Baculoviruses are common pathogens in lepidopteran populations and studies have documented population level impacts of different baculoviruses on several host species (Dwyer et al. 2000; Van Frankenhuyzen et al. 2002; Cory & Myers 2003; Myers & Cory 2013). These effects, in combination with the virus’ ability to replicate exponentially within the host (White et al. 2012), make baculoviruses an ideal candidate for the biological control of lepidopteran pests (Moscardi 1999). Relatively few insect viruses have been studied with an ecological focus, and the majority of information stems from the *Drosophila melanogaster* model system and RNA viruses (Breitenbach et al. 2011), with emphasis on anti-viral defences and molecular mechanisms. Some symbiotic microorganisms, such as *Wolbachia* spp., can reduce or impede virus infection (Haine 2008; Hoffmann et al. 2011). Although the mechanisms underlying this interaction are not well known, this draws attention to the ways that viruses interact with other microorganisms in their host.

**Aims and hypotheses**

We used the insect host *Trichoplusia ni* (Hübner) and its obligate baculovirus, *T. ni* Single Nucleopolyhedrovirus (TnSNPV). *T. ni* is a generalist herbivore and pest of greenhouse and field crops in British Columbia (Franklin et al. 2010) and across North America, Asia and Europe (Capinera 2008). I wanted to know whether TnSNPV was affected either positively or negatively by a co-infecting pathogen. I investigated this by challenging hosts to TnSNPV, and subsequently exposing them to a second pathogen,
either bacteria or fungi. The bacterium used was *Bacillus thuringiensis* (*Bt*), and the fungus was *Beauvaria bassiana*; both of these are licensed biological control agents and live pathogens that are widely used for pest management in greenhouses. By measuring host mortality and cause of death, as well as within-host replication of the virus, I could conclude whether and how the baculovirus was affected by a competitor.

While TnSNPV and *Bt* are orally ingested pathogens (Keddie et al. 1989; Schnepf et al. 1998), *B. bassiana* enters via the host cuticle (Ferron 1978). I hypothesised that pathogens with different modes of entry would compete via a different mechanism within the host and that this would alter the outcome of baculovirus infection. A host that detects a virus in the gut is likely to focus its immune resources to this region (Ulrich & Schmid-Hempel 2012) requiring a trade-off either between different components of the immune response, such as specific pathways or molecules associated with viral versus fungal defences (Wang et al. 2010), or an area specific trade-off, with immune resources being concentrated at the gut and reduced elsewhere (Cotter et al. 2004). The fungus could therefore enter a compromised host and would be able to compete more strongly against the baculovirus, resulting in the fungus benefitting from the interaction whereas the virus would be negatively affected. The bacterium, on the other hand, enters through the same route as the baculovirus and therefore may face increased immune defence from a host that is already challenged in the gut region. This would negatively impact the bacteria in treatments where virus and bacteria infect together. In summary, I hypothesized that host mortality to baculovirus would be reduced when the fungus was present and increased when the bacterium was present.

### 3.2. Methods

#### 3.2.1. Study organisms

Cabbage looper (*Trichoplusia ni* Hübner) eggs were collected from a long term laboratory colony originally established from a greenhouse population in 2001 and maintained on a wheat-germ based diet. Colony larvae were maintained individually in 1oz plastic soufflé cups (Solo Cup Company, Lake Forest, IL) from neonate until pupation and were then mass mated with up to 200 moths per cage. Temperature was
kept constant at 25°C with a 16:8 hour light:dark cycle. *Trichoplusia ni* single nucleopolyhedrovirus (TnSNPV) suspension was prepared by isolating occlusion bodies from infected cadavers through multiple rounds of centrifugation to remove debris from the suspension. The concentration of viral occlusion bodies (OBs) was estimated using a compound microscope (400X magnification) and an improved Neubauer haemocytometer (Hausser Scientific). I used *Bt* subsp. *kurstaki* in the form of the commercial product Dipel® (Valent Biosciences, Guelph, Ontario, Canada) and Botanigard ES (Bioworks, Victor, NY) was the source of *B. bassiana*.

3.2.2. Experimental rearing

One thousand larvae were reared individually on wheat-germ based diet in bioassay trays (128 cells, Bio-serv, Frenchtown, NJ) until pathogen exposure. Insects were reared at 24°C and a 16:8 light: dark cycle. After six days, 500 moulting third instar larvae were randomly selected, weighed and placed in 12-well tissue culture plates (Becton Dickinson Labware, Franklin Lakes, NJ) for starvation up to 24 hours prior to pathogen exposure. The experiment was carried out twice, referred to as blocks. Due to timing of larval development, block 1 larvae were exposed to pathogens late in the day and block 2 larvae early in the day.

The experiment consisted of exposure to two different pathogens, 24 hours apart. Larvae were first exposed to a low or high dose of baculovirus or a control (dH2O), followed by a low or high dose of either the bacterium, *B. thuringiensis*, or the fungus, *B. bassiana*, or no treatment (Fig. 3.1).

3.2.3. Treatment 1:

*Baculovirus exposure*

Virus suspensions of desired dose (Table 3.1) were prepared by serial dilution and 1% concentration blue food colouring (Club House®, London, ON) was added to ensure the solution was visible both in the well plate and inside the insect gut. Larvae were dosed using a technique modified from Hughes & Wood (1981). A 1µl droplet of the suspension was placed directly in front of each larva in the 12-well plates. Having been starved for
**Figure 3.1** Experimental design for *T. ni* exposed to a control or baculovirus, followed by either a control, bacteria or fungi, with a timeline along the bottom of the figure.

**Table 3.1** Concentrations of the high and low doses of TnSNPV, *Bt*, and *B. bassiana* that were used to dose *T. ni* larvae. All pathogens were administered in 1µl per larva.

<table>
<thead>
<tr>
<th>Pathogen</th>
<th>Dose</th>
<th>Concentration (per 1µl)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Virus – <em>T. ni</em> Single Nucleopolyhedrovirus</strong></td>
<td>High</td>
<td>1000 occlusion bodies (OBs)</td>
</tr>
<tr>
<td></td>
<td>Low</td>
<td>100 OBs</td>
</tr>
<tr>
<td><strong>Bacteria - <em>Bacillus thuringiensis</em></strong></td>
<td>High</td>
<td>2 cabbage looper units (CLU)</td>
</tr>
<tr>
<td></td>
<td>Low</td>
<td>0.4 CLU</td>
</tr>
<tr>
<td><strong>Fungus - <em>Beauvaria bassiana</em></strong></td>
<td>High</td>
<td>1,300,000 spores</td>
</tr>
<tr>
<td></td>
<td>Low</td>
<td>100,000 spores</td>
</tr>
</tbody>
</table>
24 hours, most larvae immediately ingested the droplet. This allowed for very accurate timing of dosage. Larvae that did not consume the entire droplet, or who refused it, were discarded. Once the droplet was consumed, larvae were given a block of artificial diet to feed on *ad libitum* for 12 hours.

All larvae, including controls, were weighed again and starved for 12 hours before the second exposure to ensure that they would ingest the diet containing bacteria. This was required as *Bt* has an inhibitory effect on feeding (Schnepf et al. 1998; Martinez-Ramirez et al. 1999) and a well-fed larva would be less likely to ingest it. Appropriate doses for the second pathogen were selected based on multiple mortality trials conducted prior to the experiments. The low dose or concentration was intended to cause minimal mortality whilst the high dose aimed for 50% host mortality (*LD*$_{50}$/*LC*$_{50}$).

