The Evolutionary Ecology of Antiviral Resistance in Insects

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Abstract

Infectious disease is ubiquitous and potentially devastating to individual hosts and populations as a whole. Understanding host resistance is therefore a key challenge. More specifically, understanding insect-virus interactions is crucial due to the role of insects as vectors of harmful human viruses, the potential role of insect viruses in the biological control of insect pest species and the impact which viruses have on beneficial insects such as the honey bee. Despite its importance, our understanding of resistance against viruses in insects and other invertebrates is less comprehensive than our understanding of resistance to bacterial and fungal parasites.

In this thesis I investigate the resistance of the Lepidopteron host *Plodia interpunctella* to its natural viral parasite *P. interpunctella* Granulosis Virus (PiGV). I focus on two forms of antiviral resistance: (a) upregulation of an individual host's (or their offspring's) defences following previous exposure to a parasite, referred to as 'immune priming' and (b) host resistance following long term selection pressure from a parasite, referred to as 'evolved resistance'. I examine these forms of resistance from an evolutionary and ecological perspective focusing on their associated costs and specificity.

I find evidence for immune priming to virus for the first time in an insect but highlight that this form of resistance may carry costs and be context dependent in *P. interpunctella.* Using a mathematical modelling approach I also show that immune priming is likely to destabilise host populations. In addition, I show that antiviral resistance in *P. interpunctella* resulting from long term selection pressure with PiGV is non specific and localised in the gut. Furthermore, I find that resistance may be traded-off with developmental traits but that the detection of these trade-offs is dependent on the food quality on which *P. interpunctella* are raised.

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Chapter 1: General Introduction

Parasites are ubiquitous in nature and represent the largest and most diverse group of living organisms (Schmid-Hempel 2012). By definition, parasites cause harm to their hosts with significantly reduced fitness and survival in parasitised hosts widely documented (Schmid-Hempel 2012). The consequences of parasitism are seen at both the individual and population level and play an important role in shaping the ecological and evolutionary dynamics of natural host populations (Altizer et al. 2003; Anderson & May 1981; Hudson et al. 1998).

The term parasite is now often used broadly to include both macroparasites and microparasites (Anderson & May 1979). Macroparasites are typically large enough to be seen with the naked eye, often require multiple hosts to complete their lifecycle, grow rather than replicate within their hosts and transmit via the release of specialised infective stages. The term macroparasite is most commonly associated with helminth worms such as nematodes, cestodes and trematodes. In contrast microparasites are much smaller, replicating within their hosts and directly transmitting between hosts. Microparsites include viruses, bacteria, fungi and protozoa and may also be referred to as pathogens (Schmid-Hempel 2012).

In response to the strong selective force of parasites, hosts have evolved a diverse array of defences including avoidance behaviours, physical and chemical barriers and a complex immune system. These defences act to reduce the risk of parasite exposure, minimize parasite entry and establishment, and destroy and clear parasites. The defence system of vertebrates is well studied, its hallmark being the adaptive or acquired immune response (Murphy et al. 2011). While the understanding of invertebrate antiparasitic defence is less comprehensive, its study has increased

markedly over recent years (Rolff & Reynolds 2009; Schmid-Hempel 2005a; Schmid-Hempel 2012; Siva-Jothy et al. 2005). In particular, the immune response of the fruit fly, *Drosophila melanogaster*, to injected bacterial parasites is well explored (Govind 2008; Hoffmann 2003). However, we still lack a detailed knowledge and understanding of defences and immune components integral to antiviral defence in insects (Imler & Elftherianos 2009; Kemp & Imler 2009).

A greater understanding of insect-virus interactions is essential for many areas of research. Firstly, insects act as vectors of many human viruses including Dengue Virus and West Nile Virus (Hemingway & Ranson 2000; Turell et al. 2005). In addition, viral infections continue to severely reduce populations of beneficial insects such as the honey bee, *Apis mellifera* (Cox-Foster et al. 2007) and the silkworm *Bombyx mori* (Ponnuvel et al. 2003). Their potential to devastate insect populations has also lead insect viruses to be used in the biological control of insect pest species which damage crops causing extensive economic damage world wide (Alexandre et al. 2010; Tinsley 1977).

With the importance of viruses and their insect hosts clear, the mechanisms by which insects defend themselves against viruses are becoming more widely investigated. The vast majority of research on the insect antiviral response has been carried out within two insect orders: Diptera and Lepidoptera. Within the dipterans, the most commonly studied species are fruit flies and mosquitoes. The fruit fly, *D. melanogaster*, is generally used as an insect model of the immune system (Tzou et al. 2002) given the wealth of biochemical and genetic information available. Mosquitoes have also been well studied given their role as vectors of many harmful human diseases (Hemingway & Ranson 2000). However a number of species in the order Lepidoptera have also become models for the study of host-virus interactions due to their numerous naturally occurring viral parasites and the ease with which their natural environment

can be mimicked in the laboratory (e.g.Boots & Begon 1993; Levy et al. 2011; Ponnuvel et al. 2003; Saejeng et al. 2010; Terenius 2008; Trudeau et al. 2001). From these insect model systems we are beginning to understand the complexity and often lack of generality in defence against viruses in insects.

In this introduction I start with a review of the mechanisms of insect antiviral defences focusing mainly on examples from Lepidopteron insect hosts and their defences against DNA baculoviruses. These studies give us an insight into the types of mechanisms that may underpin the phenomenon that I examine in the rest of the thesis. The main focus of this thesis is the evolution and ecology of resistance to virus. Next, I therefore introduce the concept of host resistance and outline the two distinct types of resistance that I examine: 1. the upregulation of an individual host's (or their offsprings) defences following previous exposure to a parasite, referred to as immune priming and 2. host resistance following long term selection pressure from a parasite, referred to as evolved resistance. In addition, I briefly describe the two aspects of antiviral resistance on which I focus: the specificity and the costs. This is followed by an introduction to the empirical model system that I use: The Indian meal moth, *Plodia interpunctella* and the baculoviruses: *Plodia interpunctella* Granulosis Virus (PiGV) and *Ephestia cautella* Nucleopolyhedrosis Virus (EcNPV). I then briefly introduce the mathematical modelling approaches that I use in chapter 4 of this thesis. I finish with a description of the overall aims of the thesis and a brief summary of each chapter.

1.1 Mechanisms of Insect Antiviral immunity

1.1.1 Recognition and immune activation pathways

Recognition of specific extracellular pathogens and activation of appropriate effector systems relies on pathogen recognition proteins, which can recognise non-self based on

pathogen associated molecular patterns (PAMPS) (Schmid-Hempel 2005a). A well studied pathogen-recognition protein is peptidoglycan-recognition protein which activates a number of immune effector systems, including phenoloxidase (PO) and phagocyctosis, on recognition of PAMPS of bacteria (Siva-Jothy et al. 2005). As recognition of a pathogen by the host relies on the detection of a non-self surface, viral pathogens pose more of a challenge to the host given that they undergo much of their lifecycle within a host cell. Although the mechanisms which insects use to identify viral infection are poorly understood, it is possible that they rely on general cell disruption as an indicator of viral infection rather than a specific pathogen cell surface cue (Beckage 2008).

Hemolin, an immunoglobulin-like protein found in Lepidoptera, is strongly induced by bacterial challenge (Ladendorff & Kanost 1991; Yu & Kanost 2002), but has also been proposed to function in antiviral defence (Terenius 2008). The specific role of hemolin in antiviral immune function is not fully understood but several hypotheses have been investigated. Hemolin has general homophillic binding properties and a specific ability to bind to haemocytes (Bettencourt et al. 1997) which suggests that hemolin functions in viral recognition and could aid phagocytosis by functioning as an opsonin (Terenius 2008). Perhaps the most promising evidence for the involvement of hemolin in antiviral defence in Lepidoptera is that in the Chinese oak silkmoth, *Antherea pernyi*, both infection with its NPV (ApNPV) and exposure to double stranded RNA (dsRNA) of this virus resulted in enhanced hemolin expression (Hirai et al. 2004).

Signaling pathways are vital to the execution of an efficient immune response. Little is known about the cell signaling pathways involved in antiviral response and much of the work on cell signaling pathways has been conducted using the *Drosophila* model system. The Toll pathway, mainly associated with defence against $Gram^+$ bacteria, was involved in the response of *D. melanogaster* to *Drosophila* X Virus

(DXV) (Zambon et al. 2005). This pathway was also shown to be important in the response of the mosquito, *Aedes aegypti*, to Dengue Virus (Xi et al. 2008). However, the Imd pathway, involved in the activation of immune defence against Gram$^-$ bacteria has also been implicated in the response of *D. melanogaster* to virus (Costa et al. 2009).

1.1.2 Cellular immunity

The cellular immune response is a key component of the insect immune system and involves the direct interaction between haemoytes and the invading parasite. Immune functions of haemocytes include phagocytosis, encapsulation and clotting (Lavine & Strand 2002). However, the role of haemocytes in the antiviral response remains questionable. In the Lepidopteron hosts, *Helicoverpa zea* (corn earworm) and *Manduca sexta* (tobacco hornworm) and *P. interpunctella*, haemocytes have been found to directly clear cells infected with virus via encapsulation (Begon et al. 1993; Trudeau et al. 2001; Washburn et al. 1996). However, *Autographa californica* multiple NPV (AcMNPV) can infect haemocytes of the cabbage looper, *Trichoplusia ni*, and thus contribute to the replication and spread of the virus while also reducing the collective capacity of these cells to combat viral infection (Barrett et al. 1998)

1.1.3 Humoral immunity

Humoral immunity in insects is characterized by the production of antimicrobial peptides and the phenoloxidase (PO) cascade. The role of PO in antiviral immunity has been the subject of a number of studies. PO is a copper-containing compound, produced via the activation of proPO, which generates melanin via the conversion of phenols to quinines (Soderhall & Cerenius 1998). Melanin is important in many insect processes such as cuticular screlotization and has also been reported to play a central role in insect immune defence. A number of studies have suggested that PO represents an antiviral

defence mechanism in Lepidoptera (Ourth & Renis 1993; Popham et al. 2004; Shelby & Popham 2006). For example, Popham et al. (2004) suggested that reduced infectivity of *H. zea* NPV (HzNPV) when incubated in *Heliothis virescens* (tobacco budworm) plasma was due to the action of PO and PO from *H. virescens* plasma promoted antiviral activity against a number of vertebrate viruses *in vitro* (Ourth & Renis 1993). In contrast however, a recent study demonstrated that PO may not have a direct role in antiviral defence in Lepidopteron hosts. PO levels in *P. interpunctella* larvae exposed to PiGV through oral inoculation and intrahaemocoelic injection were not significantly different to those found in control larvae during the infection time course and had no effect on susceptibility to this virus (Saejeng et al. 2010).

1.1.4 Intracellular Processes

Apoptosis: Insect cells may respond to cell disruption that is typical of intracellular pathogen attack by undergoing apoptosis, or programmed cell death. By killing infected cells, this process terminates viral replication and decreases the yield of viral progeny (Clarke & Clem 2003b). There is great support for apoptosis as an important form of antiviral defence in Lepidoptera. The evolution of anti-apoptotic viral genes in baculoviruses (Clem et al. 1996) indicates that the effect of apoptosis is strong enough to select for counter mechanisms. Much of the studies investigating the role of apoptosis in antiviral defence has focused on the AcMNPV mutant, a baculovirus which lacks the p35 gene (Clarke & Clem 2003a). The p35 gene actively blocks apoptosis by binding to proteases, called capases, that function as the main executers of apoptosis (Clarke & Clem 2003a). In support for the role of apoptosis in antiviral defence virus production was significantly reduced following inoculation of cells with the AcMNPV mutant lacking p35 compared to the wildtype form of this virus (Clem & Miller 1993; Hershberger et al. 1992).

RNA interference (RNAi): RNA interference (RNAi), a process found in all organisms, regulates gene expression by halting transcription of a specific gene during the translation stage of its expression. It is also thought to play a role in antiviral defence across invertebrates and plants (Cherry & Silverman 2006; Settles & Friesen 2008). The immune function of RNAi is triggered by viral replication and the associated accumulation of dsRNA. This mechanism of antiviral defence is completely independent of cellular and humoral effector mechanisms as it relies on nucleic acid base pairing between siRNA and target RNA not peptide recognition (Saumet & Lecellier 2006). RNAi is considered the main defence mechanism in *D. melanogaster* against RNA virus as well as in many species of plants and nematodes (Kemp & Imler 2009; Saumet & Lecellier 2006; Wang et al. 2006). Support for the role of RNAi as an antiviral defence mechanism in *Drosophila* includes enhanced susceptibility of *Drosophila* mutants lacking Dicer-2, an enzyme necessary for the RNAi process, to three different RNA viruses (Galiana-Arnoux et al. 2006) and the presence of an RNAi suppressor in *Drosophila* C Virus (DCV) (van Rij et al. 2006). The importance of RNAi in *Drosophila* is also emphasised by the fact that genes involved in the RNAi process are among the fastest evolving genes in the genome, which suggests they are under strong selection pressure (Obbard et al. 2006). However, the role of RNAi in defence against DNA viruses is not well explored. It is possible that insect DNA viruses such as baculoviruses can produce dsRNA necessary to activate the RNAi process as plant DNA viruses do (Ding & Voinnet 2007; Obbard et al. 2009) but the role of RNAi in Lepidopteron host defence against baculoviruses in insects requires investigation.

1.1.5 Midgut-based defence

Insect viruses, along with bacteria and protozoa are typically ingested and enter the host tissue via midgut cells, thus defence strategies in this area are crucial in preventing the

establishment and spread of infection. The inhospitable environment of the midgut likely represents the first line of antiviral defence. Here the virus is faced with a high pH (Keating et al. 1990) and a diverse array of digestive enzymes including proteases and lipases (Nakazawa et al. 2004; Ponnuvel et al. 2003). For example, a lipase enzyme isolated from the digestive juices of the silkworm, *B. mori* was found to actively suppress infectivity of occluded *B. mori* NPV (BmNPV) and at high concentrations, inhibit viral propagation completely (Ponnuvel et al. 2003).

The peritrophic membrane and basement membrane, which line the midgut epithelial cells, provide barriers inhibiting the establishment and spread of viral infection. The peritrophic membrane, composed of sugars, proteins and chitin, overlays the midgut epithelial cells and therefore prevents contact between these cells and the contents of the gut (Levy et al. 2011). It is porous and allows the transfer of digestive fluid and nutrients between the midgut and the cells, but simultaneously prevents the passage of pathogens. In support for the role of the peritrophic membrane in antiviral defence, velvetbean moth, *Anticarsia gemmatalis*, larvae resistant to *A. gemmatalis* multicapsid NPV (AgMNPV) were found to have a thicker stronger membrane compared to susceptible strains of *A. gemmatalis* (Levy et al. 2011).

The basement membrane (also referred to as the basal lamina) is a proteinaceous membrane which surrounds all insect tissue, including the midgut. This membrane may also act as a barrier to viruses. However it is clear that baculoviruses can overcome this membrane barrier by passing through it at sites of weakness or damage (Granados & Lawler 1981) or bypassing the membrane using the host tracheal system as a conduit (Engelhard et al. 1994).

1.2 The Evolutionary Ecology of Antiviral Resistance

So, although our understanding of insect defences against viruses is less comprehensive than our understanding of insect responses to other parasites, we are beginning to appreciate the diversity and complexity of antiviral defence components. However, as important as the understanding of individual antiviral defence components and mechanisms, is the understanding of host resistance to virus. The term resistance is often used broadly to include any host strategy that results in a reduced probability of host infection, a reduction in parasite replication within the host or an accelerated clearance of the parasite (Boots et al. 2009). Fundamentally resistance mechanisms improve host fitness but reduce parasite fitness. Antiviral resistance therefore refers to the functional output of the action of potentially multiple and often interacting antiviral defence components. The nature of host resistance to a virus will shape the outcome of host-virus interactions and in turn the population level impact of the virus. In this thesis I approach the study of insect antiviral resistance from an ecological and evolutionary perspective. In particular, I examine two forms of resistance: 1. the upregulation of an individual host's (or their offspring's) defences following previous exposure to a parasite, referred to as immune priming and 2. host resistance following long term selection pressure from a parasite, referred to as evolved resistance and the associated costs of resistance and the specificity of protection which they provide. I introduce these themes here and also provide more detailed information within the relevant thesis chapters.

1.2.1 Immune priming and evolved antiviral resistance

Traditionally, due to the absence of immune cells necessary for the vertebrate adaptive or acquired immune response, defences of invertebrates were thought to lack any

capacity for memory. However, increasingly, examples in invertebrates where exposure to immune elicitors and parasites increase resistance to parasite infection later in life (within generation priming) (e.g. Moret & Siva-Jothy 2003; Roth et al. 2009; Schmid-Hempel 2005b) and in offspring (transgenerational immune priming) have been documented (e.g. Little et al. 2003; Moret 2006; Sadd et al. 2005). This phenomenon, while functionally similar to vertebrate adaptive immunity is mechanistically distinct and termed 'immune priming'.

The occurrence of immune priming illustrates that insects may be capable of adapting their defences (within their genetic constraints) in response to their current environment. The generality of immune priming across insect species and in response to different parasites is not yet fully understood. Immune priming protection following exposure to bacterial parasites or bacteria derived immune elicitors are widely documented in the literature (e.g. Little et al. 2003; Moret 2006; Moret & Siva-Jothy 2003; Roth et al. 2009; Sadd & Schmid-Hempel 2006). The only evidence for within generation immune priming to virus in invertebrates involves increased protection against White Spot Syndrome Virus (WSSV) following previous exposure to virus proteins in the crustaceans *Penaeus monodon and Penaeus japonicus* (Witteveldt et al. 2004; Wu et al. 2002). The occurrence of within generation immune priming and transgenerational immune priming to virus in insects has not been explored prior to this thesis.

The occurrence of immune priming illustrates that insects may be capable of adapting their defences in response to their current environment. Host resistance may also arise in response to parasite selection over multiple generations. The evolution of resistance in response to parasite selection pressure is documented for natural invertebrate host populations (Duncan & Little 2007; Hasu et al. 2009) and in the

laboratory (Boots & Begon 1993; Fellowes et al. 1999b; Fuxa et al. 1988; Milks & Myers 2000).

Host resistance to parasites, resulting from immune priming or long term selection will clearly impact host-parasite interactions and communities. However, costs associated with these forms of resistance and their effectiveness against multiple parasites strains, i.e. their specificity, will ultimately determine their wider implications and consequence for host parasite population dynamics.

1.2.2 Specificity of resistance

Specific immune defence is a trait strongly associated with vertebrates and the antigen specific binding properties of antibodies (Murphy et al. 2011). Despite the lack of obvious mechanism, specificity in invertebrate resistance to parasite has been investigated. Specificity in invertebrate resistance can be thought of as the effectiveness of host resistance to one parasite against other parasite strains or types. Specificity has been found in immune priming protection with for example, strain specific protection following immune priming with bacteria shown in the woodlouse *Porcellio scaber* (Roth & Kurtz 2009). Also, *Daphnia magna* offspring from primed mothers were most protected against the strain of bacteria their mothers were primed with (Little et al. 2003). However, specificity in immune priming protection is not ubiquitous with previous exposure to lipopolysaccharide (LPS), a component of bacterial cell walls, providing increased protection against infection with fungus in the mealworm beetle *Tenebrio molitor* (Moret & Siva-Jothy 2003).

Specificity in resistance which results in host populations following long term selective forces from a parasite has also been investigated. For example, *Biomphalaria glabrata* snails selected for and showing resistance to particular strains of the trematode *Schistosoma mansoni*, to which it is an intermediate host, did not show increased

resistance to other strains of this trematode parasite thereby demonstrating the evolution of a parasite specific resistance (Webster & Woolhouse 1998). The specificity of *D. melanogaster* resistance to parasitoids has also been explored. For example, cross resistance to the generalist parasitoid *Asobara tabida* in *D. melanogaster* resistant to the more host specific parasitoid *Leptopilina boulardi* was found but no significant increase in resistance against *L. boulardi* was found in flies resistant to *Asobara tabida* (Fellowes et al. 1999b). This study therefore highlights that the effectiveness of defences against different parasites may be highly dependent on the specific parasite in question. An investigation into the effectiveness of antiviral resistance against multiple viruses further highlights the variation in cross resistance between different parasites. While cross resistance to *Pieris rapae* (small white butterfly) GV (PrGV) and *T. ni* GV (TnGV) in *T. ni* larvae selected for resistance to *T. ni* Single NPV (TnSNPV) was found, no increase in resistance to AcMNPV was found in these Lepidopteron insects (Milks & Myers 2003).

The specificity in antiviral defence has a number of important implications. In theory, newly emerging viral infections may impact host populations that show specific protection following immune priming and long term evolution with a native viral parasite to a greater extent than hosts which exhibit cross resistance to multiple parasite strains or types. Specificity in resistance may also have consequences for the evolution of parasite virulence (Gandon et al. 2001; Mackinnon et al. 2008). For example, a mostly resistant population will select for parasites capable of challenging host defences and may result in greater within-host parasite competition in the fewer susceptible hosts present (Alizon & van Baalen 2008; May & Nowak 1995). Specificity in immune defence is also crucial to the maintenance of genetic diversity in coevolving hosts and parasites by frequency dependent selection (Haldane 1949; Sadd & Schmid-Hempel 2009c). For example, specific host resistance to the most prevalent parasite results in

selection for a rare parasite species capable of evading the host's resistance mechanism, which in turn selects for rare hosts which are resistant to the now common parasite. It is clear therefore that a greater understanding of specificity in antiviral resistance is important for predicting the epidemiology of disease and the consequences of viral infection for host parasite population dynamics.

1.2.3 Costs of resistance

Costs may constrain resistance gained via immune priming and via long term selection (Schmid-Hempel 2003; Sheldon & Verhulst 1996; Stearns 1989). These costs may take a number of forms. Activation of defence may be energetically demanding limiting resources available for other fitness traits. For example, growth and fecundity was reduced in *D. melanogaster* females which had successfully activated a cellular immune response following parasitoid attack (Fellowes et al. 1999a). Upregulation of PO activity in the red flour beetle, *Tribolium castaneum* offspring from fathers primed with *Escherichia coli* has also been shown to reduce fecundity significantly (Roth et al. 2010). The action of immune components may also directly harm host tissue, a phenomenon known as immunopathology (Graham et al. 2005; Long & Boots 2011). For example, in *T. molitor* activation of the PO cascade resulted in melanisation of malpighian tubules (Sadd & Siva-Jothy 2006). In addition, hosts may also suffer costs as a consequence of genetic based changes resulting from the evolution of resistance. These genotype trade-offs result when genes linked to resistance traits have a negative effect on other host fitness traits, through antagonistic pleiotropy. For example, a genetic trade-off between trematode resistance and fecundity was found in snails (Webster & Woolhouse 1999) and genetic based trade-offs between resistance to virus and development and fecundity have been documented in Lepidopteron insect hosts (Boots & Begon 1993; Fuxa & Richter 1998). Environmental heterogeneity is an

important consideration in the study of costs associated with resistance. In some instances, costs associated with the evolution or the activation of resistance are only detected when hosts are in a poor environment. For example in *D. melanogaster*, the genetic based trade-off found between resistance to the bacterial parasite *Providencia rettgeri* and fecundity was only seen in insects fed a low protein diet (McKean et al. 2008).

Overall, costs shape the optimal investment in resistance gained via immune priming and may maintain genetic based variation in resistance in host populations. A greater understanding of costs associated with antiviral resistance is therefore crucial for predicting host-virus interactions and the evolution of host resistance to virus.

1.3 The Empirical Model System

1.3.1 The host

The Indian meal moth, *Plodia interpunctella,* is a small (approximately 1cm) moth belonging to the family Pyralidae. It is a considered a major pest of stored grain causing huge economic damage worldwide with larvae known to feed on grains, grain products, dried fruits, nuts and cereals (Mohandass et al. 2007). The ability to mimic their natural environment closely in the lab and their insect pest status means we have a great deal of information about the life history of this insect. *P. interpunctella's* lifecycle comprises an egg stage, five larval instar stages (distinguished by the size of the head capsule), a pupal stage and an adult stage and takes approximately 40 days on average (Bjornstad et al. 1998) but is dependent on the quality of resource on which it is maintained (Boots & Begon 1994) (figure 1.1). Adults do not eat and mated female adults can lay up to 300 eggs.

The *P. interpunctella* used in these experiments were maintained as a large outbred stock at The University of Sheffield for approximately 9 years. Insects were reared on a cereal based diet consisting of 50% Ready Brek ©, 30% bran and 20% rice, with 20g yeast, 0.2g sorbic acid, 0.2g methyl paraben, 25ml honey and 25ml glycerol added to 100g of cereal mix. When low quality food was required the resource was mixed with a given percentage of methyl cellulose, an inert bulking agent. Food was frozen before use to ensure there was no insect contamination. Insects were kept at 27°C in a 16:8 h light dark regime. Stock and experimental populations of this host were generated by placing 35 adults on 40g food in a 1 litre ventilated nalgene tub.

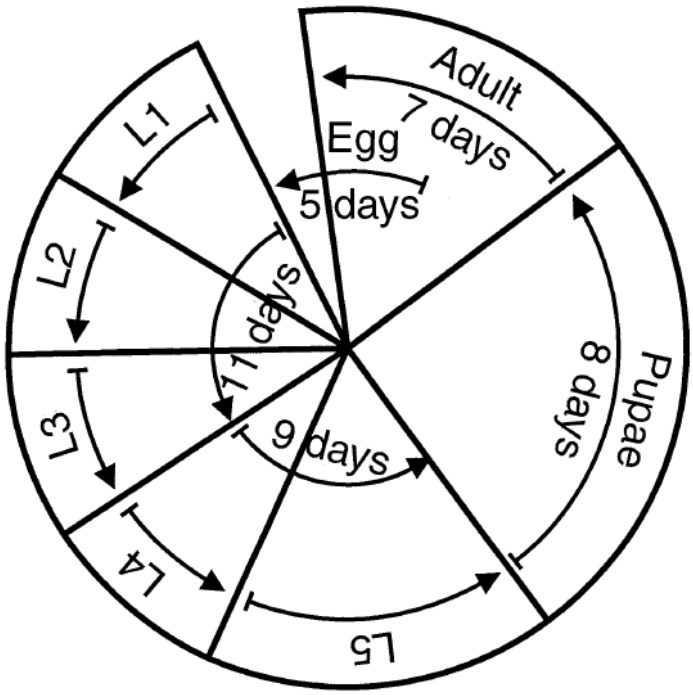
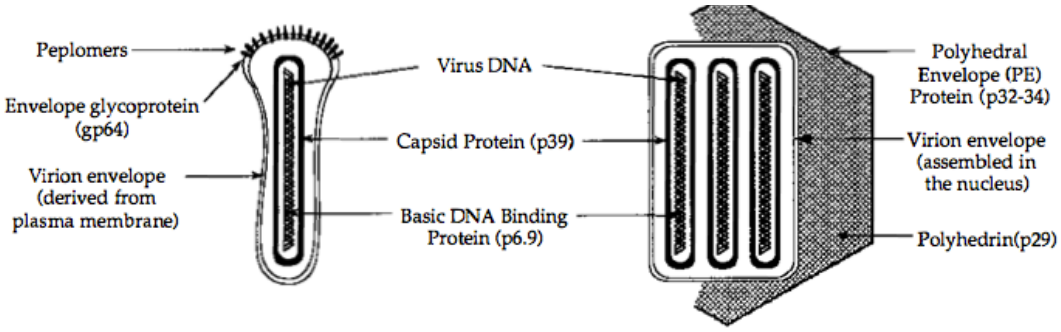


Figure 1.1. The life-cycle of the Indian Meal Moth. L1 – L5 refer to larval instars 1 to 5. (Taken from Bjornstad et al. (1998)).

1.3.2 The virus

Baculoviruses are large rod shaped double stranded DNA viruses. Most infect Lepidopteron hosts only, although some baculoviruses infect other insect orders including Hymenoptera and Coleoptera (Cory & Bishop 1997). Baculoviruses can be divided into two main subgroups; the Granulosis Viruses (GVs) which have only one virion (containing a single virus nucleocapsid) within the granulin envelope and the Nucleopolyhedrosis Viruses (NPVs) which have multiple virions (containing single or multiple virus nucleocapsids) within their polyhedral envelope.

P. interpunctella Ganulosis Virus (PiGV), as its name suggests, is a Granulosis Virus and is specific to the host *P. interpunctella*. The combination of *P. interpunctella* and PiGV therefore provides a system for examining a coevolved natural host-virus interaction. The interaction between *Ephestia cautella* Nucleopolyhedrosis Virus (EcNPV) and *P. interpunctella* is also investigated in chapter 3 and chapter 6. EcNPV naturally infects the Lepidopteron host *E. cautella*, a closely related moth that also belongs to the Pyralidae family. However, in the lab it is possible to infect



Budded Virus (BV) Occluded Virus (OCV)

Figure 1.2. The structural components of the budded and occluded phenotype of baculoviruses. The occluded virus here represents a multicapsid NPV and includes details of one of multiple virions which are present within the polyhedrin matrix. (Taken from Blissard & Rohrmann (1990)).

P. interpunctella with EcNPV (Hunter et al. 1973) and as such the combination of *P. interpunctella* and EcNPV is used to represent a novel host-virus interaction.

Baculoviruses have two phenotypes that are necessary for the completion of their lifecycle; the occluded virus and the budded virus (figure 1.2). The occluded virus consists of virions (either single for GVs or multiple for NPVs) encased within a protein matrix (which is composed of granulin for GVs or polyhedrin for NPVs). The occluded virus is the transmission form and is shed from the insect on death. Prior to ingestion by larvae and the initiation of the lifecycle, the protein coat of the occlusion virus protects the infectious virions from the environment making the occluded virus very stable. On ingestion, the protein coating of the occluded virus dissolves in the alkaline conditions of the midgut and the infectious virions are released. These virions then enter the midgut epithelial cells by fusing with epithelial villi. Once within the midgut cells, the nucleocapsids move to the nucleus where the viral DNA is uncoated and replication is initiated. The virus progeny produced are in the form of budded virus particles which cross through the nuclear and cytoplasmic membrane into the haemocoel. Infection of secondary tissue (including the fat body, haemocytes and tracheal matrix) by these budded virions then occurs. In latter stages of infection, occluded virus is produced in addition to budded virus. The occlusion body is formed when a protein coat crystalises around the single (in the case of GVs) or multiple (in the case of NPVs) virions. Occluded virus particles are not usually produced in the midgut cells. Occluded and budded virus differ both in their cell entry and their cell specificity. Occluded virus targets midgut cells and enters via fusion with the microvilli of the midgut epithelia while budded virus targets secondary tissue in the haemocoel entering these cells by endocytosis (Blissard & Rohrmann 1990).

PiGV infected *P. interpunctella* larvae have a characteristic opaque white colour and EcNPV infected *P. interpunctella* have a characteristic brown colouration. Infected

insects are therefore easily distinguishable from healthy individuals. Symptoms of PiGV infection in susceptible *P. interpunctella* occur 6-8 days post exposure and symptoms of EcNPV in susceptible *P. interpunctella* occur 12-14 days post exposure.

1.4 Mathematical modelling

In addition to empirical work, theoretical study, by means of mathematical modelling, has been vital to our understanding of host parasite interactions. Indeed epidemiology of infectious disease is one of the best developed areas of theoretical biology. The application of mathematical models to understand host parasite interactions was popularised by Roy Anderson and Robert May in the late 70s and early 80s (Anderson 1978; Anderson & May 1980; Anderson & May 1981; Anderson & May 1985) and remains vital for understanding the mechanisms that drive the spread of disease and for predicting the most successful measures for their control and eradication (Ferguson et al. 2001a; Ferguson et al. 2001b; Keeling et al. 2003).

Mathematical models aim to simplify complex processes into a series of mathematical equations. The aim is to gain general insights into how different processes may affect the population dynamics of the host or the parasite. Models are deliberately simplistic in order to give general predictions. A classical example of the use of mathematical modeling is in determining the simple criteria for the eradication of disease through vaccination (Greenhalgh 1992; Keeling et al. 2003). This has proven vital to the design of management strategies in the face of human and livestock disease.

Mathematical models can also make predictions of how different processes, such as host immunity and parasite transmission may influence the population dynamics of the disease and the host. Often we are interested in what processes lead to epidemics and how often epidemics are likely to occur. The classic and very successful example

of this is the detailed modeling of childhood disease that has shown that the different epidemic periods of diseases such as measles and whooping cough can be understood with simple Susceptible-Infectious-Recovered, referred to as SIR models, with term time forcing and stochasticity (Fine & Clarkson 1982; Keeling & Rohani 2008; Rohani et al. 1999) . In the thesis I build on this well developed theory and use these standard mathematical modelling techniques to examine the population dynamic implications of immune priming.

1.5 Aims of the thesis

The aim of this thesis was to gain a better understanding of the evolutionary ecology of antiviral resistance in insects using a natural insect host-virus model system and mathematical modeling. Specifically, my aims were to:

1. Investigate whether immune priming is a feature of antiviral resistance in insects and if so, whether protection gained from immune priming is specific and carries costs.
2. Explore the implications of immune priming for the dynamics of host-parasite populations using a mathematical model.
3. Determine whether evolution of antiviral resistance is associated with developmental costs and the influence of host food quality on detection of these costs.
4. Examine the specificity of evolved antiviral resistance.
5. Investigate whether antiviral resistance is affected by the entry route of the virus.

1.6 Thesis outline

The first part of my thesis focuses on immune priming. While immune priming to bacteria in insects is well documented, immune priming to virus has not been investigated. I use a natural insect-virus combination (*P. interpunctella* and PiGV), and prime insects with a very small amount of live virus via the natural transmission route. In chapter 2, I examine infection following viral challenge in insects previously primed with virus and in offspring from primed parents. In chapter 3, I build on chapter 2 and investigate whether immune priming protection is specific by challenging PiGV primed insects with EcNPV and PiGV later in life and offspring from PiGV primed parents with EcNPV and PiGV. In chapter 3 I also examine the development time and development weight of insects previously primed with PiGV in order to determine whether immune priming carries developmental costs. My final piece of work on immune priming is undertaken in chapter 4 and involves the development and analysis of a simple mathematical model to investigate the implications of immune priming for parasite prevalence, parasite persistence and host-parasite populations dynamics.

The second part of my thesis concerns the nature of evolved antiviral resistance. I produce *P. interpunctella* insect lines that show increased resistance to PiGV by using an artificial evolution experiment. In chapter 5, I assess development time and developmental weight in insect subjected to18 generations of selection and control insects. In order to determine whether food quality affects the cost of resistance I raise insects on both high and low quality food prior to developmental trait assay. To investigate the consequences of costs of resistance for insect populations, in chapter 5 I also assess the stability of resistance in insects in the absence of selection pressure. In chapter 6 I examine the specificity of evolved resistance by assessing infection following challenge with two different strains of PiGV and EcNPV. In chapter 7, my

focus is on investigating whether resistance is localised and if resistance to oral inoculation of virus also confers resistance to intrahaemocoelic injection. Finally, in chapter 8 I present a general discussion of my results and in light of my findings propose future avenues of research.

I also include details of empirical techniques and protocols that are used throughout the thesis and integral to my empirical work (Appendix 1). In addition, I include brief details of extension projects and collaborations from chapter 2 (Appendix 2) and chapter 4 (Appendix 3) and finally a publication of the work in chapter 2 (Appendix 4) and as detailed below.

Publications from this thesis:
Chapter 2: Tidbury, H. J., Pedersen, A. B. & Boots, M. 2011 Within and transgenerational immune priming in an insect to a DNA virus. *Proceedings of the Royal Society B-Biological Sciences* **278**, 871-876. (Appendix 4).

Submissions from this thesis:
Chapter 4: Tidbury, H. J., Best, A. & Boots, M. 2012 The Epidemiological Consequences of Immune Priming. *Proceedings of the Royal Society B-Biological Sciences*.
Awaiting decision following resubmission in response to initial review.

Additional work which has lead from this thesis:
Gene Expression profiling of immune primed *Plodia interpunctella* using De Novo Transcriptome Assembly in collaboration with Dr. S. McTaggart, University of Edinburgh. (Appendix 2).

The evolution of immune priming in invertebrate hosts in collaboration with Dr. Alex Best and Prof. M Boots, University of Exeter. (Appendix 3).

Chapter 2: Within and Transgenerational Immune Priming in an Insect to a DNA Virus

2.1 Abstract

Invertebrates mount a sophisticated immune response with the potential to exhibit a form of immune memory through 'priming'. Increased immune protection following early exposure to bacteria has been found both later in life (within generation priming) and in the next generation (transgenerational priming) in a number of invertebrates. However, it is unclear how general immune priming is and whether immune priming occurs in response to different parasites, including viruses. Here, using *Plodia interpunctella* (Lepidoptera) and its natural DNA virus, *Plodia interpunctella* Granulosis Virus (PiGV), I find evidence for both within generation and transgenerational immune priming. Individuals previously exposed to low doses of virus, as well as the offspring of exposed individuals, are subsequently less susceptible to viral challenge. Relatively little is known about the mechanisms that underpin viral immunity in insects but it is likely that the viral immune response is somewhat different to that of bacteria. I show that immune priming may however be a characteristic of host responses to both bacteria and virus, mediated through different mechanisms, suggesting that immune memory may be a general phenomenon of insect immunity. This is important because immune priming may influence host-parasite population dynamics.

2.2 Introduction

Parasites, broadly defined to include both macroparasites and microparasites such as bacteria and viruses, have pronounced effects on host fitness and life history and as a result shape host evolution (Anderson & May 1981; Boots & Begon 1993), population dynamics (Hudson et al. 1998; Pedersen & Greives 2008) and community structure

(Hatcher et al. 2006; Lafferty et al. 2006; Wood et al. 2007). Generally, it is assumed that there will be an optimal level of immune defence against parasites, determined by the associated costs of resistance and the risk of infection (Boots & Begon 1993; Hoang 2001; Kraaijeveld & Godfray 1997). In nature, hosts are faced with attack from a range of different parasites, but in many circumstances they may be more likely to be repeatedly exposed to the same parasites either within one generation or across consecutive generations. The likelihood of such future exposure to a parasite will clearly determine the cost to benefit balance of eliciting an immune response and influence the type, specificity, and length of the response.

The acquired immune system of vertebrates is well understood, and its primary role is to provide long lasting protection against parasitic infections (Murphy et al. 2011). In the early 80s, short term memory was found in the American cockroach, *Periplaneta americana*, following a series of cuticle transplant experiments (Lackie 1983), and more recently, examples in invertebrates where previous exposure to parasites has led to increased protection on subsequent challenge exist (e.g. Little et al. 2003; Moret & Siva-Jothy 2003; Pham et al. 2007; Roth et al. 2009; Sadd & Schmid-Hempel 2006). This increased protection against parasitic infection in invertebrates following an initial exposure to the same parasite, a different parasite or an immune response elicitor has been termed 'immune priming'.

In some cases this protection seems to be broad. For example, previous exposure to lipopolysaccharides (LPS), bacterial cell wall components, increased protection against a fungal parasite in the mealworm beetle, *Tenebrio molitor* (Moret & Siva-Jothy 2003). However, there are a number of cases where the protection provided by the initial exposure is more pronounced when the parasite is of the same taxonomic type, species or even strain (Pham et al. 2007; Roth et al. 2009; Sadd & Schmid-Hempel 2006). It is also increasingly apparent that exposure of mothers to parasites may

influence offspring resistance in invertebrates. For example, in the freshwater crustacean, *Daphnia magna*, offspring from mothers primed with the bacteria *Pasteuria ramosa* suffered less of a reduction in fitness, in terms of reproductive output, when subsequently infected with this bacteria compared to offspring from naïve mothers (Little et al. 2003). This protection was also found to be specific, such that offspring exposed to the same parasite strain as their mother had a greater fitness advantage than offspring exposed to a different parasite strain to their mother. In addition, in the cabbage semilooper, *Trichoplusia ni*, offspring from mothers that had been raised on a bacteria rich diet showed increased immune enzyme activity and increased expression of immune related proteins (Freitak et al. 2009). The phenomenon of transferring protection to parasites from mother to offspring in invertebrates is termed 'transgenerational immune priming'. While most studies focus on maternal transgenerational immune priming, there is now evidence that paternal transgenerational immune priming can occur (Roth et al. 2010) and in *T. molitor,* may provide protection by a different mechanism and for a shorter time period compared to maternal priming (Zanchi et al. 2011).