### 3.2.4. Treatment 2:

**Bt exposure**

Initially, a master solution with 20,000 cabbage looper units (CLU) per ml was prepared (0.025g Dipel® in 40ml dH$_2$O). Once suspended, experimental solutions were serially diluted from this mixture (Table 3.1). A 1µl droplet of *Bt* was placed on a very small block of diet (approx. 2x4mm). The insect was given 4 hours to feed on this and was then given a block of clean diet. Ingestion of *Bt* inhibits feeding due to gut damage caused by Cry toxins (Schnepf et al. 1998; Martinez-Ramirez et al. 1999) therefore some hosts did not completely consume the diet containing the *Bt*. However, all larvae fed on it for a specific amount of time so any variation in dosing can be assumed to be random across treatments. *Bt* exposed larvae were left in the plates with clean diet for one day to avoid handling side effects immediately after dosing. They were then moved individually into 1 oz cups containing diet *ad libitum* and monitored daily for mortality.

**B. bassiana exposure**

Desired concentrations (Table 3.1) were achieved by serial dilution from the original product concentration of 2.11x10$^{10}$ spores/ml. Before exposure, larvae were transferred from the 12-well plates where they had been starved, into 1 oz plastic cups containing diet *ad libitum*. Larvae were immediately exposed to the pathogen by placing
a 1µl droplet of the fungal suspension on their central dorsal abdomen. This allowed the fungal spores to enter the host through the cuticle. All 1 oz cups were maintained under experimental conditions (24°C; 16:8 light:dark cycle).

3.2.5. Measures of mortality and weight at death

Larvae were checked daily for mortality or signs of infection. All dead larvae were collected, weighed where possible, and stored at -20°C. Virus infected larvae turned a pale, milky colour (instead of green) and became moribund. They also tended not to moult into the fifth instar. Death due to baculovirus infection usually causes larvae to liquefy. In order to collect virus infected cadavers for occlusion body counts, larvae that were no longer feeding and were judged to be near death were placed in 1.5 ml Eppendorf tubes, weighed and monitored daily for mortality. Time to death was recorded in hours post exposure. *Bt* killed larvae died within 4 days of exposure and were identified by their appearance (shrivelled) and size (small). Larvae killed by *B. bassiana* hardened at death, turned a purple colour, and were kept in a growth chamber at 24°C to allow the fungus time to sporulate. The appearance of the white fungal spores was used to confirm the fungal infection.

Occasionally, larvae were clearly co-infected, although this never constituted more than 10% of exposed insects per treatment. They displayed symptoms of virus infection but the cadaver did not fully lyse, and it was partially sporulated. If a virus-infected larva died and showed signs of fungal infection it was kept in the 24°C growth chamber to allow sporulation. If fungal spores did not appear the larva was marked as virus infected only, as sporulation of the fungus is required for successful transmission to a new host. If cause of death could not be identified, a larva was marked as ‘unknown’.

3.2.6. Virus occlusion body counts – virus yield

Water was added to virus infected cadavers to a total volume of 1ml. The sample was then macerated using a pellet pestle (VWR, Edmonton, AB, Canada). A 100X diluted sample was loaded into a 0.100mm deep Neubauer haemocytometer (Hausser Scientific) and OBs across five big squares of the counting chamber were counted, at a
magnification of 400X. Two separate counts were taken and averaged. Between 15 and 20 replicates (larvae) per treatment were randomly selected for counting, apart from the virus low-fungus high treatment where only four individuals died from virus and were available for OB counts.

3.2.7. Statistical Analyses

Data were analysed using JMP® (Version 10.0, SAS Institute Inc, Cary, NC) and figures were made using R (Version 3.1.0, The R Foundation for Statistical Computing). Overall mortality and mortality due to each pathogen individually were analysed by running generalized linear models with a binomial distribution and a logit link function. For example, when testing for mortality due to virus, I included the virus alone treatments and co-exposed treatments but excluded the second pathogens alone; whereas for mortality due to fungus, I included the fungus alone treatments and the fungus combined with virus treatments, but excluded the virus alone treatments. This method avoided over-dispersion of the data that would have been caused by including treatments that did not receive the pathogen of interest and therefore contained no deaths due to that pathogen. Larvae with unknown causes of death constituted a small percentage of each treatment and were excluded from all analyses. Co-infected larvae were included in mortality analyses and were classified as being infected by both pathogens.

Virus yield and conversion efficiency data (yield per unit weight at death) residuals were not normally distributed. Therefore, the data were log_{10} transformed and the distribution of the residuals was again assessed for normality. Data were analysed with the same terms as the mortality analysis but using a linear model. Here, all doses and controls were included, to compare across virus doses. Time to death of virus-killed larvae was analysed by running a linear model with terms as described below.

Virus dose, bacterial dose, fungal dose and block were treated as independent, categorical factors in the model, and weight was included as a covariate. Therefore the maximum model consisted of: dependent variable ~ virus dose + bacteria dose + fungi dose + weight at virus exposure + virus dose*bacteria dose + virus dose*fungi dose +
block. For the analysis of mortality due to fungus, bacteria treatments (‘bacteria dose’) were not included, and vice versa. Non-significant interactions were removed first and then the least significant main effects were removed sequentially until the minimal model was reached. Full models are reported in Appendix B.

3.3. Results

3.3.1. Overall mortality

Overall mortality was significantly affected by both virus dose, as expected, and fungal dose, with treatments that had both these pathogens causing higher larval mortality compared to virus alone (Table 3.2/Table B1/Fig. 3.2). Mortality increased by ~10% in all treatments that included fungi, but this effect was not seen with bacteria.

3.3.2. Host mortality due to virus

The proportion of deaths caused by virus in co-exposed treatments varied depending on whether the second pathogen was the bacterium or the fungus. When hosts received bacteria after virus, the proportion of hosts that died from virus infection was unchanged. However hosts exposed to virus followed by a high dose of the fungal pathogen had ~30% reduced mortality due to virus (Table 3.2/Table B1/Fig. 3.3). Co-infected hosts are not shown separately (but were included in analyses) as they represented fewer than 10% of the larval deaths in each treatment.

3.3.3. Host mortality due to bacteria

Levels of mortality due to bacteria alone were unexpectedly low in the high Bt dose, possibly due to under-dosing which is discussed below. Unfortunately, differences were therefore not detectable in statistical analysis (Fig. 3.4).
3.3.4. Host mortality due to fungi

*T. ni* mortality resulting from fungal infection was positively affected by previous exposure to the baculovirus (Table 3.2/Table B1/Fig. 3.4). This effect was more prominent in treatments with a high fungal dose and complimented the pattern seen with virus mortality in co-exposed insects. Interestingly, the positive effect on mortality was most noticeable in hosts exposed to a high virus dose prior to fungal treatment.

Table 3.2 Minimal model output from the generalized linear models for overall *T. ni* mortality, or specific mortality due to the virus, TnSNPV, or the fungus, *B. bassiana*. Maximal model outputs are shown in Table B1 in Appendix B. Horizontal lines delineate separate models.

<table>
<thead>
<tr>
<th>Response</th>
<th>Predictor variable</th>
<th>Degrees of Freedom</th>
<th>Chi-squared value</th>
<th>Pr(&gt;Chi)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Overall mortality</td>
<td>Fungi dose</td>
<td>2</td>
<td>7.05</td>
<td>0.0295</td>
</tr>
<tr>
<td></td>
<td>Virus dose</td>
<td>1</td>
<td>87.38</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Host mortality due to virus</td>
<td>Virus Dose</td>
<td>1</td>
<td>74.80</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td></td>
<td>Fungi Dose</td>
<td>2</td>
<td>24.70</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Host mortality due to fungi</td>
<td>Virus Dose</td>
<td>2</td>
<td>16.41</td>
<td>0.0003</td>
</tr>
<tr>
<td></td>
<td>Fungi Dose</td>
<td>2</td>
<td>43.07</td>
<td>&lt;0.0001</td>
</tr>
</tbody>
</table>
Figure 3.2  Overall mortality of *T. ni* larvae in virus alone treatments and treatments where virus was combined with either bacteria or fungi. Low dose virus treatments are shown in the left panel, with high dose virus treatments in the right panel. The black bar indicates virus alone, the light grey bars indicate virus plus bacteria, at a low and high bacterial dose, and dark grey bars indicate virus plus fungi, at the two fungal doses.
Figure 3.3  Proportion of *T. ni* mortality due to baculovirus across treatments with virus alone and virus plus a second pathogen. Treatments that received virus alone are indicated by black bars, at a low (left panel) or a high (right panel) virus dose. Treatments where virus was combined with a second pathogen are in light grey bars for bacteria, or dark grey bars for fungi, with a low and high dose in each case.
Figure 3.4  Proportion of *T. ni* mortality due to either the bacterium, *Bt*, or the fungus, *B. bassiana* in single exposed treatments, and treatments where insects received TnSNPV prior to the second pathogen. Bacterial treatments are in the left panel and fungal treatments are on the right. Bar colours indicate the second pathogen infecting the host alone (black), at a low or high dose, or the second pathogen at each respective dose combined with a low (light grey) or a high dose of baculovirus (dark grey).

3.3.5.  Instar at death and time to death

Time to death due to baculovirus did not vary significantly by treatment (Fig. B1); however there was a significant difference between the two experimental blocks (Table B2). Whilst time to death did not vary between treatments, the instar that larvae died at was significantly affected by virus dose (Table 3.3/Table B3/Fig. 3.5). Larvae exposed to a high virus dose were more likely to die as fourth instars, that is, they did not moult after exposure to baculovirus. The presence of bacteria was more likely to cause a larva to die of virus as a fourth instar than moult into the fifth, whereas the presence of fungi did not significantly affect instar at death (Table 3.3/Table B3).
Output from the minimal nominal logistic model to measure the effect of pathogen treatment(s) on *T. ni* larval instar at death. Maximal model output in shown in Table B3 in Appendix B.

<table>
<thead>
<tr>
<th>Response</th>
<th>Predictor variable</th>
<th>Degrees of Freedom</th>
<th>Chi-squared value</th>
<th>Pr(&gt;Chi)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Instar at death</td>
<td>Weight at exposure</td>
<td>1</td>
<td>9.36</td>
<td>0.0022</td>
</tr>
<tr>
<td></td>
<td>Bacteria dose</td>
<td>2</td>
<td>15.35</td>
<td>0.0005</td>
</tr>
<tr>
<td></td>
<td>Virus dose</td>
<td>1</td>
<td>14.19</td>
<td>0.0002</td>
</tr>
</tbody>
</table>

Figure 3.5  Percentage composition of the instar upon death of *T. ni* hosts killed by baculovirus. As individuals were exposed as fourth instars, they had either successfully moulted into the fifth instar or died as fourth instars.
3.3.6. **Virus yield**

The number of virus OBs produced per host differed between virus doses; hosts that received a low dose of virus produced more occlusion bodies than those that were treated with the high virus dose (Table 3.4/Table B4/Fig. 3.6a). Additionally, treatments where larvae received bacteria as well as virus produced fewer OBs than virus alone treatments. Fungi treatments did not significantly affect virus yield however there was a difference in the trend between low and high virus doses. Low virus dose treatments displayed a similar trend to that seen in the bacterial treatments, whereas there is an indication that the high virus-high fungi combination produced more OBs than the high virus dose alone (Fig. 3.6a). Only four individuals in the virus low-fungi high treatment succumbed to virus resulting in a very small sample size for OB counts in this treatment and therefore reduced statistical power.

Virus yield per milligram of host is a measure of virus efficiency where a host that has a higher number of OBs per mg has been more efficiently converted into virus. Again, we see a negative effect of bacteria on virus efficiency at both virus doses, but no significant effect of fungi (Table 3.4/Table B4/Fig. 3.6b). There is a significant effect of block in both models – block 1 larvae produced slightly more OBs on average than block 2 larvae.

3.3.7. **Virus yield and time to death**

Larvae that lived longer produced more occlusion bodies than those that died quickly, independent of treatment (Fig. 3.7/Table B5).
Table 3.4 Minimal model output for linear models on TnSNPV yield and efficiency from *T. ni* hosts across singly and multiply exposed treatments. Maximal model output can be seen in Table B4 in Appendix B.

<table>
<thead>
<tr>
<th>Response</th>
<th>Predictor variable</th>
<th>Degrees of Freedom</th>
<th>Sum of Squares</th>
<th>F Ratio</th>
<th>Prob &gt; F</th>
</tr>
</thead>
<tbody>
<tr>
<td>Virus yield – count per cadaver</td>
<td>Virus dose</td>
<td>1</td>
<td>0.96</td>
<td>4.86</td>
<td>0.0288</td>
</tr>
<tr>
<td></td>
<td>Block</td>
<td>1</td>
<td>1.05</td>
<td>5.33</td>
<td>0.0222</td>
</tr>
<tr>
<td></td>
<td>Bacteria dose</td>
<td>2</td>
<td>2.40</td>
<td>6.08</td>
<td>0.0028</td>
</tr>
<tr>
<td>Virus efficiency – count per mg cadaver</td>
<td>Bacteria dose</td>
<td>2</td>
<td>0.95</td>
<td>5.44</td>
<td>0.0052</td>
</tr>
<tr>
<td></td>
<td>Block</td>
<td>1</td>
<td>0.84</td>
<td>10.09</td>
<td>0.0018</td>
</tr>
</tbody>
</table>
Figure 3.6 Mean ± standard error of log transformed virus yield and virus efficiency in virus-killed *T. ni* hosts exposed to virus alone or virus plus a second pathogen. Treatments are divided into low virus dose on the left and high virus dose on the right. Solid circles indicate treatments where the host was exposed to virus alone, squares are for hosts that received bacteria after virus exposure, and triangles are for treatments that included the fungal pathogen after virus exposure. Sample size range from 15-20 larvae in every treatment except for the low virus-low fungus combination where just four larvae died from virus.
Figure 3.7  Virus yield by time to death of T. ni larvae killed by the baculovirus, TnSNPV. Virus yield data has been log$_{10}$ transformed for normality. The line of fit ($y = 7.39 + 0.007x$) is calculated from a regression analysis which can be seen in Table B5 in Appendix B.

3.4. Discussion

We demonstrated that a baculovirus can be negatively impacted by a competing pathogen, both in terms of host mortality and within-host replication (potential transmission success). Despite a 24 hour advantage in initiating infection, the proportion of hosts killed by baculovirus was lower when larvae additionally received a high dose of the fungal pathogen than when they were treated with just baculovirus. Conversely, mortality due to baculovirus did not change in treatments where hosts received bacteria following baculovirus. However, bacteria did affect baculovirus replication as hosts that received bacteria following virus tended to die as fourth instars and produced fewer viral OBs than hosts that received the same dose of virus alone. One would expect hosts that received a higher virus dose to die as fourth instars, and to therefore be killed more rapidly (Cory et al. 2004). Here, however, mortality was only checked once daily and there would therefore have to be big differences in time to death between treatment groups to be distinguishable. There is also a difference in time to death between blocks,
as a result of larvae being exposed at different times of day (block one in the evening, block two in the morning) but mortality being checked at the same time of day (morning) across both blocks. A more robust measure of time to death is warranted in future studies to fully elucidate the relationship between time to death and virus yield between single and co-exposed treatments. However, when all treatments were combined, there was a positive relationship between virus yield and time to death, with larvae that lived longer producing more OBs. The negative effects on virus yield seen with bacteria were not present when fungus was the competing pathogen.