The interactions between insects and their bacterial and fungal parasites are becoming increasingly well understood, while our knowledge of insect-virus interactions remains much more limited (Imler & Elftherianos 2009; Strand 2008). Potential mechanisms of viral resistance in insects may include essential defence processes such as RNA interference (RNAi) (Kemp & Imler 2009; Saleh et al. 2009; Wang et al. 2006) and apoptosis (Clarke & Clem 2003b). Support for the role of RNAi as an antiviral defence mechanism in *Drosophila* includes the enhanced susceptibility of *Drosophila* mutants lacking Dicer-2, an enzyme necessary for the RNAi process, to three different RNA viruses (Galiana-Arnoux et al. 2006) and the presence of an RNAi suppressor in *Drosophila* C Virus (DCV) (van Rij et al. 2006). However, there is a lack

of generality and consistency in viral resistance mechanisms across insect taxa and there is debate as to whether the immune pathways and effectors which are responsible for clearing viral infections are similar (Imler & Elftherianos 2009; Sabatier et al. 2003; Xi et al. 2008; Zambon et al. 2005) or different (Dostert et al. 2005; Imler & Elftherianos 2009) to those important in the antibacterial response. For example, Toll, an immune pathway involved in defence against $Gram^+$ bacteria, is important in the response of *Drosophila melanogaster* to *Drosophila* X Virus (DXV) (Zambon et al. 2005) and *Aedes aegypti* to Dengue virus (Xi et al. 2008). In *D. melanogaster,* the Imd immune pathway, which is involved in the defence against $Gram^-$ bacteria, has also been shown to be involved in antiviral immune responses (Costa et al. 2009). However, haemolymph from *D. melanogaster* infected with DCV contained none of the molecules which are hallmark of the response to bacterial challenge (Sabatier et al. 2003). Given that there may be differences between antibacterial and antiviral immune mechanisms, it is unclear whether the immune priming that occurs in response to bacterial exposure will also occur in response to viral exposure in invertebrates. Evidence for within generation immune priming to White Spot Syndrome Virus (WSSV) in the crustaceans *Penaeus monodon and Penaeus japonicus* has been found (Witteveldt et al. 2004; Wu et al. 2002) but within generation and transgenerational immune priming to viruses in insects has not been examined in detail. A greater understanding of insect-virus interactions, and antiviral resistance in particular is not only important for the control of human viral diseases vectored by insects, including Dengue Fever and West Nile Virus which are vectored by mosquitoes (Hemingway & Ranson 2000; Turell et al. 2005) but also because insect viruses may be used in the biological control of insect pests (Alexandre et al. 2010; Tinsley 1977).

In this chapter I assess whether early exposure to virus leads to immune priming either within a generation or transgenerationally in an insect. I use the well developed

host-parasite laboratory model system, *Plodia interpunctella* (Lepidoptera) and its natural virus *Plodia interpunctella* Granulosis Virus (PiGV) (Baculovirus). In particular, I examine the effect of viral exposure in early life, and viral exposure in the previous generation on rates of infection following subsequent challenge with the virus. I demonstrate for the first time in insects, that previous exposure to a low dose of live virus increases resistance to a lethal challenge both later in life and in the next generation.

2.3 Materials and methods

2.3.1 The insect-virus system

The Indian meal moth, *P. interpunctella,* is a pest of stored agricultural products, with a natural environment that is very similar to the one in which it is maintained in the laboratory. Insects were reared on a cereal based diet consisting of 50% Ready Brek ©, 30% bran and 20% rice, with 20g yeast, 0.2g sorbic acid, 0.2g methyl paraben, 25ml honey and 25ml glycerol added to 100g of cereal mix and were kept at 27°C in a 16:8 h light dark regime. I used the naturally occurring DNA virus, PiGV that infects larvae through the oral ingestion of viral particles. When the virus occlusion bodies enter the midgut their protein coat is dissolved, and virions are released into the midgut cavity and enter midgut epithelium cells. Once the virus has passed through the midgut, virus proliferation in secondary tissue such as the fat body leads to cell lysis, tissue destruction and eventual host death. Infected individuals are a characteristic opaque white colour and easily distinguishable from healthy individuals. Once symptomatic, larvae die before pupation. Purified virus solution was produced by centrifugation of a homogenate of infected insect cadavers (Smith & Crook 1988, Appendix 1a, 1b) and diluted in 75% blue food dye in double distilled water with 5% sucrose to the required viral concentration. For virus priming inoculation and virus challenge inoculation,

droplets of virus/dye solution were orally administered to the larvae using a pipette following the standard droplet feeding method (Boots & Begon 1993, Appendix 1d). Only insects successfully inoculated (indicated by the presence of dye in half of the length of the gut) were used in the experiments. The same inoculation procedure was used for control larvae but using virus-free dye/sucrose solution. Prior to these experiments, I conducted several oral dose response bioassays to identify the dose response of *P. interpunctella* to PiGV. Specifically, I calculated the lethal dose of 1% (LD_1) for second and third instar larvae and the lethal dose of 50% (LD_{50}) for third and fourth instar larvae. Here, I used this LD_1 dose as the 'priming' dose and the LD_{50} as the subsequent 'challenge' dose.

2.3.2 Within generation immune priming to virus

Experimental insects were established by placing 30 newly emerged adults, from a large outbred stock population, onto 40g of food in ventilated 1litre Nalgene tubs. Adults were left to mate and lay eggs for 24 hours. Second instar larvae (8 days) were collected from the food and starved for two hours. Approximately 75 larvae were orally primed with virus solution of a LD_1 concentration while 75 larvae were inoculated with control solution. Successfully inoculated larvae were given abundant food resources and virus primed and control primed larvae were kept separate and monitored. When the larvae reached fourth instar (14 days) they were removed from the food and starved for two hours. No larvae showed viral symptoms at this stage. Approximately 25 virus primed larvae and 25 control primed larvae were inoculated with the LD_{50} virus challenge solution and to control for infection resulting from virus priming alone, some virus primed larvae were inoculated with a control, virus-free solution. In addition, to control for contamination of insect treatment groups, control primed larvae were inoculated with control, virus free solution. All inoculated larvae were then kept individually with

abundant food and examined for the presence of viral infection 7-8 days post challenge. The experiment was repeated 6 times, each on a different day and the number of infected and non-infected larvae was recorded for each treatment group. (Figure 2.1a).

2.3.3 Transgenerational immune priming to virus

Experimental insects were established from newly emerged adults taken from a large outbred stock. 30 adults were placed on 40g of food and left to mate and lay eggs for 24 hours. In total, six containers per block were established from the same large outbred stock. Third instar larvae (11 days) were taken from the food and starved for 2 hours. Individuals from three containers were inoculated with a LD_1 virus prime solution, while individuals from the remaining three containers were inoculated with control, virus-free dye solution. Approximately 200 successfully inoculated larvae from each container were transferred to separate clean containers with abundant food. Larvae were left to develop, pupate, and emerge as adults. The small number of larvae that became infected as a result of the priming inoculation (between 0.5 - 8.5% across replicates) were removed immediately on presentation of symptoms. 30 newly emerged adults were then transferred to separate clean containers with 40g fresh food and allowed to mate and lay eggs for 24 hours (F2 generation). Third instar larvae (11 days) from each F2 container were picked out from the food, starved for 2 hours and challenged with an LD_{50} virus challenge solution. Successfully inoculated larvae (~100 insects/treatment replicate) were kept individually with abundant food resource and examined for the presence of viral infection 7-8 days post viral challenge. The number of infected and non-infected larvae was recorded for each treatment group. F2 generation larvae from virus primed parents were orally inoculated with control solution and checked for symptoms to confirm that the virus did not pass vertically. This

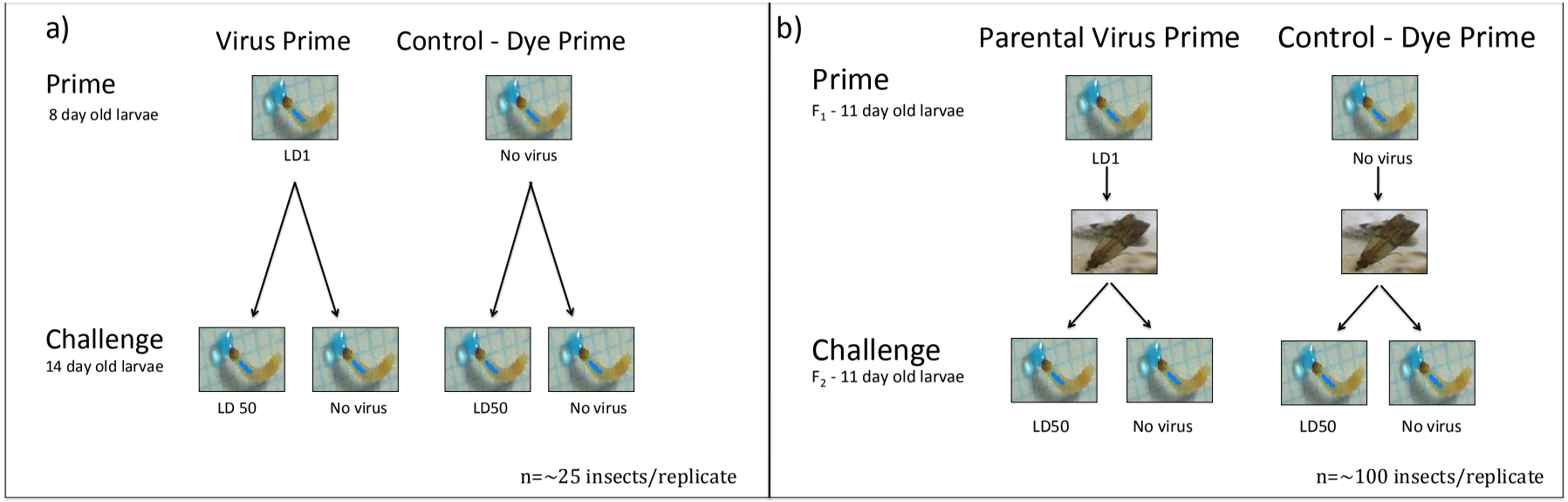

a)
Virus Prime
Control - Dye Prime
Prime
8 day old larvae
LD1
No virus
Challenge
14 day old larvae
LD 50
No virus
LD50
No virus
n=~25 insects/replicate
b)
Parental Virus Prime
Control - Dye Prime
Prime
F1 - 11 day old larvae
LD1
No virus
Challenge
F2 - 11 day old larvae
LD50
No virus
LD50
No virus
n=~100 insects/replicate

Figure 2.1

Figure 2.1. Experimental design of the (a) Within generation priming experiment and (b) Transgenerational priming experiment. In (a) second instar larvae (8 days) were collected from the food and starved. 75 larvae were orally primed with virus solution of concentration equivalent to LD_1 and 75 larvae were primed with control solution. When these larvae reached fourth instar (14 days) 25 were orally challenged with virus solution of concentration equivalent to LD_{50}. To determine the level of infection that resulted from prime inoculation some larvae primed with virus were challenged with control solution. This figure outlines the procedure for one experimental replicate. The experiment was repeated six times in total. In the transgenerational priming experiment (b), approximately 200 third instar (11days) F1 generation larvae were removed from six containers established from the same large outbred insect stock, kept separately and starved. Larvae from three containers were primed with LD_1 virus prime solution and larvae from the other three containers were primed with a control solution. These primed larvae were left to develop and make six F2 generation populations. The small number of larvae that became infected following the virus prime treatment were removed immediately once they showed symptoms. 100 third instar (11 days) F2 generation larvae from each container were then challenged with LD_{50} virus solution. In addition, F2 generation larvae from virus primed parents were orally inoculated with control, virus-free solution. This figure outlines the procedure for one treatment replicate. This study was carried out six times, each on a different day with three virus prime treatment replicates and three control treatment replicates per experimental replicate.

experiment was repeated six times, each on a different day. (Figure 2.1b).

To determine whether any effect of virus prime treatment lasted longer than one generation, infection in F3 generation insects was assessed. F3 generation insects were set up by placing 30 F2 adults from each container onto 40g virus free food. Third instar F3 larvae (11 days) were picked out from each container, starved for two hours and challenged with an LD_{50} virus solution. Successfully inoculated larvae (~100 insects/treatment replicate) were kept individually with abundant food resource and

examined for the presence of viral infection 7-8 days post viral challenge. The number of infected and non-infected larvae was recorded for each treatment group.

2.3.4 Statistical analysis

The within generation priming experiment was analysed separately to the transgenerational priming experiment. Firstly, for the within generational priming experiment, the effect of prime treatment (previous viral exposure early in life) on subsequent susceptibility to viral challenge (proportion infection) was analysed using a generalised linear model (GLM) in R (version 2.14). Quasibinomial errors were incorporated into the model given that the data were proportions which showed dispersion above which binomial errors account for. Experimental replicate (of which there were 6) was excluded from the final model as it was non-significant ($F_{5,6}$=0.176, p=0.96).

Similarly, for the trangenerational priming experiment, the effect of prime treatment (parental viral exposure) on offspring susceptibility to viral challenge (proportion infection) was analysed using a GLM. One experimental replicate was removed from the analysis as no F2 generation larvae were produced. Again, quasibinomial errors were incorporated into the model as the data showed overdispersion. In addition to prime treatment, the explanatory variables: experimental replicate (of which there were 6) and treatment replicate (of which there were 3), which was nested within experimental replicate, were incorporated into this model. A separate GLM was used to determine whether infection in F3 insects originating from control F1s differed significantly from infection in F3s originating from virus primed F1s. Treatment replicate was removed from this GLM as it was non-significant ($F_{11,16}$=1.14, p=0.39), leaving only the explanatory variables: experimental replicate and prime treatment in the final model.

2.4 Results

2.4.1 Within generation immune priming to virus

Previous exposure to a low dose of virus significantly reduced the susceptibility of insects to subsequent virus challenge ($F_{1,10}$=9.22, p=0.013, figure 2.2). Thus we provide evidence that priming with a low dose of virus confers lasting protection against virus challenge later in life. The number of infections resulting from priming dose varied between 2.7% and 18% across the 6 experimental replicates. Excluding the experimental replicate where 18% infection resulted from the priming dose did not change the result, with the effect of priming treatment becoming stronger ($F_{1,8}$=13.4, p=0.0064). The mean number of infections resulting from virus priming alone, with this replicate excluded was higher than expected at 3.6%. Mortality was negligible and no insects exposed to only control dye solution became infected, demonstrating that there was no contamination throughout the experiment.

2.4.2 Transgenerational immune priming to virus

Offspring from parents exposed to a low dose of virus were less susceptible to viral challenge when compared to offspring from parents exposed to control dye solution ($F_{1,27}$=25.8, p<0.001, figure 2.3). Exposing parents to virus confers protection in offspring challenged with the same virus, providing evidence for transgenerational immune priming. Proportion infection varied significantly between treatment replicates ($F_{11,16}$=3.75, p=0.0084) and experimental replicates ($F_{5,28}$=10.5, p<0.001). The number

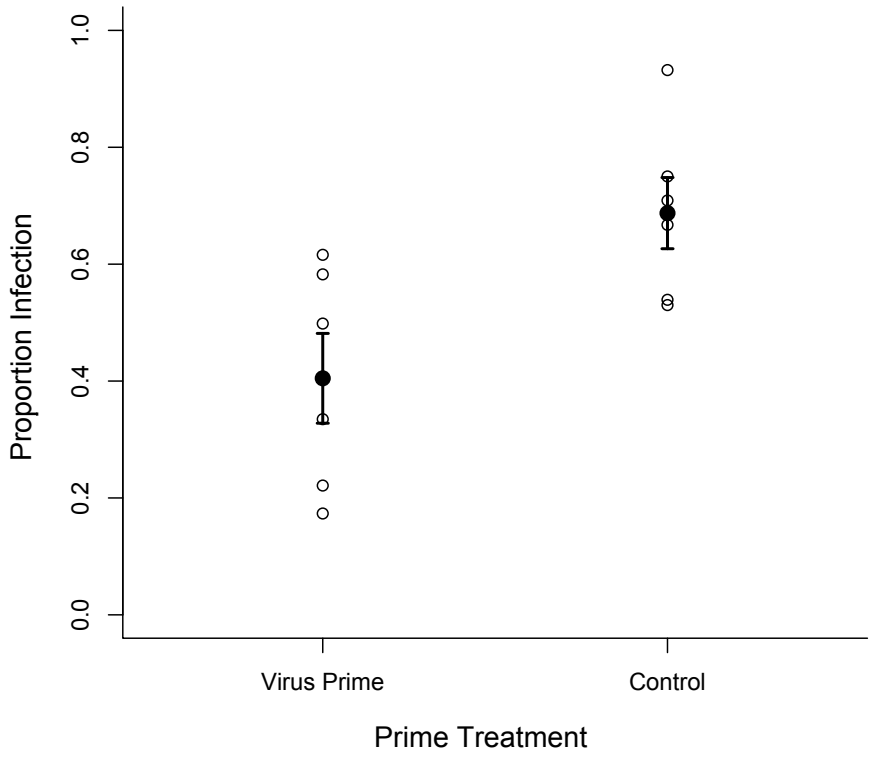


Figure 2.2. Within generation priming, by exposure to low level virus, significantly reduced the susceptibility of insects to a lethal virus challenge later in life ($F_{1,10}$=9.22, p=0.013). Open circles represent experimental replicates, filled circles represent means ± s.e.

of insects which became infected from the priming dose varied between 0.5% and 8.5% with a mean of 5.1% and was higher than expected.

I examined the F3 generation in order to rule out the possibility of selection for resistance and found that the transgenerational priming effect only lasted one generation. There was no significant difference in viral infection between F3 larvae originating from virus primed F1s and F3 larvae originating from control F1s ($F_{1,27}$=2.30, p=0.15)

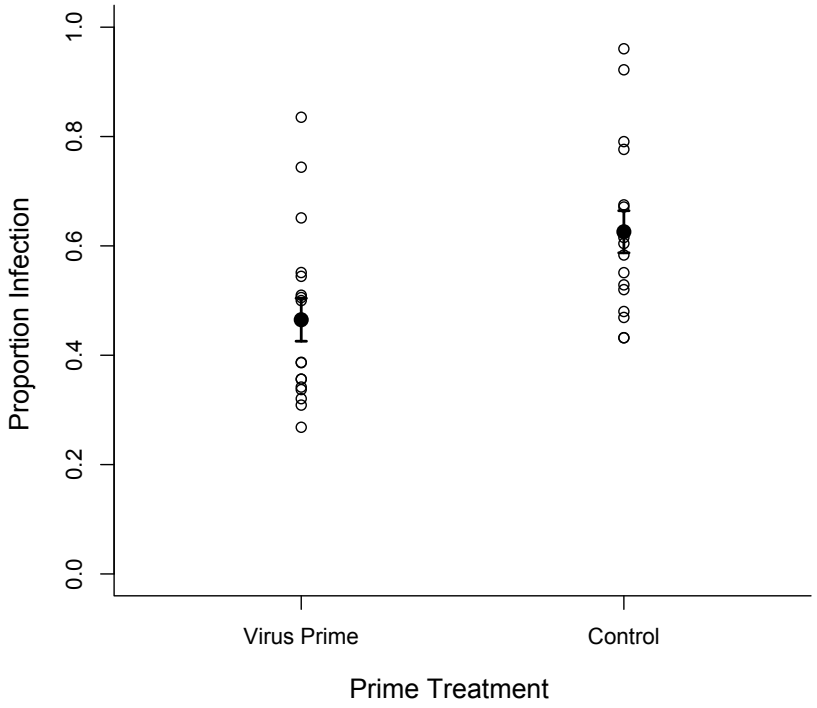


Figure 2.3. Transgenerational immune priming, by exposing parents to low level virus, significantly reduced the susceptibility of offspring to lethal virus challenge ($F_{1,27}$=25.8, p<0.001). Open circles represent experimental replicates, filled circles represent means ± s.e.

although infection in F3 insects did differ significantly between experimental replicates ($F_{5,28}$=19.8, p<0.001). This confirms that the reduction in infection seen in insects from primed parents was not a result of selection. Mortality was negligible and there was no vertical transmission and no virus contamination of control insects.