3.4.1. The effect of competing pathogens on host mortality

Interestingly, overall host mortality was affected by the presence of a fungal pathogen at both virus doses, although the increase in mortality was small, around 10% in all treatments. An additive design was used here, so hosts in treatments with two pathogens effectively received a double dose as they were given the same amount of each pathogen as in the single pathogen treatments. With this design, we would expect overall mortality to increase given the increase in pathogen load that each host is receiving. Additionally, theory predicts that overall mortality should be higher in co-infected rather than singly-infected hosts, due to increased competition that leads to increased pathogen growth (Nowak & May 1994; May & Nowak 1995). These are known as synergistic effects, that is, host mortality to the co-infection is higher than the sum of mortality to the two pathogens when infecting alone. An additive effect was seen in a study using two fungal species, Beauvaria bassiana and Metarhizium anisopliae, in a locust host, although it only became apparent at high temperatures (Thomas et al. 2003). Interestingly, in a different study, temperature resulted in synergism in a Lymantria dispar host infected with a baculovirus, LdNPV, and a fungus, Entomophaga maimaiga (Malakar et al. 1999). Here, the data for overall mortality in virus and fungal combined treatments show a small additive effect. However the 30% reduction in host mortality to virus when the host is also exposed to fungus supports an antagonistic interaction between these two pathogens; an outcome that is supported in other studies. In an intraspecific interaction between multiple strains of Schistosoma mansoni in a snail host, the more virulent strain suffered more in a mixed infection and the overall parasite burden was lower when the virulent and avirulent strains were present (Gower &
Reduced replication was also seen in a baculovirus-entomopoxvirus co-infection in the smaller tea tortrix, *Aدوxophyes honmai*, although the negative impact of a competitor only affected the entomopoxvirus, not the baculovirus (Ishii et al. 2002). Distinguishing differences between intra- and interspecific competition is important, particularly due to the research bias towards the former. Interspecific competition studies are considerably harder to set up and interpret and examples, particularly in invertebrates, are limited. The outcome of co-infections continue to surprise us and, as yet, there is no clear framework for predicting synergism, additive effects or antagonism between different pathogens or between genotypes of a single pathogen (Schjørring & Koella 2003).

The order of exposure of a host to an infectious agent can affect the success of pathogen or parasite establishment but the mechanisms behind this are unclear. Two studies on prior residency in the water flea *Daphnia magna* concluded that sequential introduction into the host caused reduced host mortality compared to infecting a host simultaneously, both intra-specifically between a protozoan and a fungus (Lohr et al. 2010), and inter-specifically between different genotypes of the bacteria, *Pasteuria ramosa* (Ben-Ami et al. 2008). In a study looking at competition between two fungal pathogens in the diamond back moth, *Plutella xylostella*, the fungus that infected second was always more successful (Zamora-Macorra et al. 2012). Conversely, in an amphibian host with two trematode parasites, the second parasite to enter the host had reduced infection success (Hoverman et al. 2013). Rather than within-host competition, this may be attributable to older hosts being more resistant due to development (Hoverman et al. 2013). Although this study is on a vertebrate host, the same concept applies in insects, including in my system. *T. ni* larvae show increased resistance to baculovirus with each instar and as they age within an instar (Engelhard & Volkman 1995). The second pathogen may therefore be at a disadvantage given that the host is further along in development by the time it enters. The success of the fungus over the virus in this study is surprising, but there are multiple other factors to consider in interpreting this result and these are discussed below.
3.4.2. Baculovirus infection and host development

The effect of the virus on host physiology is a key factor in determining the success of the virus within the host. Many baculoviruses express the \textit{egt} gene which arrests host development (O’Reilly & Miller 1989) and has been shown to benefit the virus by increasing virus yield (O’Reilly & Miller 1991; Cory et al. 2004) and potentially limiting host immune capability. Cory et al. (2004) demonstrated a dose effect of AcMNPV on \textit{T. ni} development with hosts dosed with fewer OBs being less likely to exhibit the phenotypic effects of \textit{egt} expression, and therefore more likely to moult into the fifth instar. Here, I found that fewer individuals in the lower virus dose experienced arrested development, supporting this result (Fig. 3.5). However it is not known how this strategy could affect the virus’ ability to compete with other pathogens inside the host. Host manipulation is a tactic employed by many pathogens and parasites (Poulin 2010). The previous chapter in this thesis documented a reduction in the presence and activity of haemocytes and two immune enzymes, phenoloxidase and glucose dehydrogenase, when \textit{T. ni} was exposed to a baculovirus. If host immune capabilities are compromised, due to pathogen suppression of the immune response (Salem et al. 2011; Jakubowska et al. 2013) or developmental arrest, this could be advantageous to the second competitor by facilitating entry due to reduced host defences. Host manipulation may be an excellent strategy in a singly infected host however it could be detrimental in competitive interactions, either by lowering host mortality to virus or by reducing virus yield.

3.4.3. Pathogen interactions and dose responses

Previous studies have shown that infectious dose can alter the outcome of a co-infection due to the within-host epidemiology of each pathogen (Fellous & Koella 2009; Fellous & Koella 2010). The within-host growth of micro-parasites in invertebrates is density dependent, in that growth is constrained as the micro-parasites reach a certain density or carrying capacity ($k$) within the host (Ebert et al. 2000). Some pathogens and parasites can manipulate this carrying capacity, for example the \textit{egt} gene in baculovirus will allow a host to grow larger thereby extending $k$ (Cory et al. 2004). Density dependent constraints are present in both single and multiple infections, as shown in \textit{Daphnia}
magna infected with a bacterium and a fungus, indicating that initial dose can have significant effects on the establishment and replication of multiple pathogens within a host (Ebert et al. 2000). Additionally, dose dependent effects on host immune parameters have been demonstrated previously in T. ni infected with TnSNPV (Chapter 2 of this thesis). Therefore initial dose may affect the types of competitive interaction that occur between pathogens depending on the state of the host, both in terms of immune capabilities and condition (i.e. resource quality for the pathogens). Here, we do not see clear evidence for differential interactions between high and low doses of competing pathogens, which may indicate that the pathogens are not competing via the host resources. Despite several studies using different doses of co-infecting pathogens and parasites, there are no firm conclusions that can be drawn and it is essential to take the ecology of the infecting organisms into account (Seppälä et al. 2012).

The two doses used for the competing pathogens aimed to tease apart the effect of two different competitive mechanisms. The ‘low’ dose of the competing pathogen was intended to be sub-lethal and thus reduce the chances of resource-based competition as the initial challenge would either be successfully repelled or only low levels of replication would occur. This is borne out by the low levels of mortality seen in the bacterial and fungal treatments (<5%). However, even a small challenge should stimulate the host immune response (Sadd & Schmid-Hempel 2006) and alter the host environment for co-infecting pathogens. The ‘high’ dose treatment of the second pathogens allowed for resource-based competition but did not exclude the potential for immune-mediated competition.

Unfortunately, mortality due to bacteria was very low across all treatments and therefore it was unlikely that even the high dose could outcompete the baculovirus. Larvae were dosed with Bt using the diet plug method, however the Cry toxins in Bt inhibit feeding (Schnepf et al. 1998; Martinez-Ramirez et al. 1999), so it is possible that larvae were under-dosed. Further work with higher Bt doses will help to confirm how baculovirus and this widely applied microbial agent (Bailey et al. 2010) might affect host mortality in the field.
3.4.4. Within-host development of pathogens

The timing of development of co-infecting pathogens within the host could affect the degree of competition between them. For example, two pathogens that replicate at similar rates, such as the baculovirus and the fungus used here, may have increased competition compared with two pathogens with very different development rates. Alternatively, whilst two pathogens that develop at different rates inside the host may not compete as directly, the faster growing pathogen will usually be more successful (Ishii et al. 2002). At the temperature used here, host mortality due to TnSNPV infection occurred 5-7 days after exposure (Fig. B1, mortality due to baculovirus only) and insects killed by B. bassiana died in a similar timeframe, whereas Bt killed its host very quickly, usually within 3 days of infection (data not shown). This suggests that the competitive interaction between the fungus and the baculovirus may be greater than the competition between the bacterium and baculovirus, however further work with higher Bt doses would need to be done to confirm this result.

As hypothesised in my study, different modes of entry of two pathogens may result in immune-mediated competition. A host that is attacked via the gut could concentrate its immune resources to this region, trading-off with defences elsewhere in the body (Sadd & Schmid-Hempel 2009). This may facilitate entry of the fungi through the host cuticle. Easier entry will result in more fungal hyphae entering the host and replicating, increasing the chances for the fungus competing against the baculovirus. Evidence for compartmentalisation of the immune response has been shown in damselflies. There is a relationship between the burden of a gut parasite, Hoplorhynchus polyhamatus, and mid-gut phenoloxidase (PO) but not between parasite burden and haemolymph PO indicating that it is costly to maintain certain immune functions across multiple body compartments simultaneously (Siva-Jothy et al. 2001). This localisation of the immune response may explain why I see an increase in infection success of the fungus in this study.

3.4.5. The effect of competitors on virus yield

While Bt did not affect host mortality to baculovirus, it did negatively impact the virus’ ability to replicate inside the host, resulting in fewer occlusion bodies per host and
lower efficiency of host tissue conversion in treatments that included bacteria. The main mechanism behind this may be the gut damage caused by *Bt*’s Cry and VIP toxins (Schnepf et al. 1998). Hosts that received *Bt* were prevented from feeding for some time following treatment, and they died smaller than the ‘virus alone’ treatments. Another explanation is that the bacterium and the baculovirus were competing directly for resources, leading to reduced host growth. The Cry1Ac toxin of *Bt* and a baculovirus, AcMNPV, interacted antagonistically at low toxin concentrations in the diamondback moth, *Plutella xylostella*, resulting in an overall reduction in host mortality, but this effect was not seen at high toxin levels (Raymond et al. 2006). A second study on interactions between *Bt* strains in the same host reported reduced *Bt* growth in mixed strain infections (Garbutt et al. 2011). My result demonstrates the importance of measuring pathogen yield as well as host mortality in order to gain a deeper understanding of the consequences of within-host interactions. In some individuals this difference is several hundred million OBs and it could have significant biological consequences for the transmission of baculovirus. This has important implications for the transmission of the virus to new hosts and could result in significant fitness differences between baculoviruses that infect alone and those that encounter competition from other pathogens. However, other factors may be as or more important in transmission, such as the location of the cadaver at death (Hails et al. 2002). UV light can severely degrade baculovirus (Griego et al. 1985) but it is well known that OBs can persist for years in the environment to be transmitted to new hosts, although the extent of this persistence is not fully understood (Jaques 1967; Fuller et al. 2012).

Although there was no overall effect of fungi on virus yield, there was a difference in the trend seen between virus doses. In low virus dose treatments, fungi may reduce virus yield whereas the high virus-high fungus treatment may increase it. If immune-mediated competition is the key mechanism by which baculovirus and fungi interact, this could affect both virus infection success and replication. This potential difference between virus doses may be caused by hosts exposed to a high virus dose being forced to trade-off between two different aspects of immunity resulting in a weakened host defence against the virus (Cotter et al. 2004). This is particularly relevant if the two activate different immune pathways, as is possible with baculovirus and fungi (Wang et al. 2010). Whilst humoral enzymes such as phenoloxidase and glucose dehydrogenase
are not thought to be an important component of viral defence in Lepidoptera (Saejeng et al. 2010; Myers et al. 2011; Chapter 2 of this thesis), it is likely that they have a significant role in the melanisation of fungal pathogens (Barnes & Siva-Jothy 2000; Lee et al. 2005; Dubovskiy et al. 2013). For example, a melanic morph of *Galleria mellonella*, has increased resistance to *B. bassiana* which is correlated with a thicker cuticle, higher encapsulation ability, and increased phenoloxidase activity in relation to a non-melanic morph (Dubovskiy et al. 2013). Additionally, higher virus doses form more foci of infection in the gut (Washburn et al. 1996) and could therefore require a larger, and more costly, response from the host thereby increasing the pressure on the host immune system.

Competitive interactions can also be expected to vary across different life stages of the pathogen, as well as the host. A pathogen that encounters competition within the host may switch from replication to preparing for transmission in order to escape the competitor. However, a study on *Plasmodium chabaudi* found that a competitive advantage within the host also meant a transmission advantage to new hosts (de Roode et al. 2005), although this is a vector-borne pathogen and therefore does not require host mobility in order to be dispersed. This result may differ in obligate killers that need to form spores or occlusion bodies prior to entering the environment and where transmission does not occur until after host death (Ebert & Weisser 1997). As a result, a species or strain that ‘wins’ the outcome of within-host competition may not necessarily be as successful during transmission, particularly considering the trade-off hypothesis between within-host virulence and transmission to a new host (Anderson & May 1982; Bull 1994; Alizon et al. 2009). As discussed previously, host development has significant consequences for pathogen success, usually with increased age inferring increased resistance (Kirkpatrick et al. 1998; Hoverman et al. 2013). As the immune capability of the host and host condition change with development, the interactions within the pathogen community may also change and addressing these complexities is a challenge for future studies.
3.4.6. Conclusion

This study showed that a baculovirus can be differentially affected by competing pathogens from different groups. Whilst host mortality to baculovirus was negatively impacted by co-exposure to fungi, virus yield was negatively affected by bacteria. We additionally saw a small increase in overall host mortality when the virus and the fungus exposures were combined. We continue to improve our understanding of the ways that invertebrate hosts defend themselves against viruses. Focusing on the potential costs and trade-offs of these defences will enable a better approach to understanding within-host interactions between viruses and other pathogens. Understanding co-infections from an ecological perspective will allow for significant advances in the ways that we manage infectious disease, as well as the utilisation of pathogens in biological control.

References


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Chapter 4. Thesis conclusion

This thesis investigated the interactions between a baculovirus and other pathogens within an insect host, with a particular focus on the role of the immune response in mediating these interactions. The results add to our fundamental understanding of within-host pathogen communities, specifically the ecology of baculoviruses, but also have significant applications in the microbial control of insect pests.

4.1. Ecology of the insect immune response

Although we have a good understanding of general invertebrate immunity, we have only recently been capable of exploring the mechanisms underlying the insect immune response to viruses. Firstly, I investigated the cellular and humoral immune response to baculovirus measuring across different doses and at three time points post exposure. All the parameters measured were reduced as virus dose increased, indicating that *T. ni* do not utilise these immune components in defence against virus. Additionally, two out of three haemocyte types showed a dose response in the last two time points, but not the first. Many studies in eco-immunology do not take measurements at multiple times post-exposure and it will be important for future studies to incorporate the variation in responses over time in order to gain a more accurate picture of the immune response, and associated costs incurred, by the host to a given pathogen. The reduction in haemocytes and immune enzyme activity with increasing dose was surprising and may have been due to a trade-off with an unmeasured immune component (Siva-Jothy et al. 2001; Cotter et al. 2004), a physiological response to illness, or manipulation of the host response by the virus (Jakubowska et al. 2013). I focused on the final option, as manipulation or suppression by the host immune response could affect subsequent co-infecting pathogens or parasites. I tested this further by challenging virus-exposed hosts to a cocktail of Gram-positive and Gram-
negative bacteria and tested their antibacterial response by measuring the activity of four antimicrobial peptides. Only one of the four AMPs measured, cecropin, had reduced activity in bacteria-challenged virus-exposed versus naïve hosts. This indicates that, in this system, baculovirus exposure does not significantly alter the host’s antibacterial response. However, a more thorough conclusion could be made if a wider variety of immune components were measured, including the cellular and immune parameters measured in the initial experiment. Further investigation into how virus exposure changes defence against other pathogen groups, will also be interesting, particularly with regards to the negative impact of fungus on mortality due to baculovirus seen in chapter 3.