2.5 Discussion

I have demonstrated both within generation and transgenerational immune priming with a DNA virus in its natural insect host. Exposure to a low dose of virus (not leading to systemic infection) reduces subsequent susceptibility to a lethal viral challenge both

later in life and in the offspring of exposed parents. There have been a number of studies demonstrating immune priming to bacteria both within generation (e.g. Roth et al. 2009; Sadd & Schmid-Hempel 2006) and transgenerationally (Little et al. 2003; Moret 2006; Sadd & Schmid-Hempel 2006). However immune priming to viruses is not well studied with only very limited evidence for its existence in invertebrates (Witteveldt et al. 2004; Wu et al. 2002). This is the first study to investigate and report this phenomenon in response to a virus in an insect. The immune response of invertebrates to virus may be different to the immune response to other parasites but this study suggests that priming may be a general phenomenon of the invertebrate immune system.

Here, I used a natural host-virus combination, challenged the insects through the natural route of infection and used live, infectious virus to prime individuals. I exposed the hosts to a very low dose of virus through oral inoculation and subsequently challenged those primed hosts, or offspring of primed hosts, by oral inoculation with the same viral stock at a higher concentration. I therefore build on previous studies where immune priming has been found in response to heat killed pathogens (e.g. Roth et al. 2009) or immune elicitors (e.g. Moret & Siva-Jothy 2003). The obvious advantage of using heat killed pathogens or immune elicitors is that it ensures the absence of live pathogen, which may alter the immune response in the insect on subsequent pathogen challenge. It also means that there is little chance of selecting the host for increased immune function over a single generation. My approach however, directly examines a natural host-pathogen interaction by priming with live virus using the natural route of infection. The likelihood of live virus from the initial exposure still being present in the midgut of the primed insects is very minimal, given that two instars of development occur between the initial priming exposure and the subsequent challenge in the within generation study. In addition, no difference was found in susceptibility between F3

insects from virus primed F1s and F3 insects from control F1s, showing that the protection only lasted a single generation. Therefore it is unlikely that I selected for increased viral resistance in the transgenerational priming experiment. These results suggest that the reduced susceptibility to lethal viral challenge both within and across a generation is due to immune priming.

It is possible that the increase in resistance seen both within a generation and across a generation is a result of multiple components of the antiviral response, however the specific mechanisms that underpin immune priming in invertebrates are not understood. There is some evidence to suggest that phagocytosis by haemocytes may contribute to protection against bacteria gained from immune priming and the specificity of this protection. For example, increased survival and reduced bacterial load resulting from previous exposure to the same strain of bacteria was linked to an increase in phagocytic activity in *Drosophila* (Pham et al. 2007) and increased phagocytosis of bacteria which the woodlouse, *Porcellio scaber* was previously exposed to was found (Roth & Kurtz 2009). Although not fully understood, it has been suggested that the increase and specificity in phagocyte function following previous parasite encounters is mediated by differential pathogen recognition by receptors present on the surface of haemocytes. One potential receptor is the Down syndrome cell adhesion molecule (Dscam) (Neves et al. 2004; Schmucker & Chen 2009). The mechanism underlying transgenerational immune priming to bacteria is even more intriguing, and even less understood. Elevated antibacterial activity in offspring of the bumble bee, *Bombus terrestris,* following priming of queens was shown to be dependent on factors transferred to the offspring in the egg, and not based on rearing (Sadd & Schmid-Hempel 2007). In principle, exposure of mothers to parasites may speed up the production of immune components in offspring and/or increase the efficiency of immune components in offspring. In theory this could be the result of

transmission of immune proteins or small amounts of pathogen from parents to offspring but as yet there is little experimental evidence of the mechanisms that underlie the phenotypic responses that I measure.

The mechanisms involved in immune priming to viruses are even less well understood. It is possible that haemocytes are involved in immune priming to virus, as well as bacteria, given that haemocyte activity may play a role in clearing baculovirus in Lepidoptera (Trudeau et al. 2001) including specifically, PiGV in *P. interpunctella* (Begon et al. 1993). Clearly greater knowledge of the components that confer resistance to viruses in insects in general will give insight into the specific immune mechanisms, pathways or general defences that may lead to immune priming.

While many studies have found evidence for immune priming in invertebrates, it is not ubiquitous. For example, studies of mosquitoes have found that priming the melanisation response, an immune defence important in malarial parasite infections, did not increase the melanisation response in offspring (Voordouw et al. 2008) and in male damsel flies *Hetaerina americana*, previous exposure to bacteria did not increase protection against bacterial challenge later in life (Gonzalez-Tokman et al. 2010). Within generation and transgenerational priming is likely to be a plastic trait dependent on specific ecological and evolutionary conditions (Little & Kraaijeveld 2004). It may also be dependent on the host or pathogen life history and the specific host-pathogen combination. Immune priming has been demonstrated in *B. terrestris* (e.g. Sadd et al. 2005; Sadd & Schmid-Hempel 2006). This is a social insect and therefore immune priming may be more beneficial as repeated exposure to the same pathogen is very likely. In addition, the life history of the parasite will also be important. Here, the pathogen used is an obligate killer and therefore the cost of infection is high and may lead to strong selection pressure for resistance mechanisms in general.

Immune priming may be costly both at the individual and population level. Long lasting protection against one pathogen strain may result in selection for different strains of the parasite with different effects on the host (Kurtz 2004). Sadd and Schmid-Hempel (2009b) found that offspring were more resistant when exposed to the same parasite as their mothers, but that these same offspring had increased susceptibility to different pathogens. This suggests that priming may be specific and costly in terms of resistance to other parasites, and that there may be trade-offs between resistance to multiple pathogens. Further work on immune priming in invertebrates needs to examine both the costs and specificity of immune priming in more detail (chapter 3). It is also interesting that the specificity of immune priming seems to vary between host-pathogen combinations. For example, priming by LPS in *T. molitor* protects against fungal infection (Moret & Siva-Jothy 2003), while in other interactions protection is highly specific even to the level of pathogen strain (e.g. Little et al. 2003; Roth et al. 2009). This difference in specificity may indicate that priming protection is due to a range of different mechanisms that are not necessarily mutually exclusive.

Although mechanistically very different to vertebrate adaptive immunity, this work suggests that the insect innate immune system has the capacity to adapt in response to previous encounters with a virus. The fact that immune priming occurs in response to both viruses and bacteria in insects suggests that similar evolutionary pressures have shaped these responses, even though they may involve different components of the immune system. Immune priming may have many wider implications, such as altering the dynamics of host and pathogen populations (Tate and Rudolf (2011), chapter 4). It may also be important when considering the long term success of using viral pathogens as biological control agents and when predicting the severity of viral disease outbreak.

Chapter 3: The Specificity and Developmental Costs of Immune Priming in an Insect to a DNA Virus

3.1 Abstract

Despite invertebrates lacking components fundamental to the vertebrate adaptive immune response, increasing evidence suggests that the immune defences of invertebrates may be 'primed' by previous parasite exposure (within generation priming) and parental parasite exposure (transgenerational priming), providing increased protection on subsequent challenge. However, there is still little understanding of the nature of immune priming, in particular the specificity of immune priming protection and the costs which immune primed hosts may incur. In this study, I use *Plodia interpunctella*, a Lepidopteron host in which immune priming to virus has been demonstrated, to investigate the specificity of within generation and transgenerational immune priming to virus. I orally prime *P. interpunctella* with its natural viral pathogen *P. interpunctella* Granulosis Virus (PiGV) and then challenge primed insects later in life or offspring of primed insects with PiGV or a distinct baculovirus from a related host species, *Ephestia cautella* Nucloepolyhedrosis Virus (EcNPV). To investigate the costs of within generation immune priming I examine the development time and development weight of *P. interpunctella* previously primed with PiGV compared to control insects. In contrast to previous work I find no evidence for increased protection against virus (within a generation or transgenerationally) following immune priming in this study and therefore gain no insight into the specificity of immune priming to virus. However, I find that insects primed with virus take significantly longer to reach maturity compared to control insects suggesting that a cost of immune priming in *P. interpunctella* may be increased development time. This study highlights the complexity of the insect antiviral response and the variability in immune

priming in this insect-virus system and emphasises the need to further study the interactions between insects and viruses and the likelihood of immune priming.

3.2 Introduction

Parasites, broadly defined to include both macroparasites, such as helminths and microparasites (also called pathogens), such as viruses and bacteria, are ubiquitous in nature. As a consequence hosts have evolved a range of antiparasitic defences. These include avoidance behaviours, which act to reduce the risk of exposure to a parasite. For example, sheep have been shown to avoid grazing on highly nutritious long grass which is more likely to be contaminated with nematode larvae (Hutchings et al. 2001; Hutchings et al. 2002) and social insects defecate away from their main habitat improving colony hygiene and reducing the risk of infection (Weiss 2006; Wilson-Rich et al. 2009). Parasite defences also include physiological and chemical barriers which function to inhibit the entry and establishment of parasite infection. For example, the peritrophic membrane, which separates the midgut epithelial cells from the gut lumen, may prevent the entry of micoparasites into host cells (Levy et al. 2011) and in leaf cutting ants, the antimicrobial secretions produced by the metapleural gland may provide a chemical barrier against the soil inhabiting fungus *Metarhizium anisopliae* (Poulsen et al. 2002). Another important aspect of antiparasitic defence is the highly complex, dynamic immune system. The immune system of vertebrates is very well studied (Murphy et al. 2011) whereas the immune system of invertebrates is less well understood (Rolff & Reynolds 2009). The hallmark of the vertebrate immune system is adaptive, or acquired, immunity. Acquired immunity relies on the production of memory cells, in addition to specific antibodies by B lymphocytes, during the primary response to an invading pathogen. These memory cells are stable and long lasting and enable rapid production of specific antibodies if the same pathogen is subsequently

encountered (Murphy et al. 2011). This arm of the vertebrate immune system therefore functions to provide long-lived, specific immunity to pathogens previously encountered. This acquired immune response is not only vital to an individual's survival but has been fundamental to the eradication of many devastating vertebrate infectious diseases, with its artificial activation via vaccination providing life time protection against targeted pathogens (Ada 2007; Kieny & Girard 2005).

Invertebrates do not have lymphocytes and do not show an antibody-based immune response. They therefore lack the mechanistic components fundamental to the vertebrate adaptive immune response. However, it is becoming increasingly evident that invertebrates can show a form of memory in their immune response, a phenomenon termed 'immune priming' (Little & Kraaijeveld 2004; Sadd & Schmid-Hempel 2009a; Schmid-Hempel 2005a). Priming refers to the occurrence of increased protection against a parasite later in life following previous exposure to a parasite or immune elicitor, or in the case of transgenerational immune priming, an increase in protection against parasite challenge in offspring from parents exposed to a parasite or immune elicitor. However, the level of specificity in protection gained from immune priming is not yet fully understood.

Immune priming has been inferred using a number of different experimental techniques. Some studies have documented prolonged upregulation of immune effectors indicative of the immune system responding to previous attack. For example, elevated Prophenoloxidase (ProPO) levels, an inactive proenzyme necessary for the production of melanin, and increased antibacterial activity, determined by a lytic zone assay, was found in adult male field crickets, *Gryllus campestris*, injected with LPS as nymphs (Jacot et al. 2005). Other studies have examined a direct link between previous parasite exposure and survival following subsequent attack. For example, mealworm beetles, *Tenebrio molitor*, previously pricked with lipopolysaccharide, LPS, a component of

bacteria cells walls, showed increased survival against an entomopathogenic fungus *M. anisopliae*, in addition to increased levels of haemocoelic antimicrobial activity (Moret & Siva-Jothy 2003). More recently, several studies have explicitly tested for specificity in immune priming. For example, Sadd & Schmid-Hempel (2006) found that the bumble bee, *Bombus terrestris,* showed greatest survival and clearance of bacteria of the same species as previously primed with. In addition, primed red flour beetles, *Tribolium casteneum* showed greatest survival on subsequent challenge with homologous bacteria compared to a different strain of bacteria (Roth et al. 2009). However this level of specificity was not found across all host-parasite combinations (Roth et al. 2009). It remains uncertain therefore as to whether specificity in immune priming protection in invertebrates is widespread, or whether the level of specificity seen is dependent on the individual host-parasite combination.

As with all life-history traits, all aspects of immune function, including specificity, may be shaped by associated costs (Schmid-Hempel 2003; Sheldon & Verhulst 1996; Stearns 1989). Understanding the immune response therefore requires further knowledge of potential costs, which may take a number of forms. Costs may result from the action of the immune response itself. For example, the encapsulation response of *T. molitor* causes significant harm to the malpighian tubules (Sadd & Siva-Jothy 2006). Alternatively, indirect physiological energy related costs may occur, such that when activation or maintenance of an immune response is required, other energy demanding traits cannot be sustained and the overall fitness of the host is reduced. For example, *Drosophila melanogaster* larvae which survive attack from the parasitoid wasp *Asobara tabida* show reduced capacity to withstand starvation and desiccation (Hoang 2002). In addition, reduction in immune performance and parasite resistance when resources are limited provides further evidence that the immune response is energetically costly (Boots 2011; Moret & Schmid-Hempel 2000). Costs may also take

the form of genetically based trade-offs, for example between immune traits and other life-history traits, or between different traits of the immune system. Cotter & Wilson (2002) found a negative genetic correlation between phenoloxidase (PO) activity and haemocyte density in the Egyptian cotton leafworm, *Spodoptera littloralis*. Costs of immune priming in insects are not well studied, however, there is some evidence to suggest that immune priming may be costly (Sadd & Schmid-Hempel 2009b) and the relative costs of immune priming in males and females could differ (Zanchi et al. 2011). While empirical evidence of immune priming is expanding, without knowledge of its specificity or costs, predicting its ecological and evolutionary impact on host-parasite systems is difficult.

The immune response of invertebrates, specifically insects, to bacteria is well documented, but knowledge of the antiviral immune response is more limited (Imler & Elftherianos 2009; Kemp & Imler 2009). Viruses represent a large proportion of the parasites which threaten invertebrates causing chronic and lethal infections in their hosts (Imler & Elftherianos 2009) and extensive damage to beneficial insects (Cox-Foster et al. 2007; Ilyinykh 2011). The threat of emergence of new viral infections is also great, given their high replication rate, and therefore high mutation potential (Cleaveland et al. 2001; Woolhouse et al. 2005). Insect-virus interactions also impact on vertebrate disease dynamics given that insects act as vectors of harmful human viruses such as West Nile Virus (Turell et al. 2005). In addition, naturally occurring insect viruses offer a potential solution in the control of insect pest species which causes huge economic damage (Alexandre et al. 2010). It is clear therefore that the study of insect-virus interactions is very important.

Immune priming in *Plodia interpucntella* (Lepidoptera) to its natural viral pathogen *P. interpucntella* Granulosis virus, PiGV (Baculovirus), has been previously found (Tidbury et al. (2011), chapter 2). However, the specificity of immune priming

and the costs associated with immune priming in this system are not currently known. In this study, I determine whether within generation immune priming and transgenerational immune priming to virus is specific and whether within generation immune priming to virus carries developmental costs. I use the insect host *P. interpuntella* its natural viral pathogen PiGV and a different baculovirus *Ephestia cautella* Nucloepolyhedrosis Virus (EcNPV) which in nature infects a related Lepidopteron host *Ephestia cautella,* but in the lab will infect *P. interpunctella* (Hunter et al. 1973). Specifically, I examine whether priming of *P. interpunctella* with PiGV confers protection both within a generation and transgenerationally against challenge with EcNPV in addition to PiGV. I also investigate development time and weight of *P. interpunctella* primed with PiGV compared to control insects. My results contrast aprevious work on this system and imply that immune priming may be a variable response in *P. interpunctella*, therefore highlighting the complexity of the insect antiviral response and the need to further study the interactions between insects and viruses and the likelihood of immune priming.

3.3 Materials and methods

3.3.1 The insect-virus system

The Indian meal moth, *P. interpunctella,* is a pest of stored agricultural products, whose natural environment and ecology can be closely mimicked in the laboratory. Here, insects were reared in 1L Nalgene screw top tubs, on a cereal based diet consisting of 50% Ready Brek ©, 30% bran and 20% rice, with 20g yeast, 0.2g sorbic acid, 0.2g methyl paraben, 25ml honey and 25ml glycerol added to 100g of cereal mix. Insects were housed at 27°C in a 16:8 h light dark regime. I used the naturally occurring DNA baculoviruses: (1) PiGV which naturally infects *P. interpunctella* and (2) EcNPV which infects *E. cautella*, a Lepidopteron host belonging to the same Family (Pyralidae) as *P.*

interpunctella. EcNPV infected *P. interpunctella* are not found in nature but *P. interpunctella* can be infected with EcNPV in the lab by oral inoculation (Hunter et al. 1973). PiGV belongs to the Granulovsis Virus (GV) subgroup of the baculoviruses and EcNPV belongs to the Nucleopolyhedrosis Virus (NPV) subgroup of the baculoviruses. The major difference between GVs and NPVs is that GVs contain one virion within their virus capsule and NPVs contain multiple virions within their virus capsule (Blissard & Rohrmann 1990, chapter 1). Both viruses are transmitted through oral ingestion of virus particles. When the virus occlusion bodies enter the midgut their protein coat is dissolved, and virions are released into the midgut cavity and enter midgut epithelial cells. Once the virus has passed through the midgut, virus proliferation in secondary tissue such as the fat body leads to cell lysis, tissue destruction and eventual host death. *P. interpunctella* larvae infected with PiGV become white/translucent and *P.interpunctella* larvae infected with EcNPV turn brown and are therefore easily distinguishable from each other and healthy larvae. PiGV symptoms in *P. interpunctella* show 7-8 days following oral challenge with virus at third instar, while EcNPV symptoms in *P. interpunctella* show 12-14 days following oral challenge at third instar. Once symptomatic, larvae die before pupation. Purified virus solution for virus priming and virus challenge was produced by centrifugation of a homogenate of infected individuals (Smith & Crook 1988, Appendix 1a, 1b) and then diluted in 75% blue food dye in double distilled water with 5% sucrose to the required viral concentration. PiGV was extracted from PiGV infected *P. interpunctella* and EcNPV was extracted from EcNPV infected *E. cautella.* For both prime and challenge oral inoculations, droplets of virus/dye solution were orally administered to the larvae using a pipette following the standard droplet feeding method adapted from Boots & Begon (1993) (Appendix 1d). Only insects successfully inoculated (indicated by the presence of dye in half of the length of the gut) were used in the experiments. The same

inoculation procedure was used for control larvae but using only the dye/sucrose solution. Prior to the experiments a number of dose response assays were carried out from which the lethal dose of 1% (LD_1) of PiGV for second and third instar larvae and the lethal dose of 50% (LD_{50}) of PiGV and EcNPV for third and fourth instar larvae were estimated.

3.3.2 Specificity of within generation immune priming to virus

Experimental insect populations were established by placing 30 newly emerged *P. interpunctella* adults, from a large outbred stock population, onto 40g of food. Adults were left to mate and lay eggs for 24 hours. Second instar larvae (8 days) were then collected from the food and starved for two hours. Larvae were orally primed with LD_1 concentration PiGV solution or control, dye solution. 100 larvae that successfully consumed the virus prime solution and 100 that consumed the control prime solution were kept separately in petri dishes with abundant food. When the larvae reached fourth instar (14 days) they were removed from the food, and a proportion were starved for two hours then orally challenged with either PiGV or EcNPV. Before challenge, no larvae showed viral symptoms. The remaining larvae were used to investigate the developmental costs of immune priming (see 3.3.4). Virus primed and control primed larvae were kept separate and approximately 20 virus primed larvae and 20 control larvae were inoculated with LD_{50} PiGV solution and approximately 20 virus primed larvae and 20 control larvae were inoculated with LD_{50} EcNPV solution. In addition, 20 virus primed insects were challenged with control dye solution to determine the level of infection resulting from the priming treatment alone, and control primed insects were inoculated with control solution to check for contamination. Successfully inoculated larvae were kept individually with abundant food. PiGV challenged insects were examined for the presence of viral infection 7-8 days post challenge and EcNPV

challenged insects were examined for the presence of viral infection 13-14 days post viral challenge. I recorded the number of infected and non-infected larvae, and this experiment was repeated twelve times, each on a different day. (Figure 3.1a).

3.3.3 Specificity of transgenerational immune priming to virus

Experimental insects were established from newly emerged adults taken from a large outbred stock. 30 adults were placed on 40g of food and left to mate and lay eggs for 24 hours. Third instar larvae (11 days) were taken from the food and starved for two hours. Individuals were either primed with a LD_1 PiGV solution, or a control dye solution. Approximately 250 larvae that successfully consumed the virus prime solution and 250 that consumed the control prime solution were transferred to separate clean containers with abundant food. Larvae were left to develop, pupate, and emerge as adults. The small number of larvae that became infected as a result of the priming inoculation (between 3% and 8.5%) were removed immediately on presentation of symptoms. Upon emergence, thirty adults from each container were then transferred to separate clean containers with 40g fresh food and allowed to mate and lay eggs for 24 hours (F2 generation). Third instar larvae (11 days) from each F2 container were picked out from the food, starved for two hours and approximately 80 larvae from virus primed parents and 80 larvae from control parents were orally challenged with a LD_{50} PiGV solution and approximately 80 larvae from virus primed parents and 80 larvae from control parents were orally challenged with LD_{50} EcNPV solution. In addition, third instar F2 generation larvae from virus primed parents were orally inoculated with control solution and checked for symptoms to confirm that the virus did not pass vertically. Third instar F2 larvae from control primed parents were also inoculated with control solution to

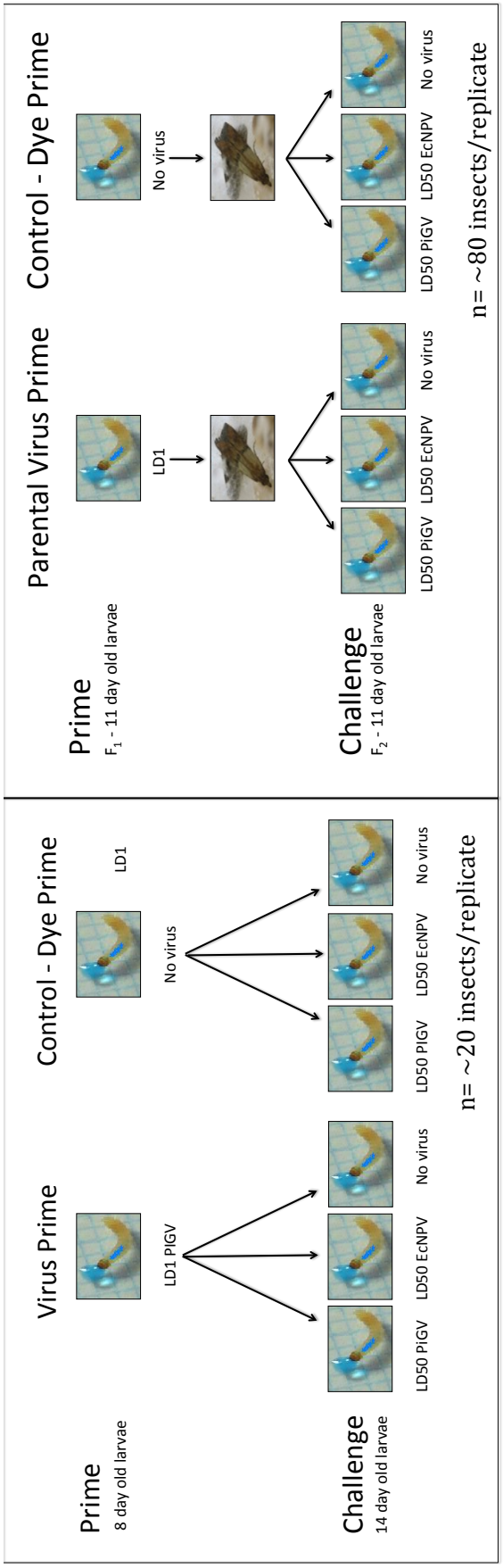

Parental Virus Prime
Control - Dye Prime
Prime
F1 - 11 day old larvae
LD1
No virus
Challenge
F2 - 11 day old larvae
LD50 PiGV
LD50 EcNPV
No virus
n= ~80 insects/replicate
Virus Prime
Control - Dye Prime
Prime
8 day old larvae
LD1 PIGV
No virus
LD1
Challenge
14 day old larvae
LD50 PiGV
LD50 EcNPV
No virus
LD50 PIGV
LD50 EcNPV
No virus
n= ~20 insects/replicate

Figure 3.1

Figure 3.1 Specificity of priming experimental design. a) Within generation priming experiment. Second instar larvae (8 days) were collected from the food and starved. 100 larvae were orally primed with PiGV solution of concentration equivalent to LD_1 and 100 larvae were primed with control solution. At fourth instar (14 days) 20 virus primed and 20 control insects were orally challenged with PiGV solution of concentration equivalent to LD_{50} and 20 virus primed and 20 control insects were orally inoculated with EcNPV solution equivalent to LD_{50}. To determine the level of infection that resulted from prime inoculation primed larvae were also challenged with control solution. This figure outlines the procedure for one experimental replicate. The experiment was repeated twelve times in total. b) Transgenerational priming experiment. Approximately 250 third instar (11days) F1 generation larvae were removed from containers established from the same large outbred insect stock, kept separately and starved. Half of the larvae were primed with PiGV of concentration equivalent to LD_1 and larvae from the other three containers were primed with a control solution. These primed larvae were left to develop and make F2 generations. The small number of larvae that became infected following the virus prime treatment were removed immediately once they showed symptoms. 80 third instar (11 days) F2 generation larvae from virus primed parents and 80 third instar larvae from control primed parents were then challenged with PiGV solution of concentration equivalent to LD_{50} and 80 third instar (11 days) F2 generation larvae from virus primed and control primed parents were challenged with EcNPV solution of concentration equivalent to LD_{50}. In addition, F2 generation larvae were orally inoculated with control, virus-free solution. This figure outlines the procedure for one experimental replicate. This study was carried out six times.

check that there was no contamination. Larvae were kept individually with abundant food after inoculation. PiGV challenged larvae were examined for the presence of viral infection 7-8 days post viral challenge and EcNPV challenged larvae were examined for the presence of virus infection 13-14 days post viral challenge and the number of infected and non-infected larvae recorded. This experiment was repeated six times, each on a different day. (Figure 3.4b).

3.3.4 Developmental costs of immune priming

Fourth instar larvae (day 14) from the within generation priming experiment (therefore inoculated with either LD_1 PiGV solution or a control dye solution at second instar) were picked out from the food and placed in individual cells of a 25 compartmented petri dish with abundant food. Virus primed insects and control primed insects were kept in separate petri dishes. Cells were examined for pupae from day 17 onwards, and the age and weight at pupation was recorded for insects from each of the twelve experimental replicates carried out on different days.

3.3.5 Statistical analysis

The within generation priming experiment was analysed separately to the transgenerational priming experiment. Firstly, for the within generation priming experiment, the effect of previous PiGV exposure early in life (prime treatment) on subsequent susceptibility to infection with PiGV or EcNPV (virus type) was analysed using a generalised linear model (GLM) in R (version 2.14). Quasibinomial errors were incorporated into the model given that the data were proportions, which showed overdispersion. In addition to the key explanatory variables: prime treatment and virus type, the effect of experimental replicate (n=12) and an interaction between prime treatment and virus type on proportion infection were examined. Experimental replicate did not explain a significant amount of variation in proportion infection ($F_{11,36}$=1.74, p=0.11) and infection with PiGV and EcNPV did not differ between virus primed and control primed insects (prime treatment*virus type: $F_{1,33}$=0.351, p=0.56). Therefore, experimental replicate and the interaction between prime treatment and virus type were removed from the final model.

A post hoc power analysis (using the library pwr in R, version 2.14) was performed to ensure that the non-significant findings in this study were not due to a lack

of statistical power, using a sample size of 206 (the lowest total treatment sample size across all blocks in the within generation priming data set), an alpha level of $p<0.05$ and an effect size, h=0.576 (based on a 28.3% lower mean infection in virus primed insects compared to control insects seen in Tidbury et al. (2011) (chapter 2).

For the transgenerational priming experiment, the effect of parental exposure to virus (prime treatment) on offspring susceptibility to infection with PiGV or EcNPV (virus type) was analysed using a GLM in R. Again, quasibinomial errors were incorporated into the model given that the data were proportions which showed overdispersion. In addition to the key explanatory variables: prime treatment and virus type, the effect of experimental replicate (n= 6) and an interaction between prime treatment and virus type were examined. There was no significant effect of experimental replicate on proportion infection ($F_{5,18}=0.97$, $p=0.47$) and no interaction between prime treatment and virus type ($F_{1,15}=0.023$, $p=0.87$). Therefore experimental replicate and the interaction between prime treatment and virus type were removed from the final model.

A power analysis was performed to ensure that the non-significant findings in this study were not due to a lack of statistical power, using a sample size of 415 (the lowest total treatment sample size across all blocks in the transgenerational immune priming data set), an alpha level of $p<0.05$ and an effect size, h=0.32 (based on a 15.6% lower mean infection in virus insects from virus primed parents compared to insects from control parents seen in Tidbury et al. (2011) (chapter 2).

The effect of previous virus exposure (prime treatment) on development time and pupal weight was assessed using a general linear model in R. In addition to prime treatment, experimental replicate (n=12) was included in the model as an explanatory variable.

3.4 Results

3.4.1 Specificity of within generation immune priming to virus

Previous exposure to PiGV earlier in life did not affect resistance to virus challenge (with PiGV or EcNPV) later in life ($F_{1,46}$=0.153, p=0.70) (figure 3.2). The power in this experiment to detect an effect size of magnitude, h=0.58, seen in a previous study with the same system was 1, eliminating lack of power as a reason for the non-significant effect of prime treatment on proportion infection. Infection with PiGV was significantly lower than infection with EcNPV ($F_{1,45}$=9.81, p=0.0031).

3.4.2 Specificity of transgeneration immune priming to virus

Resistance of offspring to virus challenge with PiGV or EcNPV was not affected by parental exposure to PiGV ($F_{1,22}$=0.807, p=0.38) (figure 3.3). The power in this experiment to detect an effect of prime treatment of magnitude, h=0.32, seen in Tidbury et al (2010) (chapter 2) was 0.995, therefore eliminating lack of power as a reason for the non-significant effect of parental priming treatment on viral infection in offspring. Infection with PiGV did not differ significantly from infection with EcNPV ($F_{1,21}$=2.02, p=0.17).

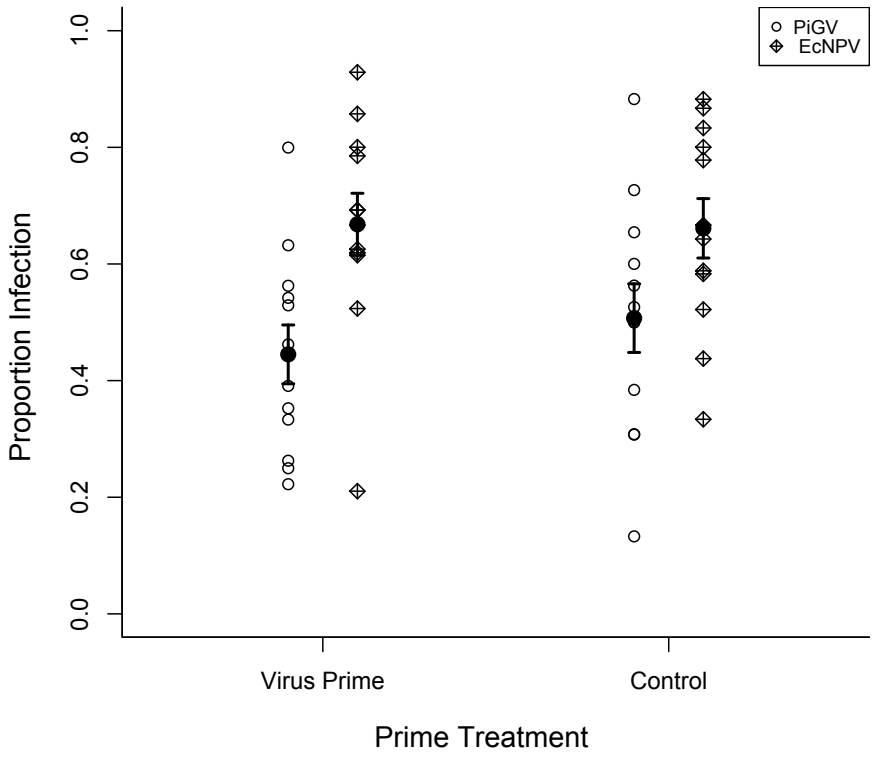


Figure 3.2. Infection with PiGV and EcNPV in insects previously exposed to PiGV and control insects. Previous exposure to virus earlier in life did not affect resistance to virus challenge later in life ($F_{1,46}$=0.153, p=0.70). Infection with PiGV was significantly lower than infection with EcNPV ($F_{1,45}$=9.81, p=0.0031). Points represent experimental replicates and filled circles are means ± s.e.

3.4.3 Developmental costs of immune priming

Insects primed with virus earlier in life had a significantly longer development time compared to control insects ($F_{1,259}$=11.93, p<0.001) (figure 3.4) and development time varied significantly between each experimental replicate across virus primed and control insects ($F_{11,260}$=4.78, p<0.001). However, insects primed with virus did not differ significantly in their mass at pupation compared to control insects ($F_{1,259}$=2.29, p=0.13) (figure 3.5) although mass at pupation did vary across both virus primed insects and control insects between different experimental blocks ($F_{11,260}$=2.14, p=0.018).

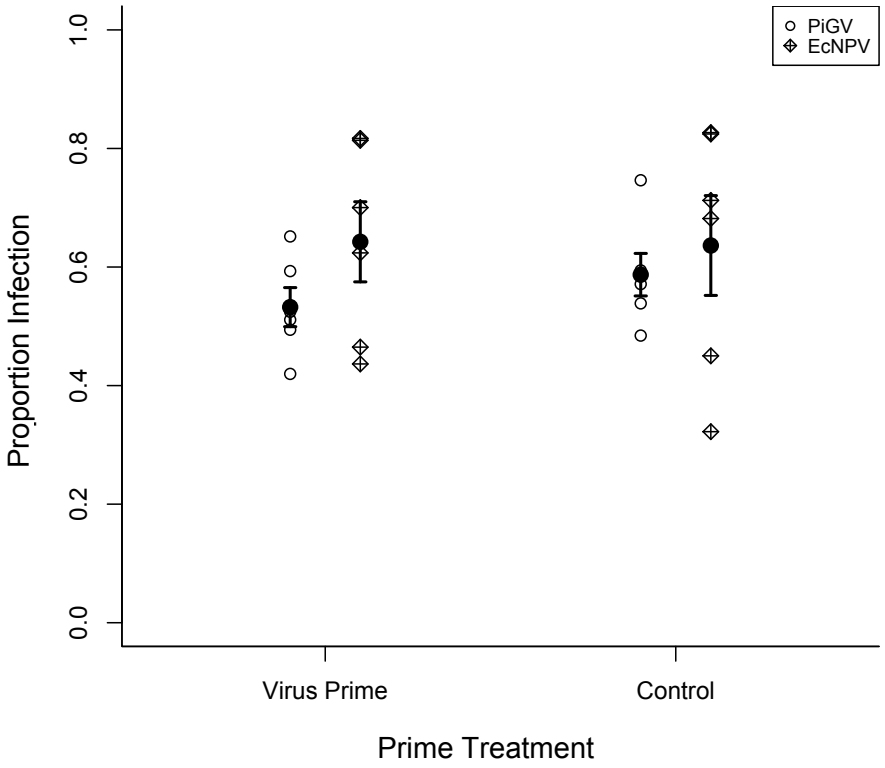


Figure 3.3. Infection with PiGV and EcNPV in offspring of parents exposed to PiGV and control parents. Resistance of offspring to virus challenge was not affected by parental exposure to virus ($F_{1,22}$=0.807, p=0.38). Infection with PiGV did not differ significantly from infection with EcNPV ($F_{1,21}$=2.02, p=0.17). Points represent experimental replicates and filled circles represent means ± s.e.

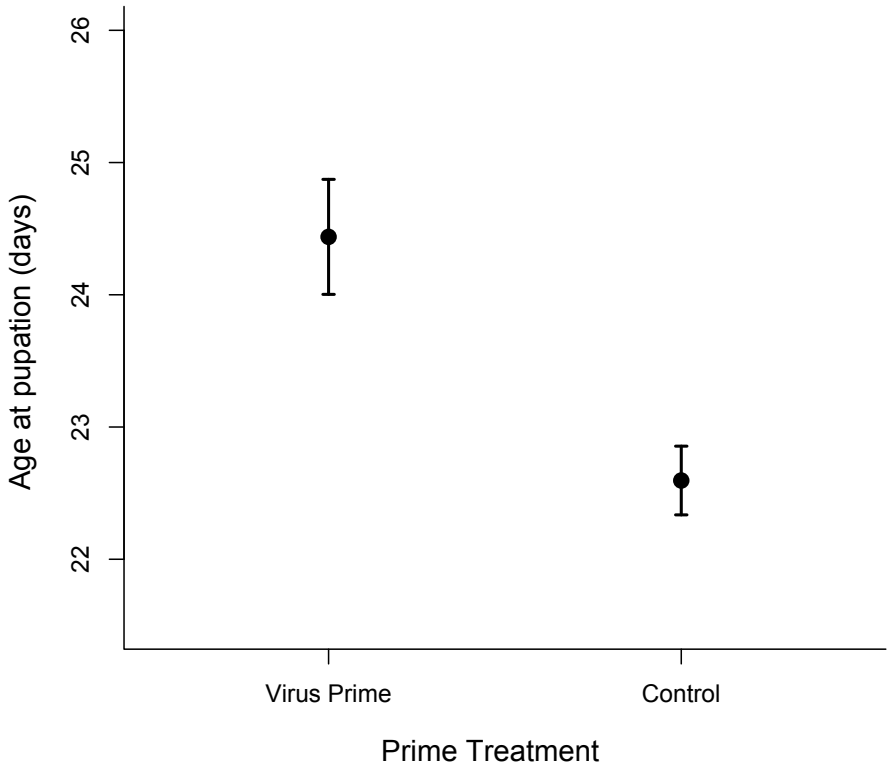


Figure 3.4. Mean age at pupation of insects previously exposed to PiGV and control insects. Insects primed with virus earlier in life had a significantly longer development time ($F_{1,259}$=11.9, p<0.001). Filled circles represent experimental treatment means ± s.e.