Advances in molecular techniques have encouraged studies on immune gene activity (Riddell et al. 2011; Choi et al. 2012) and RNA interference (Gammon & Mello 2015), but there are still many areas in invertebrate immunity that need to be addressed. Most importantly, perhaps, is investigating these responses in the context of host and pathogen ecology and evolution. As discussed here, a change in an immune parameter may alter the host for a subsequent pathogen. Whilst these scenarios have been discussed in the literature, there is a missing link between comprehensive immune studies and host-pathogen ecology studies. It will be important to address this gap in the future both by addressing host defences to co-infections and communities of pathogens, rather than a single pathogen, and also by focusing on the costs induced from these immune responses, in terms of within immune system trade-offs (Ulrich & Schmid-Hempel 2012).

Although the invertebrate immune response is not adaptive, several insects have shown specificity to different pathogen groups (Loker et al. 2004). This indicates the potential for trade-offs within the immune system as a host responds differently depending on the pathogen group or species that is attacking it (Siva-Jothy et al. 2001). We are now able to measure immune gene activity as well as downstream components, including antimicrobial peptides, haemocytes and immune enzymes. Future studies that can incorporate immune responses at both of these levels will give a more robust picture of how an invertebrate responds to infection. In a multiply-infected host, this may allow for the investigation of within immune system trade-offs. Although the presence of trade-
offs within immune components has been shown in several studies (Cotter et al. 2004; Ulrich & Schmid-Hempel 2012), the mechanistic basis underlying the trade-offs is not known. Additionally, the notion that all immune components are costly to the host has been disputed (Labbé et al. 2010). Trade-offs are challenging to measure as the absence of a trade-off does not necessarily mean that one is not occurring, it may be that the parameter measured was not the one that was facing a trade-off or that the trade-off only becomes significant in certain environmental conditions, for example if the host lacks resources or is suffering stress (Valtonen et al. 2010). It is important to utilise new techniques in order to understand the mechanisms behind immune responses, but future work should also focus on applying ecological thinking to within-host processes to better realize the evolutionary pressures the host immune response is under (Pedersen & Fenton 2007; Graham 2008). This will include measuring immune responses in multiply-infected, rather than singly-infected, hosts and linking lab studies to field studies for a more natural picture of host-pathogen interactions.

An important distinction to recognise is that host resources are shared between competing pathogens, but also by the host itself, particularly in immune function (Pedersen & Fenton 2007). Cressler et al. (2014) identified theoretically that the degree of common resource sharing between immune system and pathogen(s) can alter the outcome of an interaction. This highlights the significance of studying host and pathogen ecology in concert and is likely to be a key area for future study, with empirical evidence needed to support this model.

4.2. Community ecology and within-host competition between pathogens

Considering the host as an ecosystem in which different organisms compete and interact, as in community ecology, has been identified as a way to move this field forward (Pedersen & Fenton 2007). Field studies in vertebrates have focused on the within-host community of pathogens, and indicate significant effects of different community interactions at the population level (Telfer et al. 2010; Johnson & Hoverman 2012). There has been less attention on these questions in invertebrates, probably due to the difficulties of taking non-fatal samples from invertebrate hosts and in identifying
specific individuals in the field, as well as the shorter lifespan of most invertebrates compared with vertebrates. However, there is a focus on the role of commensal and pathogenic gut microorganisms in invertebrate hosts. Limited studies in this area indicate that gut micro-organisms may affect the establishment of pathogens due to competition within the gut, and they may also boost host immunity (Raymond et al. 2008; Broderick et al. 2009; Jakubowska et al. 2013). The mechanisms underlying these effects are largely unknown and it is an exciting area for future research.

As seen in this thesis, within-host competition can affect host mortality and pathogen replication. In terms of host mortality, the highest levels were seen in treatments exposed to virus and fungus, however specific mortality to baculovirus was negatively affected by the fungal competitor, but not by the bacterium. On the other hand, in hosts that were killed by baculovirus, within-host replication of the virus was reduced in bacteria-exposed hosts but not in fungus-exposed hosts. Further research is required before the mechanisms that lie behind these results can be fully determined. Establishing the type of competition that occurs between two species may enable us to make predictions about the outcome of a co-infection. For example, do pathogens of certain groups always interact via the same competitive mechanism and does this vary depending on the host they are infecting? This knowledge could be useful in pest management if two products are applied together. One of the key issues facing the long-term utilisation of microbial control is a lack of evolutionary focus (Cory & Franklin 2012). Virus yield is not necessarily an effective measure of between host transmission and it would be interesting to address this by seeing if virus from co-exposed hosts transmits differently to new hosts compared with virus that infected alone. Measuring pathogen transmission in the field and the lab is extremely challenging but focusing on the relationship between yield and transmission will offer a more complete picture of baculovirus dynamics. Although empirical evidence is not extensive, evolutionary theory predicts that within-host interactions between pathogens drive the evolution of increased virulence in pathogen populations (Alizon et al. 2013). This could have significant consequences for pest management, particularly for microbial products that are able to cycle through several rounds of infection in a pest population. Experimental evolution studies that study the effect of competitors on virulence evolution are needed. However, a major drawback is our inability to naturally mimic transmission to a new host. In
pathogens that must persist in the environment before a new host ingests them, surviving environmental conditions, including UV light, is essential for their evolutionary fitness. Collecting pathogens from one host and using them to infect another (i.e. passaging) ignores any environmental impacts that may occur when the pathogen is awaiting transmission to a new host. Future research needs to take this into account in order to gain a full picture of how competition affects virulence evolution (Alizon et al. 2013). An additional understudied area is the long term effects that interactions between pathogens may have on the evolution of host resistance. Future studies that can focus on the mode of competition between pathogens and the long term effects on both host and pathogens will contribute much needed knowledge to this area.

4.3. Significance and final conclusions

Until recently, the majority of studies in ecology focused on macro-organisms and the intricate roles of micro-organisms have been underestimated. However, more and more we realise the importance of these communities in terms of biodiversity, public health, and pest management. In conservation, it is accepted that maintaining biodiversity results in more stable ecosystems (Cardinale et al. 2012). The existence of a diverse community of natural enemies, including entomopathogens, has also been promoted as an effective and sustainable pest management strategy (Crowder et al. 2010). In human medicine, taking a community perspective may enable the treatment of multiple infections at once, rather than treating a single parasite or pathogen at a time (Druilhe et al. 2005). However, drug and vaccine treatments can alter the within-host community of micro-organisms with unintended negative effects, so this perspective also needs to be considered (Galvani 2003). Overall, however, the more we understand about the interactions that occur between micro-organisms within a host, the more effectively treatment options can be applied.

In pest management, the need for sustainable options is becoming increasingly important and applying multiple microbial agents to a pest population is already a useful tool. However, until the interactions between microbial agents are fully appreciated, caution is warranted as the outcome could be unexpected. In addition, it is essential that the pest management community develops a long term perspective (Cory & Franklin
2012) and considers resistance management in the host as well as the evolution of virulence in pathogen populations. The interactions between pathogens and parasites within a host are clearly complex and striving to understand them will lead to sustainable, long term pest management in future.

References


Appendix A. Chapter 2 Supplementary Tables and Figure

Table A1  Maximal model output for generalised linear model on mortality of *T. ni* larvae to baculovirus, shown in Figure 2.2.