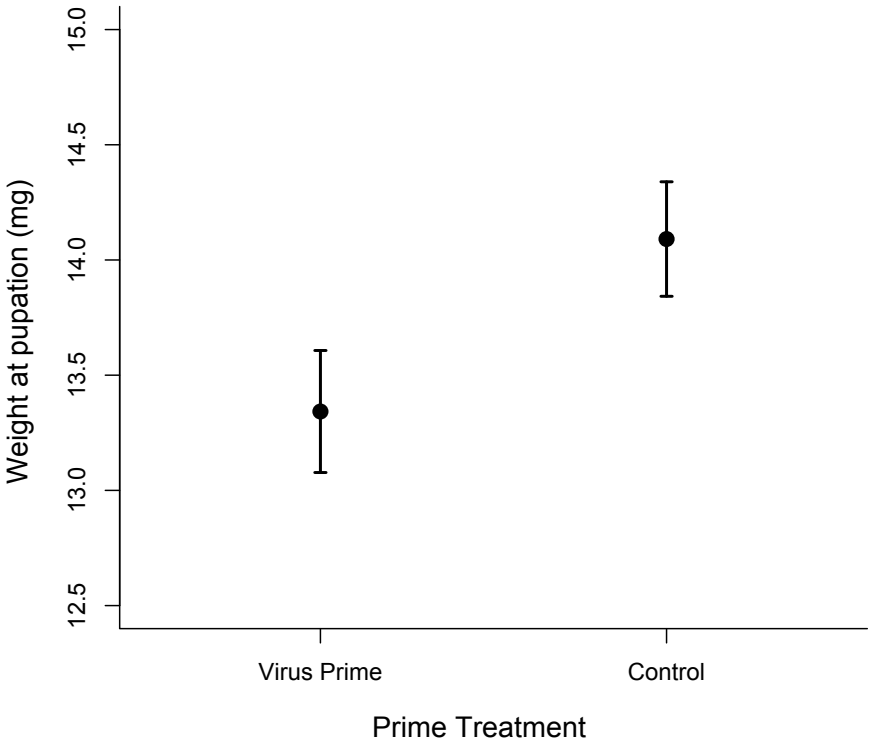


Figure 3.5. Mean weight at pupation of insects previously exposed to PiGV and control insects. Insects primed with virus did not differ significantly in their mass at pupation compared control insects ($F_{1,259}$=2.29, p=0.13). Filled circles represent experimental treatment means ± s.e.

3.5 Discussion

In contrast to previous work on immune priming in invertebrate systems and in the *P. interpunctella*-PiGV system specifically, in this experiment I find no support for the hypotheses that (i) previous exposure to a parasite earlier in life increases resistance to subsequent parasite challenge and (ii) exposure of parents to a parasite increases offspring resistance to parasite challenge. This study therefore highlights that immune priming protection following exposure to PiGV in *P. interpunctella* does not always occur, raising questions about how variable immune priming may be within a host species, as well as between different invertebrates hosts and indicates the need for further research on the context dependency of immune priming. It is unclear whether variation in immune priming protection is a characteristic of *P. interpunctella* alone or

whether it is widespread in other systems, but due to positive result publication bias, it has not been documented in other species.

While immune defence is vital for hosts to survive parasite attack, variation in immune defence is well documented (Schmid-Hempel 2003). Immune defence is energetically costly and is therefore constrained if the host has limited resources (Boots 2011; Moret & Schmid-Hempel 2000). Immune components may also directly harm host tissue, a phenomenon referred to as immunopathology (Graham et al. 2005). In addition immune defence may be negatively genetically correlated with other important fitness traits (Adamo et al. 2001; Boots & Begon 1993). Investment in immune defence may be influenced by internal and external factors with for example, a reduction in immune investment seen when resources are limited. Specifically, PO activity and encapsulation has been demonstrated to be much reduced in *T. molitor* when starved for 5 days (Siva-Jothy & Thompson 2002). Also, high host density environments may result in greater investment in immunity among individuals, due to the increase in parasite exposure and disease prevalence associated with high density populations. For example, increased PO activity was reported in the African armyworm, *Spodoptera exempta,* raised in high densities (Wilson et al. 2001). However, a recent study has highlighted that in addition to single environmental factors influencing the immune response, multiple environmental factors may interact resulting in a more complex affect of host environment on immune defense (Triggs & Knell 2012). Specifically, Triggs & Knell (2012) showed that PO activity and haemocyte numbers in *P. interpunctella* were highly sensitive to food quality, host population density and temperature but also the specific combination of these environmental factors. For example, they found a significant interaction between density and food quality, with increased investment in immunity on high quality food and at high population densities but reduced investment in immunity at low food quality, despite high density. This

highlights that even with the increased risk of infection indicated by increased density, investment in immune defense may be too costly when resources are low.

Although less is known about the costs of immune priming, there is evidence to suggest that, at least in some systems, immune priming does carry costs. For example in *T.molitor,* immune priming increases the time taken to reach adulthood (Moret 2006) and in *B. terrestris*, while priming with one parasite increases protection against that same parasite, it increases susceptibility to other parasites (Sadd & Schmid-Hempel 2009b). In this study, I examined potential developmental costs of within generation immune priming in *P. interpunctella*. Developmental costs represent a good proxy for general fitness in this system given for example, that size and weight at maturity are strongly correlated with fecundity (Calvo & Molina 2005; Honek 1993; Jervis et al. 2008; Rasotto et al. 2010) and time to maturation controls the earliest point at which offspring can be produced. Here, I found that primed insects did not differ in their mass at pupation compared to controls; however, they did take longer to reach pupation. Increased resistance in *P. interpunctella* to PiGV is also correlated with slower development (Boots & Begon 1993) suggesting that immune priming and resistance could be controlled by the same mechanisms in this insect host species. The sensitivity of immune components in *P. interpunctella* to resource quality found by Triggs & Knell (2012) and the increase in development time seen in primed insects in the present study, suggest that the lack of consistency in immune priming protection in *P. interpunctella* may be a consequence of this trait being costly.

In addition to developmental costs, the specificity of immune priming protection was also investigated in the present study. Previous research suggests that the specificity of immune priming protection may vary between different host species. In this study we find that *P. interpunctella* larvae primed with PiGV respond in the same way to PiGV challenge and EcNPV challenge relative to controls. Similarly, offspring from parents

primed with PiGV respond similarly to challenge with PiGV and EcNPV relative to controls. While this suggests that immune priming protection in *P. interpunctella* is non-specific, further study of the specificity of immune priming protection in this host species is needed as in the present study significant protection from immune priming was not found.

The specificity of immune priming and costs of immune priming are likely to be interconnected, and in part, determined by the mechanistic basis of immune priming. Non-specific immune memory, in theory, could be the result of prolonged presence of immune effectors either because of their increased upregulation or delayed degradation. For example, increased antibacterial activity in *G. campestris* and *T. molitor* following priming with LPS is suggestive of a non-specific response (Jacot et al. 2005; Moret & Siva-Jothy 2003). The benefit of a non-specific priming protection could be an overall reduction in parasite infection following priming. However, prolonged immune responses are costly to the host, draining vital resources (Kelly 2011) and resulting in immunopathology (Cerenius & Soderhall 2004; Sadd & Siva-Jothy 2006). Interestingly, Jacot et al. (2005) found increased levels of the inactive proenzyme ProPO, involved in melanzation, in primed insects but not the active enzyme PO. While non-specific prolonged upregulation of immune components may be the mechanism behind immune priming protection, the presence of an inactive form of enzyme is suggestive of adaptation by the insect to reduce costs associated with long-term upregulation of the immune components.

Specific immune memory, where immune priming to one pathogen confers protection to that pathogen only, is undoubtedly controlled by a more complex mechanism. Limited, but compelling evidence suggests haemocytes may be the key to immune priming specificity (Pham et al. 2007; Roth & Kurtz 2009). While cellular immune responses are typically classed as non-specific (Schmid-Hempel & Ebert

2003), in theory, specificity could lie at the point of pathogen recognition by haemocytes or differential action of haemocytes (Pham & Schneider 2008). The most likely molecule proposed for allowing diversity in pathogen recognition and binding is the Down syndrome cell adhesion molecule (Dscam), a functional homologue of the human Down Syndrome Cell Adhesion Molecule (DSCAM), found in *Drosophila*. It is a member of the immunoglobulin family, a group of proteins which give rise to variety of surface receptors and function as signal transduction receptors and cell adhesion molecules. Thousands of isoforms of Dscam can be produced by alternative splicing of the molecule (Schmucker & Chen 2009) with 14 different Dscam isoforms expressed on an individual haemocyte (Neves et al. 2004).

Most studies assess the specificity of immune priming protection at one or two time points post immune priming. However, the time post immune priming may determine the level of specificity seen. For example, in American cockroach, *Periplaneta Americana*, adults previously primed with bacteria, specificity of priming protection increased as time post priming exposure increased. Non-specific protection elicited by 4 different bacteria was documented 24-72 hours post priming, while 7 days post priming, the protection was highly specific (Faulhaber & Karp 1992). Distinct responses at different time points following priming are also evident in *B. terrestris* where at 8 days post priming with bacteria, protection was non-specific but at day 22 post priming, insects were only protected against the bacteria which they were promed with (Sadd & Schmid-Hempel 2006). In view of this, future work on immune priming specificity should investigate protection at multiple time points.

The mechanism and therefore the specificity and the costs of transgenerational immune priming will likely be distinct from the mechanisms determining within generation immune priming. However, the similarities and differences between these different protections are unclear and it is possible that the protection seen in offspring of

primed mothers is a result of the transfer of immune eliciting elements within the egg (Sadd & Schmid-Hempel 2007). A degree of specificity has been documented in transgenerational priming. For example, offspring of the flour moth, *Ephestia kuchinella,* from mothers primed with *Bacillus thuringiensis* toxin, but not LPS, were resistant to *B. thuringiensis* challenge (Rahman et al. 2004). In *B. terrestris* offspring from parents primed by injecting bacteria were more susceptible to an orally ingested bacteria (Sadd & Schmid-Hempel 2009b) suggesting that costs of transgenerational priming could be manifested in trade-offs between immune defences present at different locations. In *Drosophila*, offspring from mothers exposed to bacteria, while showing no increase in resistance against bacteria did have a reduced lifespan compared to controls (Linder & Promislow 2009). This highlights that the costs, in addition to the benefits of immune priming, not only effect the primed generation directly but also potentially their offspring.

While the immune response of invertebrates to bacterial and fungal pathogens is well explored, the invertebrate antiviral response is less understood (Kemp & Imler 2009). This lack of understanding may explain the relatively small number of virus immune priming studies conducted in invertebrates systems. Proposed mechanisms of antiviral defence and therefore potential virus immune priming mechanisms include essential defence processes such as apoptosis (Clarke & Clem 2003b) and RNA interference (Kemp & Imler 2009; Wang et al. 2006), cellular immune cells such as haemocytes (Begon et al. 1993; Trudeau et al. 2001) and humoral immune responses such as PO (Popham et al. 2004; Shelby & Popham 2006). However, in order to fully understand the nature of immune priming of insects to virus a greater basic knowledge of the antiviral response is crucial and will allow the examination of immune priming mechanisms in addition to the functional outcome in terms of resistance and survival of the host.

Power analyses show that the lack of a significant effect of prime treatment (within and transgenerational) on the proportion infection found in this experiment is not just a consequence of a lack of power to detect an effect. The infection data was highly overdispersed, beyond what would be expected with a binomial distribution and this is most likely the result of high variation in proportion infection between experimental repeats. In future studies, potential overdispersion could therefore be reduced, although practically difficult, by reducing the number of experimental replicates and increasing the sample size and therefore power within the repeats.

Another possible explanation for the lack of priming response could be a failure in administration of the virus dose intended to prime the insect's immune response. However, as part of the within generation immune priming experiment, a control treatment, where insects were exposed to a prime dose of virus and challenged with a control dye solution indicates that the insects had received the prime dose and the level of resulting infection was even slightly higher than predicted. In the transgenerational priming experiment infection resulting from the prime dose of virus was also slightly greater than predicted and did not differ significantly between repeats. It is therefore unlikely that the lack of priming effect documented was due to unsuccessful uptake of the priming virus dose in experimental insects. However, it is very possible that because the virus prime dose was at such a low viral concentration (LD_1) that there was variation across individuals in their level of priming exposure; such that some larvae received sufficient viral particles to prime the response, while others received too few, or no particles at all. Effort was used to minimise the heterogeneity across priming doses; however this cannot be excluded as a possibility.

The lack of evidence for immune priming to viruses found in the present study contrasts previous work done by myself and others. This study therefore highlights that the immune priming response in *P. interpunctella* is variable and may be context

dependent. Although little is known about the potential mechanism of immune priming to virus, a development cost of immune priming in *P. interpunctella* was found in this study which is likely to influence when we would expect to see immune priming. Further insight into the mechanisms underlying immune priming and the antiviral response in general is necessary to determine the nature of the variation seen in immune priming protection in this study. It is possible that the variability seen in immune priming response in *P. interpunctella* to PiGV is specific to this system but it may typical of immune priming in insects generally. Determining the variation seen in the immune response is crucial for a more accurate prediction of immune priming protection and its consequences in natural insect and invertebrate systems.

Chapter 4: The Epidemiological Consequences of Immune Priming

4.1 Abstract

Exposure to low doses of parasites that do not result in the host becoming infectious, may 'prime' the immune response and increase protection to subsequent challenge. There is increasing evidence that such immune priming is a widespread and important feature of invertebrate host-parasite interactions. Immune priming clearly has implications for individual hosts but will also have population level implications. I present a Susceptible-Primed-Infectious (SPI) model – in contrast to the classic Susceptible-Infectious-Recovered (SIR) framework - to investigate the impacts of immune priming on parasite persistence and population stability. I describe impacts of immune priming on the epidemiology of the disease in both constant and seasonal environments. A key result is that immune priming may act to destabilise population dynamics. In particular, when the proportion of individuals becoming primed rather than infected is high, but this priming does not confer full immunity, the population may be strongly destabilised through the generation of limit cycles. I discuss the implications of our model both in the context of invertebrate immunity and more widely.

4.2 Introduction

Parasites are ubiquitous in nature and by definition cause significant harm to their hosts. In response, hosts have evolved a range of resistance mechanisms including behavioural avoidance, physical barriers and complex cellular and humoral immune responses (Murphy et al. 2011; Schmid-Hempel 2005a; Siva-Jothy et al. 2005). The immune responses of invertebrates, though less well understood than that of vertebrates, are now

widely studied (Rolff & Reynolds 2009; Schmid-Hempel 2005a). Traditionally invertebrates were thought to have no capacity for memory in their immune response to parasites as they lack the immune cells which are responsible for vertebrate acquired immunity (Rowley & Powell 2007). However, despite being mechanistically distinct to the vertebrate acquired immunity, growing empirical evidence suggests that invertebrates show immune memory in that they are more resistant to parasite infection following previous exposure (Little & Kraaijeveld 2004; Schmid-Hempel 2005a). This form of memory in an invertebrate is termed 'immune priming' which I broadly define as increased protection to a parasite following previous exposure to a parasite (but which does not result in infectiousness) or an immune elicitor. Although recent work has investigated the impacts of immune priming on infection prevalence, host population size, and population age structure using a stage structured SIRS model (Tate & Rudolf 2011) it remains unclear what impact this form of immunity has on parasite persistence and the stability of host-parasite systems in general.

Evidence for immune priming has been found in a number of different insect species and in response to a diverse range of parasite species indicating that this phenomenon may be widespread. For example, immune priming has been shown in the red four beetle, *Tribolium castaneum* (Roth et al. 2009) and in the bumble bee, *Bombus terrestris*, in response to bacteria (Sadd et al. 2005; Sadd & Schmid-Hempel 2006) in the Indian meal moth, *Plodia interpiunctella*, in response to virus (Tidbury et al. 2011) and in the mealworm beetle, *Tenebrio molitor*, in response to lipolysaccharide (LPS), a component of bacterial cell walls (Moret & Siva-Jothy 2003). Furthermore there is evidence for immune priming in other invertebrates such as crustaceans including *Daphnia magna* (Little et al. 2003), *Penaeus monodon* (Witteveldt et al. 2004), *Penaeus japonicus* (Wu et al. 2002) and *Litopenaeus vannamei* (Pope et al. 2011). Both within generation (e.g. Moret & Siva-Jothy 2003; Roth et al. 2009; Tidbury et al. 2011) and

transgenerational priming (e.g. Little et al. 2003; Sadd et al. 2005; Tidbury et al. 2011), where protection is passed from parent to offspring, has been found. Although our mechanistic understanding of immune priming is limited and clearly requires further study (Hauton & Smith 2007), the phenomenon of immune priming has been demonstrated in a number of invertebrate species.

Immune responses clearly have implications for individual hosts, but they may also have implications for the host population as a whole. Mathematical modelling is a key tool in capturing infectious disease dynamics and can be used to infer the effect that different immune responses can have on host-parasite interactions and population dynamics. Traditionally, epidemiological studies are in the form of SIR (Susceptible-Infectious-Recovered) models (Anderson & May 1979; Dietz 1967), where following infectiousness hosts recover to a completely immune state. Comparison of these SIR systems with SIS (Susceptible-Infectious-Susceptible) models, where following infectiousness individuals return to being susceptible, have highlighted the impact that immunity can have on population dynamics. For example, SIR systems exhibit more prolonged and larger damped oscillations on approach to a stable equilibrium compared to SIS systems (Keeling & Rohani 2008). Factors important in disease dynamics are unlikely to be constant through time with seasonality in epidemiological and demographic parameters substantial in natural and human disease interactions (Altizer et al. 2006). Incorporating such variation into mathematical model therefore enables the more accurate representation and prediction of disease dynamics in nature. Seasonality in the SIR model tends to drive regular multi-annual epidemic disease outbreaks as is seen in childhood infections such as Measles (Fine & Clarkson 1982; Finkenstadt & Grenfell 2000; London & Yorke 1973). Vaccination has also been modelled as removal of individuals from the susceptible class directly to the immune class without an infectious stage and at a rate independent of parasite density in the population (Griffith

1973; Heffernan & Keeling 2009). However, these classic SIR-type models do not capture the population dynamics with immune priming, where a proportion of individuals do not become infectious but are primed following parasite exposure and have reduced risk of becoming infectious on subsequent exposure. The immune priming process I consider is epidemiologically distinct from the acquired immunity more commonly assumed in classical SIR models, where immunity is only gained after hosts have been infectious for some period of time. Instead, I assume that primed hosts gain immunity following exposure to an infected host but without ever becoming infectious themselves.

Here I develop and analyse a mathematical model which incorporates within generation immune priming, and transgenerational immune priming into a disease system. I describe the implications of immune priming for the persistence and prevalence of the parasite and the host-parasite population dynamics in constant environments and under seasonal forcing.

4.3 Modeling

4.3.1 Model framework

A general theoretical framework is developed to examine the impacts of both within generation and transgenerational immune priming on the dynamics and stability of a host-parasite population. The host population is divided into three distinct classes: susceptible hosts with density S, primed hosts with density P and infectious hosts with density I (total density, $H=S+P+I$) (figure 4.1). I therefore call this model 'Susceptible-Primed-Infectious' (SPI). The dynamics of the population are represented by equations 1, 2 and 3:

$$\frac{dS}{dt} = (a - hH)(S + f_I I + (1-\tau) f_P P) - bS - \beta SI + cP \quad (1)$$

$$\frac{dP}{dt} = \tau(a - hH) f_P P - bP + p\beta SI - q\beta PI - cP \quad (2)$$

$$\frac{dI}{dt} = (1-p)\beta SI + q\beta PI - (b + \alpha)I \quad (3)$$

All hosts reproduce at rate a, which is reduced by factor h, due to density dependence, so that increased population density results in a negative feedback on reproduction. The host population is therefore limited to a carrying capacity in the absence of disease. All hosts are subject to a natural death rate b. The transmission coefficient is β and is assumed to be through direct contact between susceptible and infectious hosts, and is density dependent. Following exposure to the parasite, a proportion, p, of susceptible individuals become primed, and the remainder (1-p) become infectious. Infectious individuals suffer increased death rate (pathogenecity) α, and do not recover. Primed individuals become infectious at a reduced rate q, with subsequent exposure to the parasite. The degree of transgenerational immune priming in the system is determined by τ, with primed individuals giving birth to primed offspring at rate τ and susceptible offspring at rate (1-τ). Primed and infectious individuals have reduced fecundity, f_P and f_I respectively. (See table 4.1 for a list of all parameters used in the model).

I investigate the ecological behaviour of the SPI system using the AUTO continuation software package (Doedel et al. 1997) which identifies the location and stability of equilibria as parameters of the model are varied. Specifically, I focus on areas of parameter space where the parasite cannot persist, where there is an

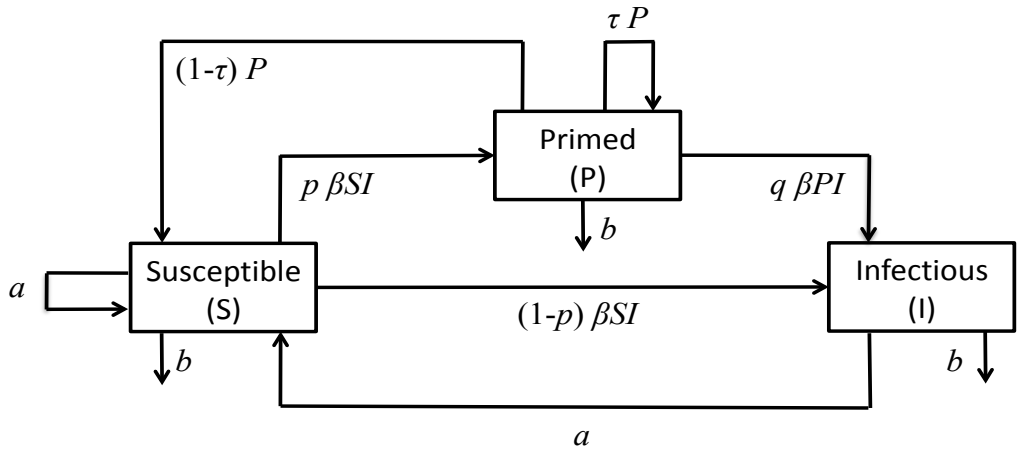


Figure 4.1. Susceptible-Primed-Infecious (SPI) model schematic.

Table 4.1. Parameters and variables in the SPI model.

Symbol	Meaning
H	Total number of hosts
S	Susceptible individuals
P	Primed individuals
I	Infectious individuals
a	Birth rate
b	Natural death rate
α	Pathogenecity
β	Transmission rate
p	Priming rate
q	Proportion of primed hosts becoming infectious
τ	Transgenerational priming
h	Density dependence
f_P	Primed host's fecundity
f_I	Infectious host's fecundity

endemic equilibrium and where there are endemic cycles. Endemic limit cycles, while considered to be mathematically stable, indicate population instability as they are characterised by periodic increases and decreases in population size. I examine the consequence of priming rate p, and degree of priming protection q, on the persistence of the parasite and stability of the endemic state with and without transgenerational priming and with varying demographic and epidemiological parameters (namely birth

rate (a), transmission (β) and pathognenecity (α)). Using the modelling package odesolve in R, I examine the dynamics of the SPI system in comparison to more traditional model frameworks and within the cyclic region. My aim is to gain a general understanding of the population dynamical effects of immune priming.

4.3.2. Seasonality

I extend this model to include seasonality in birth rate and transmission rate by setting these parameters to be a function of the sine wave so that:

$$a = a(1 + \delta \sin(2\pi t)) \quad (4)$$

$$\beta = \beta(1 + \delta \sin(2\pi t)) \quad (5)$$

where δ is the amplitude of the seasonal variation and the period of the oscillation is exactly 1 year. I explore how seasonal fluctuation in birth rate and transmission rate affect the population dynamics of a system which exhibits immune priming.

4.4. Results

4.4.1 Parasite Invasion

R_0 for a parasite is the average number of secondary cases of disease that arise from one infectious individual in an otherwise susceptible population. Theoretically, if $R_0 > 1$ then the parasite can successfully invade a population whereas if $R_0 < 1$ the parasite cannot invade (Anderson & May 1981). The expression of R_0 for the SPI model is:

$$R_0 = (1-p)\frac{\beta\hat{X}}{(\alpha+b)} \qquad (6)$$

where $\hat{X} = (a-b)/h$ is the disease-free host equilibrium density. Clearly the value of R_0 depends on priming, p. From (6) it is clear that, rather intuitively, increasing the proportion of priming (p) reduces the ability of the parasite to invade a susceptible host population since fewer hosts are becoming infected. It is important to note though that the level of protection which priming provides (q) does not influence the R_0 value.

4.4.2 Endemic Disease

Whenever $R_0 > 1$, the disease-free equilibrium is unstable and a stable endemic state exists where the host and parasite coexist. However, in this system the host and parasite may also coexist when $R_0 < 1$. Therefore when the rate of priming is high (despite $R_0 < 1$), both the disease-free and endemic equilibrium are stable, i.e the system is bistable (figure 4.2). Whether the disease-free or endemic equilibrium is reached depends on the starting conditions of the system and the protection that priming provides (q). Independent of q, when priming is high and the parasite is initially absent, the parasite cannot invade and the system settles at the disease-free equilibrium. However, if the parasite is already present it may persist depending on the value of q. When q is low so that the protection provided by priming is high the parasite cannot persist when $R_0 < 1$, but when q is high so that the priming protection is low the parasite can persist at $R_0 < 1$. Therefore in a system which experiences high rates of priming the persistence criteria and the invasion criteria for the parasite are distinct.

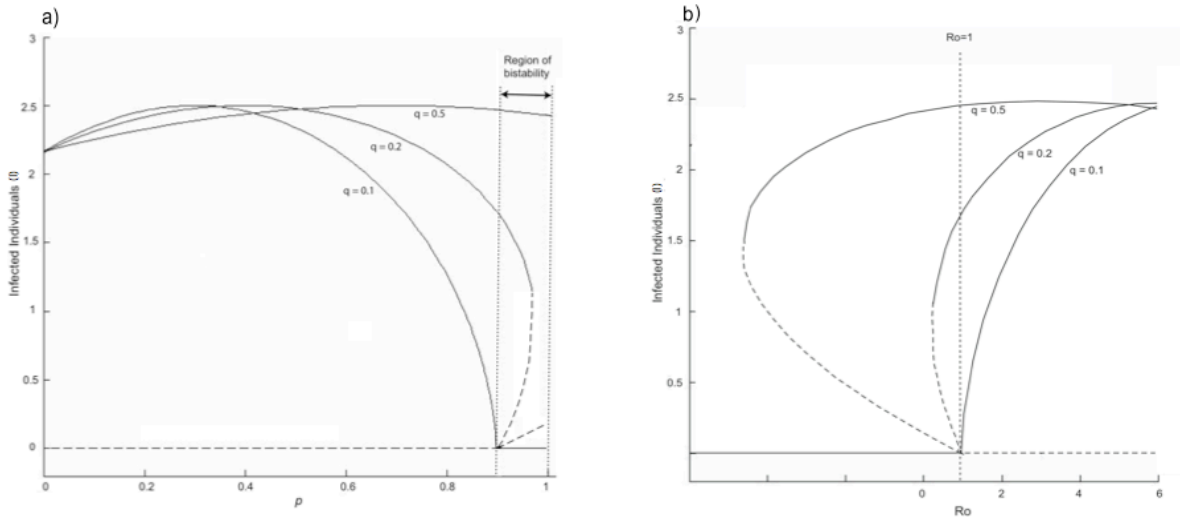


Figure 4.2. Bistability in the SPI system when there are no fecundity costs of infectiousness and priming. a) Region of bistability, when priming rate (p) is high, is dependent on priming protection (q). When priming protection is high (q=0.1) there is no bistability but when priming protection is less (q=0.5) the parasite can persist even when all susceptible individuals become primed (p=1). b) Bistability plot in terms of R_0. R_0 when p=0.9 for the model parameters used. When q>0.1, the number of infecteds can be positive, with the system at an endemic equilibrium, even if R_0 <1. Therefore, higher levels of priming protection result in persistence of the parasite below R_0=1.. (Parameters: a=2, b=1, h=0.1, α=1, β=2, τ=0, f_Y=1, and f_P=1. Solid lines represent a stable population and dashed lines represent an unstable population).

A key result from the numerical simulations performed in AUTO and R is that high levels of immune priming destabilises the host-parasite population. However the destabilising effects of priming are only seen in systems where infectiousness results in reduced fecundity and increases if primed individuals also have reduced fecundity. When the rate of immune priming is low the SPI system mimics the traditional SIR system and reaches an endemic equilibrium following damped oscillations. However, when the rate of priming is high the SPI system may exhibit prolonged limit cycles (Figure 3). Limit cycles produce repeated increases and crashes in the densities of the population classes. Therefore in a system characterised by endemic limit cycles, we see

periods of disease outbreak followed by near-absence of disease. The range of priming rates and priming protection where instability is found depends on specific parameters of the model (figure 4.4). In particular, increased fecundity costs of infectiousness and priming are important and increase the range of priming rate and priming protection over which cycles are exhibited. In addition, increased pathognenecity (figure 4.4b) and increased birth rate (figure 4.4c) lead to reduced chance of destabilisation, while increasing transmission (figure 4.4d) and the addition of trangenerational priming (figure 4.4e) result in an increase in the total cycling region and therefore further destabilisation of the population.

In addition to generating cyclic population dynamics, immune priming also modulates these cycles as it varies. Increasing the level of priming within the cycling

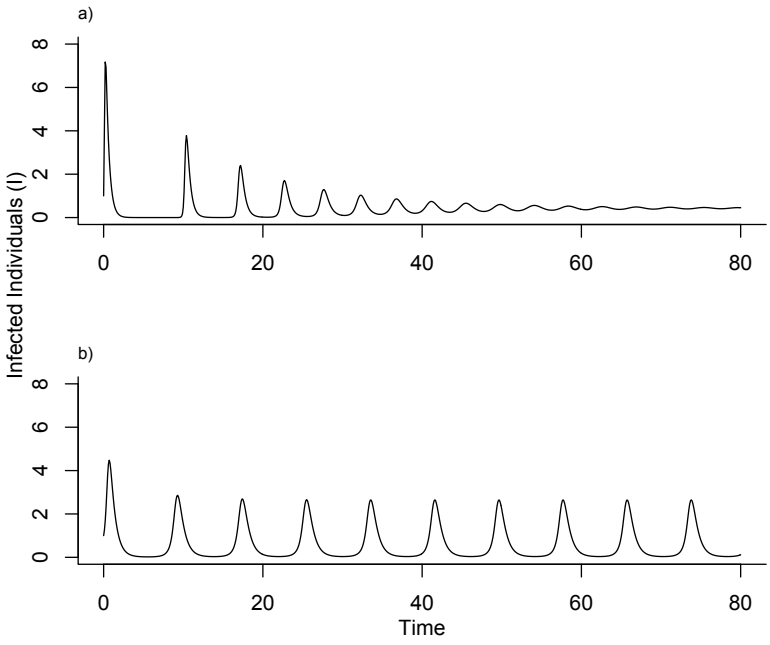


Figure 4.3. Comparison of SIR and SPI model dynamics. a) SIR model with damped oscillatory approach to stable equilibrium. (Parameters: a=2, b=1, h=0.1, α=1, β=2, γ=0.5, f_Y=0.2). b) SPI model with high priming rate showing endemic limit cycles. (Parameters: a=2, b=1, h=0.1, p=0.8, q=0.5, α=1, β=2, τ=0.5, f_Y=0.2, f_P=0.5).

region results in limit cycles of greater amplitude but reduced frequency (figure 4.5b, 4.5c) which translates into fewer, larger disease outbreaks over time. Furthermore, outside the region of limit cycles, the time taken for the cycles to dampen is longer under increased priming (figure 4.5a, 4.5b). Throughout then it is clear that in systems with high levels of immune priming, and fecundity costs of infection and priming, the population dynamics are more likely to be unstable.

4.4.3 Seasonality

The addition of seasonality into the SPI model adds complexity to the population dynamics. However the forced dynamics can be closely predicted from the non-forced dynamics (figure 4.6) so that small annual cycles occur in the forced system where there is a stable endemic equilibrium in the non-forced system (figure 4.6a), i.e. when priming rate is low. These small annual cycles are entirely due to seasonal forcing, and are seen when both birth rate and transmission rate are forced. At intermediate priming rates, for specific model parameters, the non-forced system produces prolonged damped oscillations which, as in the SIR model, when combined with seasonality produce multi-year cycles (figure 4.6b). At high priming rates, the non-forced system and the forced birth rate system produce stable limit cycles. However, when transmission is forced, quasi-periodic solutions occur (figure 4.6c). These trajectories form a torus around the existing limit cycle and appear close to a regular cycle, but in fact never return to the same point and hence do not complete a true cycle (Rinaldi et al. 1993).

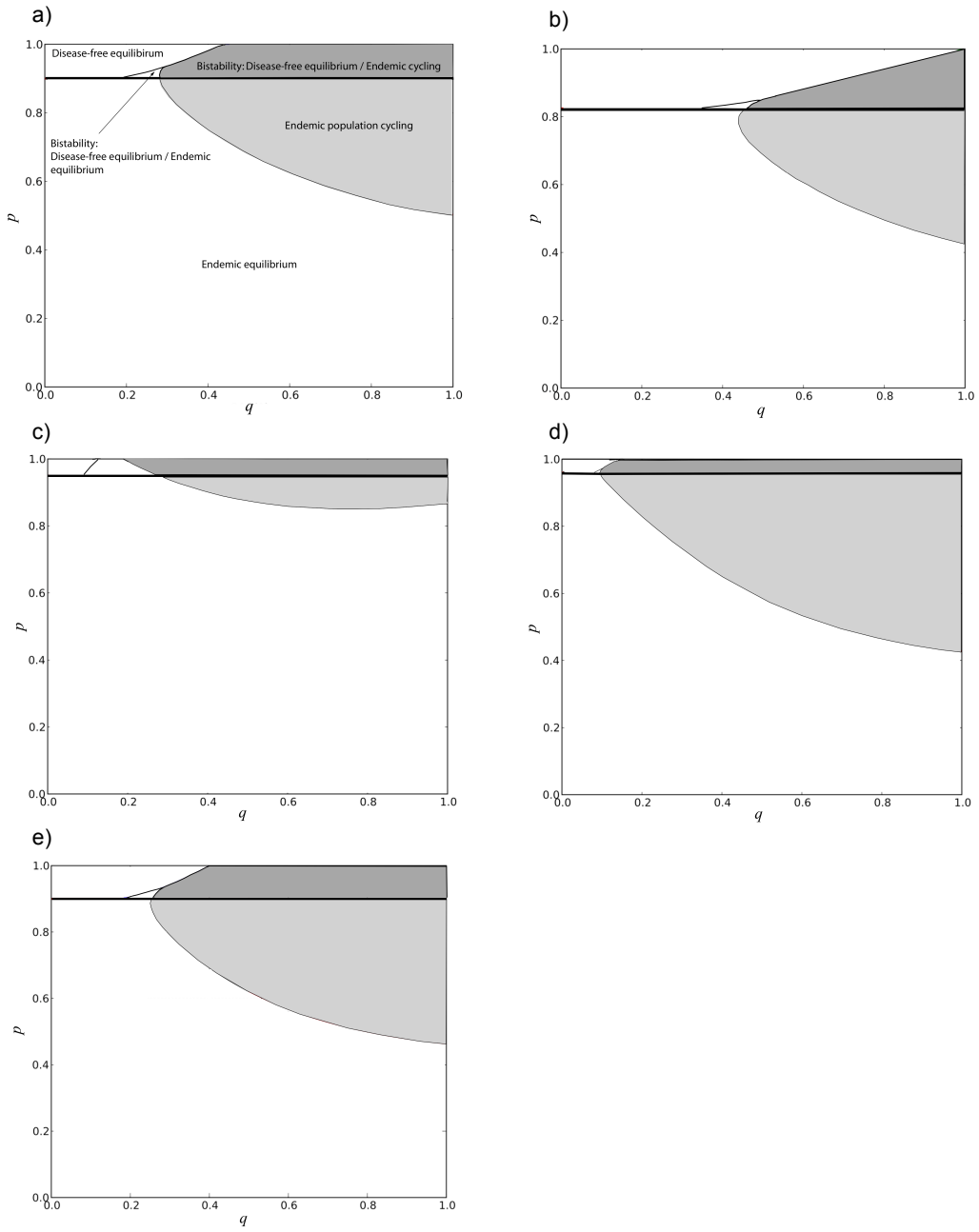


Figure 4.4. Regions in p (degree of priming), q (priming protection) parameter space where the disease–free equilibrium is stable, the endemic equilibrium is stable, the endemic population is unstable and there is bistability. a) Default parameters (a=2, b=1, h=0.1, α=1, β=2, τ=0, f_Y=0.2, f_P=0.5). b) Increased pathogenecity (α=2.5) leads to a reduced range of q over which the endemic population cycles. c) Increased birth rate (a=3) also leads to a reduced region of p over which the endemic population cycles. d) Increased transmission (β=5) leads to a greater region of p, q parameter space over which the endemic population cycles. e) The addition of transgnerational priming (τ=0.5) leads to a slight increase in endemic population cycling region.

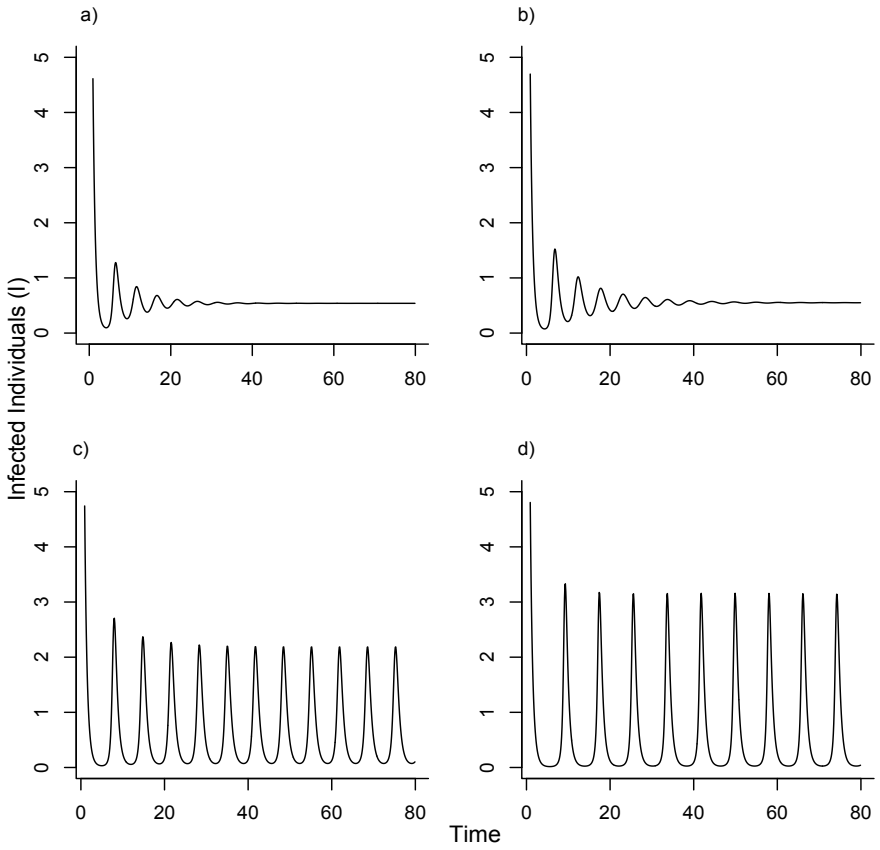


Figure 4.5. The nature of the SPI system dynamics. (Parameters: a=2, b=1, h=0.1, α=1, β=2, τ=0.5, f_Y=0.2, f_P=0.5). a) Endemic equilibrium is reached via damped oscillations when priming is low (p=0.2, q=0.5). b) More prolonged damped oscillations occur when priming is increased within the stable endemic region (p=0.4, q=0.5). c) Limit cycles occur when priming is increased again (p=0.7, q=0.6). d) Limit cycles of greater amplitude but reduced frequency are evident when priming is increased further (p=0.8, q=0.6).