<table>
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<th>ChiSquare Value</th>
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<td>Log_{10} (Virus Dose)</td>
<td>1</td>
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<td>&lt;0.0001***</td>
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Table A2  Maximal model output for general linear models on total number of haemocytes and on each haemocyte type individually in *T. ni* larvae exposed to different virus doses, with measurements taken at three times post-exposure.

<table>
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<tr>
<th>Response</th>
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<th>F ratio</th>
<th>Prob &gt; F</th>
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<td><strong>Total Haemocytes</strong></td>
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<td></td>
<td>Dose*Time</td>
<td>2</td>
<td>31.89</td>
<td>17.63</td>
<td>&lt;0.0001 ***</td>
</tr>
<tr>
<td><strong>Oenocytoids</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Weight at collection</td>
<td>1</td>
<td>9.24</td>
<td>9.26</td>
<td>0.0025 **</td>
</tr>
<tr>
<td></td>
<td>Dose</td>
<td>1</td>
<td>12.25</td>
<td>12.28</td>
<td>0.0005 **</td>
</tr>
<tr>
<td></td>
<td>Time</td>
<td>2</td>
<td>22.75</td>
<td>11.40</td>
<td>&lt;0.0001 ***</td>
</tr>
<tr>
<td><strong>Phenoloxidase</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Weight at collection</td>
<td>1</td>
<td>0.17</td>
<td>1.00</td>
<td>0.3191</td>
</tr>
<tr>
<td></td>
<td>Time</td>
<td>2</td>
<td>17.42</td>
<td>49.78</td>
<td>&lt;0.0001 ***</td>
</tr>
<tr>
<td></td>
<td>Dose*Time</td>
<td>2</td>
<td>5.83</td>
<td>33.33</td>
<td>&lt;0.0001 ***</td>
</tr>
<tr>
<td><strong>Glucose Dehydrogenase</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Weight at collection</td>
<td>1</td>
<td>12.86</td>
<td>5.68</td>
<td>0.0178 *</td>
</tr>
<tr>
<td></td>
<td>Time</td>
<td>2</td>
<td>10.58</td>
<td>4.67</td>
<td>0.0315 *</td>
</tr>
<tr>
<td></td>
<td>Dose*Time</td>
<td>2</td>
<td>10.58</td>
<td>4.67</td>
<td>0.0315 *</td>
</tr>
<tr>
<td></td>
<td>Dose</td>
<td>1</td>
<td>10.58</td>
<td>4.67</td>
<td>0.0315 *</td>
</tr>
<tr>
<td></td>
<td>Time</td>
<td>2</td>
<td>51.30</td>
<td>11.33</td>
<td>&lt;0.0001 ***</td>
</tr>
</tbody>
</table>
Table A3  
Fold change in *T. ni* antimicrobial peptide expression in all virus and bacterial treatments relative to control treatment (no pathogen or wounding). \( \Delta \Delta C_t \) values (top line) and associated ranges based on standard deviations (below in brackets) were calculated according to Livak & Schmittgen (2001).

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Antimicrobial Peptide</th>
<th>Gloverin</th>
<th>Cecropin</th>
<th>HDD</th>
<th>Defensin</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td></td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(0.1-7.3)</td>
<td>(0.1-15.4)</td>
<td>(0.0-24.2)</td>
<td>(0.2-6.5)</td>
</tr>
<tr>
<td>Virus alone</td>
<td></td>
<td>3.4</td>
<td>1.2</td>
<td>5.8</td>
<td>2.9</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(0.7-16.6)</td>
<td>(0.4-3.7)</td>
<td>(0.7-46.1)</td>
<td>(0.4-20.2)</td>
</tr>
<tr>
<td>Bacteria alone</td>
<td>2824.4</td>
<td>13.6</td>
<td>1.9</td>
<td>4.0</td>
<td></td>
</tr>
<tr>
<td></td>
<td>(110.5-72198.8)</td>
<td>(2.2-86.1)</td>
<td>(0.0-76.9)</td>
<td>(1.1-15.1)</td>
<td></td>
</tr>
<tr>
<td>Virus plus bacteria</td>
<td>2408.2</td>
<td>4.0</td>
<td>2.8</td>
<td>4.8</td>
<td></td>
</tr>
<tr>
<td></td>
<td>(54.4-106524.6)</td>
<td>(0.1-115.5)</td>
<td>(0.1-77.3)</td>
<td>(0.4-62.9)</td>
<td></td>
</tr>
<tr>
<td>Wounding alone</td>
<td>55.7</td>
<td>0.7</td>
<td>0.3</td>
<td>1.8</td>
<td></td>
</tr>
<tr>
<td></td>
<td>(0.2-12898.6)</td>
<td>(0.1-3.6)</td>
<td>(0.0-11.3)</td>
<td>(0.2-15.4)</td>
<td></td>
</tr>
<tr>
<td>Virus plus wounding</td>
<td>28.1</td>
<td>0.5</td>
<td>0.8</td>
<td>0.1</td>
<td></td>
</tr>
<tr>
<td></td>
<td>(2.9-269.4)</td>
<td>(0.1-4.1)</td>
<td>(0.0-39.2)</td>
<td>(0.0-52.9)</td>
<td></td>
</tr>
</tbody>
</table>
Figure A1  Log_{10} transformed mean fold change ± range in expression of four antimicrobial peptides across six treatments in the host *T. ni*. The left panel (a-d) shows each treatment relative to the control group (no pathogen or wounding). The right panel (e-h) shows each treatment relative to the bacteria only treatment, an internal control. Bars that fall below the zero line indicate reduced gene expression relative to the control (set at zero) on that plot, whereas bars above zero show increased gene expression.
Appendix B. Chapter 3 Supplementary Tables and Figures

Table B1  Output for the maximum overall mortality model and mortality due to virus and fungus respectively. Non-significant terms were removed sequentially starting with the least significant interaction term, and the model was rerun after each term removal to establish the next least significant term.

<table>
<thead>
<tr>
<th>Response</th>
<th>Predictor variable</th>
<th>Degrees of Freedom</th>
<th>Chi-squared value</th>
<th>Pr(&gt;Chi)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Overall host mortality</td>
<td>Virus dose x Fungi dose</td>
<td>2</td>
<td>1.42</td>
<td>0.4915</td>
</tr>
<tr>
<td></td>
<td>Virus dose x Bacteria dose</td>
<td>2</td>
<td>4.59</td>
<td>0.1009</td>
</tr>
<tr>
<td></td>
<td>Block</td>
<td>1</td>
<td>0.01</td>
<td>0.9350</td>
</tr>
<tr>
<td></td>
<td>Bacteria dose</td>
<td>2</td>
<td>0.62</td>
<td>0.7336</td>
</tr>
<tr>
<td></td>
<td>Weight at exposure</td>
<td>1</td>
<td>1.33</td>
<td>0.2494</td>
</tr>
<tr>
<td></td>
<td>Fungi dose</td>
<td>2</td>
<td>7.05</td>
<td>0.0295*</td>
</tr>
<tr>
<td></td>
<td>Virus dose</td>
<td>1</td>
<td>87.38</td>
<td>&lt;0.0001***</td>
</tr>
<tr>
<td>Mortality due to virus</td>
<td>Virus dose x Fungi dose</td>
<td>2</td>
<td>1.46</td>
<td>0.4811</td>
</tr>
<tr>
<td></td>
<td>Virus dose x Bacteria dose</td>
<td>2</td>
<td>0.76</td>
<td>0.6835</td>
</tr>
<tr>
<td></td>
<td>Bacteria dose</td>
<td>2</td>
<td>0.52</td>
<td>0.7698</td>
</tr>
<tr>
<td></td>
<td>Weight at exposure</td>
<td>1</td>
<td>0.25</td>
<td>0.6144</td>
</tr>
<tr>
<td></td>
<td>Block</td>
<td>1</td>
<td>1.46</td>
<td>0.2264</td>
</tr>
<tr>
<td></td>
<td>Virus Dose</td>
<td>1</td>
<td>74.80</td>
<td>&lt;0.0001***</td>
</tr>
<tr>
<td></td>
<td>Fungi Dose</td>
<td>2</td>
<td>24.70</td>
<td>&lt;0.0001***</td>
</tr>
<tr>
<td>Mortality due to fungus</td>
<td>Virus dose x Fungi dose</td>
<td>2</td>
<td>1.23</td>
<td>0.5417</td>
</tr>
<tr>
<td></td>
<td>Block</td>
<td>1</td>
<td>1.65</td>
<td>0.1987</td>
</tr>
<tr>
<td></td>
<td>Weight at exposure</td>
<td>1</td>
<td>3.06</td>
<td>0.0804.</td>
</tr>
<tr>
<td></td>
<td>Virus Dose</td>
<td>2</td>
<td>16.41</td>
<td>0.0003**</td>
</tr>
<tr>
<td></td>
<td>Fungi Dose</td>
<td>2</td>
<td>43.07</td>
<td>&lt;0.0001***</td>
</tr>
</tbody>
</table>
### Table B2

**Output from the maximal linear model on the time to death of virus killed *T. ni* larvae across treatments.** Non-significant terms were removed sequentially starting with the least significant interaction term, and the model was rerun after each term removal to establish the next least significant term.

<table>
<thead>
<tr>
<th>Response</th>
<th>Predictor variable</th>
<th>Degrees of Freedom</th>
<th>Sum of Squares</th>
<th>F Ratio</th>
<th>Prob &gt; F</th>
</tr>
</thead>
<tbody>
<tr>
<td>Time to death</td>
<td>Virus dose x bacteria dose</td>
<td>2</td>
<td>589.35</td>
<td>0.77</td>
<td>0.4654</td>
</tr>
<tr>
<td></td>
<td>Virus dose x fungi dose</td>
<td>2</td>
<td>784.45</td>
<td>1.02</td>
<td>0.3609</td>
</tr>
<tr>
<td></td>
<td>Bacteria dose</td>
<td>2</td>
<td>233.57</td>
<td>0.30</td>
<td>0.7377</td>
</tr>
<tr>
<td></td>
<td>Fungi dose</td>
<td>2</td>
<td>445.08</td>
<td>0.58</td>
<td>0.5586</td>
</tr>
<tr>
<td></td>
<td>Weight at exposure</td>
<td>1</td>
<td>284.98</td>
<td>0.75</td>
<td>0.3873</td>
</tr>
<tr>
<td></td>
<td>Virus dose</td>
<td>1</td>
<td>848.68</td>
<td>2.24</td>
<td>0.1361</td>
</tr>
<tr>
<td></td>
<td>Block</td>
<td>1</td>
<td>28858.03</td>
<td>75.65</td>
<td>&lt;0.0001***</td>
</tr>
</tbody>
</table>

### Table B3

**Maximum output of nominal logistic model for larval instar at death due to TnSNPV.** Non-significant terms were removed sequentially starting with the least significant interaction term, and the model was rerun after each term removal to establish the next least significant term.

<table>
<thead>
<tr>
<th>Response</th>
<th>Predictor variable</th>
<th>Degrees of Freedom</th>
<th>Chi-squared value</th>
<th>Pr(&gt;Chi)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Instar at death</td>
<td>Virus dose x bacteria dose</td>
<td>2</td>
<td>4.74</td>
<td>0.0936</td>
</tr>
<tr>
<td></td>
<td>Virus dose x fungi dose</td>
<td>2</td>
<td>5.79</td>
<td>0.0553</td>
</tr>
<tr>
<td></td>
<td>Fungi dose</td>
<td>2</td>
<td>0.50</td>
<td>0.7770</td>
</tr>
<tr>
<td></td>
<td>Block</td>
<td>1</td>
<td>0.17</td>
<td>0.6829</td>
</tr>
<tr>
<td></td>
<td>Weight at exposure</td>
<td>1</td>
<td>9.36</td>
<td>0.0022*</td>
</tr>
<tr>
<td></td>
<td>Bacteria dose</td>
<td>2</td>
<td>15.35</td>
<td>0.0005**</td>
</tr>
<tr>
<td></td>
<td>Virus dose</td>
<td>1</td>
<td>14.19</td>
<td>0.0002**</td>
</tr>
</tbody>
</table>
Table B4  Linear model output for maximal model of virus yield per cadaver by treatment. Each non-significant term that was removed is shown in sequence and the remaining marginally significant or fully significant terms are also reported.

<table>
<thead>
<tr>
<th>Response</th>
<th>Predictor variable</th>
<th>Degrees of Freedom</th>
<th>Sum of Squares</th>
<th>F Ratio</th>
<th>Prob &gt; F</th>
</tr>
</thead>
<tbody>
<tr>
<td>Virus yield – all virus doses</td>
<td>Virus dose x bacteria dose</td>
<td>2</td>
<td>0.72</td>
<td>1.84</td>
<td>0.1628</td>
</tr>
<tr>
<td></td>
<td>Virus dose x fungi dose</td>
<td>2</td>
<td>0.60</td>
<td>1.51</td>
<td>0.2239</td>
</tr>
<tr>
<td></td>
<td>Fungi dose</td>
<td>2</td>
<td>0.32</td>
<td>0.80</td>
<td>0.4504</td>
</tr>
<tr>
<td></td>
<td>Virus dose</td>
<td>1</td>
<td>0.96</td>
<td>4.86</td>
<td>0.0288*</td>
</tr>
<tr>
<td></td>
<td>Bacteria dose</td>
<td>2</td>
<td>2.40</td>
<td>6.08</td>
<td>0.0028**</td>
</tr>
<tr>
<td></td>
<td>Block</td>
<td>1</td>
<td>1.05</td>
<td>5.33</td>
<td>0.0222*</td>
</tr>
<tr>
<td>Virus efficiency – all virus doses</td>
<td>Virus dose x bacteria dose</td>
<td>2</td>
<td>0.06</td>
<td>0.36</td>
<td>0.6994</td>
</tr>
<tr>
<td></td>
<td>Virus dose x fungi dose</td>
<td>2</td>
<td>0.12</td>
<td>0.73</td>
<td>0.4854</td>
</tr>
<tr>
<td></td>
<td>Fungi dose</td>
<td>2</td>
<td>0.28</td>
<td>1.73</td>
<td>0.1811</td>
</tr>
<tr>
<td></td>
<td>Virus dose</td>
<td>1</td>
<td>0.14</td>
<td>1.71</td>
<td>0.1924</td>
</tr>
<tr>
<td></td>
<td>Bacteria dose</td>
<td>2</td>
<td>0.95</td>
<td>5.44</td>
<td>0.0052**</td>
</tr>
<tr>
<td></td>
<td>Block</td>
<td>1</td>
<td>0.84</td>
<td>10.09</td>
<td>0.0018**</td>
</tr>
</tbody>
</table>

Table B5  Regression analysis for virus yield by time to death (hours).

| Term       | Coefficient | Standard Error | t Ratio | Prob > |t| |
|------------|-------------|----------------|---------|--------|-----|
| Time to death | 0.007      | 0.002          | 4.39    | <0.0001*** |
Figure B1  Time to death in hours of *T. ni* larvae killed by baculovirus across different treatments. Larvae exposed to a low dose of virus are in the left panel and those exposed to a high virus dose are in the right panel. Virus alone treatments are designated by black bars, and co-exposed treatments are in light grey for bacteria or dark grey for fungi treatments.