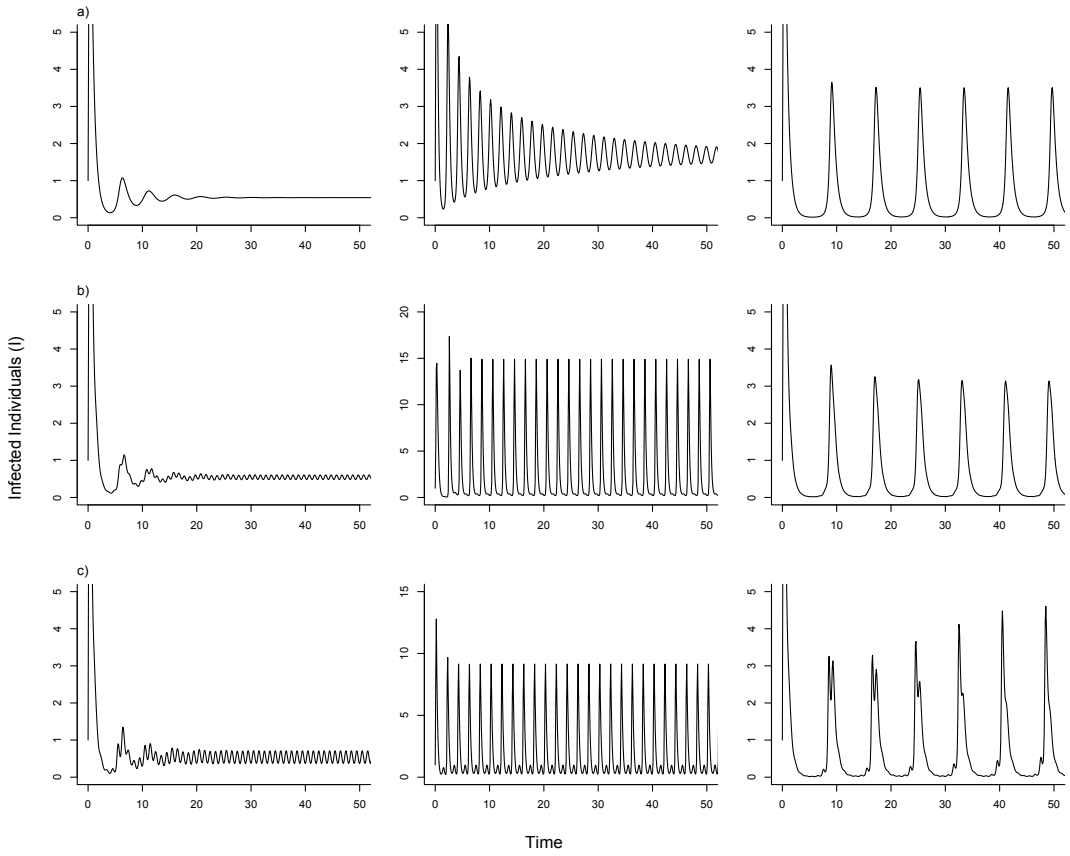


Figure 4.6. The dynamics of the SPI system with seasonality incorporated into the model. On the left, low priming rate (parameters: a=2, b=1, h=0.1, p=0.1, q=0.2, α=1, β=2, τ=0.2, f_Y=0.2, f_P=0.5). In the centre, intermediate priming rate (parameters: a=5, b=2, h=0.1, p=0.5, q=0.7, α=3.5, β=2, τ=0.5, f_Y=0.2, f_P=0.5) and on the right, high priming rate (parameters: a=2, b=1, h=0.1, p=0.8, q=0.7, α=1, β=2, τ=0.5, f_Y=0.2, f_P=0.5). a) Original SPI model with fixed transmission and birth rate. Endemic equilibrium occurs at low priming, prolonged damped oscillations to endemic equilibrium at intermediate priming and endemic limit cycles at high priming. b) SPI model with seasonal birth rate. Small annual cycles occur at low priming, multi-year cycles at intermediate priming and fewer, larger multi-year cycles at high priming. c) SPI model with seasonal transmission rate. Annual cycles occur at low priming rate, large biennial cycles at intermediate priming rate and quasi-periodic cycles at high priming rate.

4.5 Discussion

Using a theoretical model I show how immune priming alters the likelihood of persistence of a parasite and has pronounced destabilising effects on the dynamics of host-parasite populations. Furthermore, I demonstrate that the effects of immune priming depend on, (i) the proportion of susceptible individuals that become primed; (ii) the level of protection on future exposure which immune priming provides and importantly; (iii) the reproductive costs to the host of priming and infection. In addition, seasonality in birth rate and, in particular, transmission further destabilise a system with immune priming. The difference in dynamics between traditional SIR-type models and the SPI model examined here highlights the need to consider immune priming, which may be a widespread phenomenom, and the way in which immunity occurs more generally, when exploring and predicting infectious disease dynamics.

The focus of many host-parasite studies is on the critical value of $R_0 = 1$, where the parasite is able to invade a disease-free host population. In traditional SIR models, this condition is identical to the condition of parasite persistence in an endemic population. However, in the SPI system, I have found that when a large proportion of hosts are immune primed following exposure to a parasite but priming does not result in full immunity, there can be bistability such that the parasite can remain endemic when $R_0 < 1$. This is due to the ability of the parasite to infect primed as well as susceptible hosts. To invade a disease-free population, the parasite still requires $R_0 > 1$. However, if the disease is already endemic, even if the susceptible population is low such that R_0 may be less than unity, the parasite is still able to gain enough infections from primed hosts (albeit at a reduced rate) to persist in the population. This phenomenon has also been found in models which consider vaccination strategies (Arino et al. 2003; Kribs-Zaleta & Velasco-Hernandez 2000). Clearly this result has important consequences for

disease control, since in a system which exhibits immune priming, simply reducing the parasite R_0 to less than unity, may not suffice to eradicate disease.

When disease affects fecundity in the SPI model, and priming rate is high, limit cycles occur, indicating instability. As primed individuals, as well as susceptible individuals, can become infectious in the SPI system the length of a disease epidemic may be longer than expected. This combined with a reduction in replenishment of susceptibles due to fecundity costs means that instead of maintaining a stable endemic equilibrium, limit cycles occur where the population undergoes periodic crashes before increasing again. These limit cycles act as indicators of population instability as theoretically the population crashes precede epidemic fadeouts and with the addition of stochastic events, host extinction. In addition to systems exhibiting immune priming, limit cycles may also be found in systems where transmission is via free-living infectious stages (Anderson & May 1981), infection is sublethal (Boots & Norman 2000) and there is latency between exposure and infection and immunity wanes over time (Greenhalgh 1997). However, limit cycles never result from basic classic models including SI (Susceptible-Infectious), SIS or SIR even when fecundity costs of infection are present. Interestingly, while the advantages of immune priming at the individual level will probably be greatest for hosts which suffer a high cost of infection, results from the model suggest that the destabilising effects of immune priming will be greatest in systems where the host incurs a high reproductive cost of infection. Consideration of the population level effects of priming as well as individual level effects will be important when examining immune priming potential further and in an evolutionary context.

Seasonal fluctuations in environmental factors such as rainfall and temperature (Hoshen & Morse 2004; Pascual et al. 2002; Waller et al. 2004), host demographic factors such as birth and death rate (Altizer et al. 2006; Pathak et al. 2011) and

epidemiological factors such as parasite transmission and host immunity (Bjornstad et al. 2002; Cattadori et al. 2005) are well known to drive population dynamics. I have shown that the inclusion of seasonality in both birth rate and transmission rate add complexity to the population dynamics and add to the instability of the SPI system. Seasonal forcing in transmission rate has a greater impact on the dynamics than seasonal forcing in birth rate. This is concurrent with traditional human epidemiological models and is thought to be due to the fact that changes in transmission effect the system over a shorter time period than changes in birth rate (Altizer et al. 2006).

Evidence for a mechanism behind immune priming in insects is limited. The presence of immune priming has been inferred empirically using many criteria including reduced fecundity costs of the bacteria *Pasteuria ramosa* in *D. magna* from mothers exposed to this bacteria (Little et al. 2003). However, substantial evidence suggests that immune priming does result in reduced prevalence of the parasite. Roth and Kurtz (2009) showed that primed, woodlice, *Porcellio scaber* had greater phagocytotic activity leading to reduced prevalence of bacteria compared to controls. In addition, Tidbury et al. (2011) found reduced probability of infection with an obligate killer following previous exposure and therefore reduced prevalence of the parasite in the population. Also, Sadd and Schmid-Hempel (2006) show increased clearance of bacteria in the haemolymph and therefore reduced prevalence in previously exposed *B. terrestris* compared to controls. This model represents what I see as a natural form of immune priming where density dependent exposure to parasite in the environment does not always result in infectiousness but may increase protection on subsequent parasite challenge. This model is therefore distinct from traditional human epidemiology models, including SIR models, where individuals are infectious prior to developing immunity, SEIR (Susceptible-Exposed-Infectious-Recovered) where there is a time lag between being exposed and becoming infectious, and vaccination models where

individuals become immune independent of the abundance of the parasite in the population. The damped oscillatory dynamics seen with traditional models compared to the cyclic dynamics seen with high priming in the SPI model reported here further highlight how different this model is from more traditional models. I do not investigate the implications of immune priming which occurs following recovery from infectiousness then subsequent exposure to a parasite (i.e. Susceptible-Infectious-Susceptible-Primed). However, I do not expect this to change the qualitative outcome of the SPI model but it presents an interesting avenue for further extension of the model.

For greatest clarity and applicability some important features of invertebrate-parasite interactions have been excluded from the model. Often there is a strong negative correlation between host development stage and susceptibility to parasite infection, with for example, early instar insect larvae being most susceptible and adults being completely resistant (Kirkpatrick et al. 1998; Tate & Rudolf 2011). I find that transgenerational immune priming has less effect on disease prevalence than within generation immune priming but the relative importance of within and transgenerational immune priming has been found to depend on the life-history stage specific nature of the host parasite interaction (Tate & Rudolf 2011). While there is no age structure included in the SPI model future investigations may benefit from the addition of stage structure. Also, I assume, for simplicity a single host-parasite interaction but appreciate that complex multiple-host, multiple-parasite interactions are common in nature (Rigaud et al. 2010). There seems to be a lack of generality in the specificity of immune priming protection with some studies concluding that protection is general (Moret & Siva-Jothy 2003)while some studies find evidence for specificity of protection (Roth & Kurtz 2009; Sadd & Schmid-Hempel 2006). There is also evidence that immune priming to one parasite may actually increase susceptibility to another parasite (Sadd & Schmid-Hempel 2009b). The specificity of immune priming protection is likely to

determine the population level consequences of priming in complex interacting populations of hosts and parasites and requires further empirical and theoretical study.

Our primary focus has been on invertebrate systems, but priming of immunity may be more widespread. The key aspect of immune priming is that, when exposed to a parasite, hosts may quickly become immune and, crucially, without ever having become infectious. Exposure to a low dose of parasites which does not result in infectiousness is likely to feature in many host-parasite interactions across multiple taxa. Although perhaps gaining little attention, instances may exist where humans and other animals test positive for parasite antibodies without any evidence of infectiousness. There is therefore scope for this model to be applied to non-invertebrate systems including humans.

Immune priming has important implications for parasite persistence and host-parasite population dynamics. Immune priming, while beneficial for the individual host, may increase the persistence of parasite and destabilise host parasite populations. This work has made general predictions of when this destabilisation is likely to occur and I emphasise that immune priming needs consideration and inclusion into models of specific disease interactions in order to understand host-parasite dynamics accurately.

Chapter 5: The Costs and Stability of Antiviral Resistance in an Insect

5.1 Abstract

Despite the clear advantage to hosts of being able to resist parasites, striking variation in resistance remains. Costs of resistance are central in explaining this variation with one form of cost manifested as genetic based trade-offs between resistance and other fitness traits. However, despite the role of trade-offs in shaping optimal investment in resistance and maintaining genetic based variation in resistance, our understanding of the context dependence of trade-offs is relatively poor. In particular, it is important to know how the environment mediates trade-offs between fitness traits. Here, using an artificial evolution experiment I examine the evolution of resistance and trade-offs in *Plodia interpunctella* to its natural viral parasite *Plodia interpunctella* Granulosis Virus (PiGV). Specifically, I examine potential costs by comparing development time, weight at pupation and the resulting growth rate in selected insects and control insects raised on high and low quality food. The key result of this study is that I find a genetic based trade-off between antiviral resistance and growth rate that is more pronounced in insects assayed on high quality food. I also examine the stability of resistance in the absence of selection pressure and find that relaxing selection for three generations results in a significant loss of resistance which further emphasises the impact of costs on resistance traits.

5.2 Introduction

Virtually all organisms are subject to attack by micro- and macroparasites, many of which cause significant damage to their host. It is therefore unsurprising that hosts have evolved a diverse range of antiparasitic defences. Insect resistance against parasites is likely the result of a number of different, often interacting, host defences (Schmid-Hempel 2005a; Schmid-Hempel 2012; Siva-Jothy et al. 2005). These may include parasite avoiding behaviors (Lefevre et al. 2012), physiological and chemical barriers against parasite invasion (Levy et al. 2011) and the complex and dynamic immune system comprising cellular, humoral and intracellular components (Rolff & Reynolds 2009; Strand 2008). However, these parasite defences may be costly, which may explain why, despite the clear fitness advantages to the host of being able to resist parasites, there remains striking variation in levels of resistance seen in nature (Schmid-Hempel 2003; Sheldon & Verhulst 1996; Stearns 1989).

When faced with parasite attack, hosts may upregulate their defences and may incur costs as a result of physiological trade-offs, often related to limited resources. For example, reduced body size and fecundity was found in female *Drosophila melanogaster* hosts following the activation of the immune reponse (Fellowes et al. 1999a) and in the land snail *Cornu aspersum* maxima, challenge with heat killed bacteria resulted in reduced growth (Fevrier et al. 2009). In addition, activation of the antibacterial immune response using immune elicitors lead to a subsequent reduction in survival in the bumble bee *Bombus terrestris* under starvation (Moret & Schmid-Hempel 2000). Physiological costs have also been examined by assessing defence in individuals investing in other energy demanding activities. For example, mated mealworm beetles, *Tenebrio molitor*, had reduced haemolymph phenoloxidase (PO) levels compared to non mated beetles (Rolff & Siva-Jothy 2002). The defences

upregulated on parasite attack may also cause immunopathology (Graham et al. 2005; Long & Boots 2011), directly damaging host tissue. For example, in *T. molitor* a direct cost of upregulation of the PO cascade is the damage by melanisation of the malpighian tubules (Sadd & Siva-Jothy 2006).

Costs may also result from the evolution and maintenance of the machinery necessary for resistance. These costs are incurred by the host in the absence of the parasite and termed evolutionary costs (Schmid-Hempel 2003). In particular, increased resistance to parasites may be traded-off with other fitness traits due to the presence of negative genetic correlations through antagonistic pleiotropy. Breeding design experiments are one method that can be used to examine the genetic correlations between different fitness traits thereby indicating potential genetic trade-offs. For example, Cotter and Wilson (2002) used a breeding design to assess genetic correlations between a number of immune and life-history traits in the Egyptian cotton leafworm, *Spodoptera littoralis*. They demonstrated significant negative genetic correlations between development time and phenoloxidase activity and between development size and haemocyte number (Cotter & Wilson 2002).

Artificial selection experiments provide an alternative means to breeding experiments to examine potential genetic trade-offs between parasite resistance and other fitness traits (Conner 2003). This method has been used to identify a genetic trade-off between fecundity and resistance to the trematode *Schistosoma mansoni* in the fresh water snail *Biomphalaria glabrata* (Webster & Woolhouse 1999) and a genetic based trade-off between larval competitive ability and resistance to the parsitoid *Asobara tabida* in in *D. melanogastor* (Kraaijeveld & Godfray 1997). In addition, some of the first examples of artificial selection studies highlighted genetic trade-offs between resistance to virus and other fitness traits in moth hosts (Boots & Begon 1993; Fuxa et al. 1988; Fuxa & Richter 1998).

However, evidence for the presence of genetic based trade-offs in resistant hosts is not ubiquitous. For example, cabbage loopers, *Trichoplusia ni,* selected for resistance to *T. ni* NPV (TnSNPV) did not differ in either their fecundity or development compared to control insects (Milks et al. 2002) and *Daphnia magna* selected for resistance to the sterilizing bacterial parasite, *Pasteria ramosa,* did not differ in their competitive ability or other life-history traits compared to control insects (Little et al. 2002). It is well documented that phenotypic traits my be determined by both genetics and environment (G x E interaction) so that the same genotype can produce different phenotypes in different environments (Falconer 1981). So as well as physiological trade-offs, the expression of genetic based trade-offs between different traits, may be highly dependent on environment (Gillespie & Turelli 1989; Sgro & Hoffmann 2004; Stearns et al. 1991). For example, a trade-off with fecundity in *D. melanogaster* selected for resistance to the bacterial parasite *Providencia rettgeri*, was lost when flies were fed a high quality diet enriched with protein (McKean et al. 2008). Furthermore, selection for increased phenoloxidase activity was only traded-off with reduced longevity in yellow dung flies, *Scathophaga stercoraria*, under starvation (Schwarzenbach & Ward 2006). In both of these cases the genetic trade-offs are only apparent under poorer resource conditions. It is clear therefore that in order to fully understand the costs associated with the evolution of resistance we need more information on how genetic trade-offs are influenced by the host resource and environment more generally.

Costs maintain variation in traits by preventing their evolution but also because costly traits which have evolved are lost in the absence of selection pressure (Schmid-Hempel 2003; Stearns 1989), so that for example, in the absence of the parasite, host resistance is lost. However, numerous studies have highlighted that antibiotic resistance is not lost in bacteria even in the absence of antibiotics because the costs may be compensated for by further adaptation by bacteria (Lenski 1998; Schulz zur Wiesch et

al. 2010). The loss of resistance to parasites in the absence of selection pressure is not often examined in insect systems (but see Fuxa 1989) with little knowledge of potential compensation of costs or the extent to which resistance may be lost and the time it may take.

Here, using an artificial evolution experiment I examine the costs of resistance to virus in the Lepidopteron host *Plodia interpunctella*. Specifically, I examine a genetic based trade-off between resistance and growth rate by comparing growth rate in insects selected for resistance and controls insects. To determine the effect of food quality on the trade-off, growth rate was measured in selected and control insects raised to pupation on low or high quality food. In addition I examine whether resistance is lost in insects relaxed from selection pressure. I find that the trade-off between antiviral resistance and growth rate is more pronounced in insects raised on high quality food. I also find that relaxing selection for three generations resulted in a significant loss of resistance further supporting the impact of costs on resistance traits.

5.2 Materials and methods

5.2.1 The insect-virus system

The Indian meal moth, *P. interpunctella,* is a pest of stored agricultural products and an ideal insect model organism as its natural environment can be accurately mimicked in the laboratory. Insects were reared in 1 litre Nalgene screw top tubs, on a cereal based diet consisting of 50% Ready Brek ©, 30% bran and 20% rice, with 20g yeast, 0.2g sorbic acid, 0.2g methyl paraben, 25ml honey and 25ml glycerol added to 100g of cereal mix. Insects were kept at 27°C in a 16:8 h light dark regime.

P. interpunctella Granulosis Virus (PiGV) is a natural viral pathogen of *P. interpunctella* and belongs to the group of arthropod viruses called baculoviruses. PiGV is naturally transmitted through oral ingestion of virus particles. Virus occlusion bodies

enter the midgut where their protein coat is dissolved, releasing virions into the midgut cavity which then fuse with epithelial microvilli and enter the midgut epithelial cells. Once the virus has passed through the midgut, virus proliferation in secondary tissue such as the fat body occurs, leading to cell lysis, tissue destruction and ultimately host death. *P. interpunctella* larvae infected with PiGV are white and therefore easily distinguishable from healthy larvae which are pale brown. PiGV symptoms in *P. interpunctella* are present 7-8 days following oral challenge with virus at third instar. Once symptomatic, PiGV infected larvae die before pupation. Purified PiGV solution was produced by centrifugation of a homogenate of infected insects (Smith & Crook 1988, Appendix 1a, 1b).

5.2.2 Artificial selection for resistance to virus

Antiviral resistance was artificially selected for in *P. interpunctella* by raising larvae on food containing PiGV. Virus selection lines were created by placing 35 adults onto 40g food, previously mixed with PiGV aliquoted from a bulk stock, for 24 hours to mate and lay eggs. The volume of PiGV solution added to food was previously estimated to result in infection of approximately 50% of larvae. Virus solution was added to the glycerol component of the food mix, which was then combined with the remaining insect food ingredients to ensure thorough mixing of virus through the food. Each generation, the adults that were first to emerge (~10% of total) were discarded and the next 35 newly emerged insects were transferred to 40g fresh food containing virus aliquoted from the same bulk stock, for 24 hours to mate and lay eggs. Selection lines were set up at 3 different time points (3 experimental blocks) with 2 virus selection line replicates and 1 control selection line replicate (maintained in the

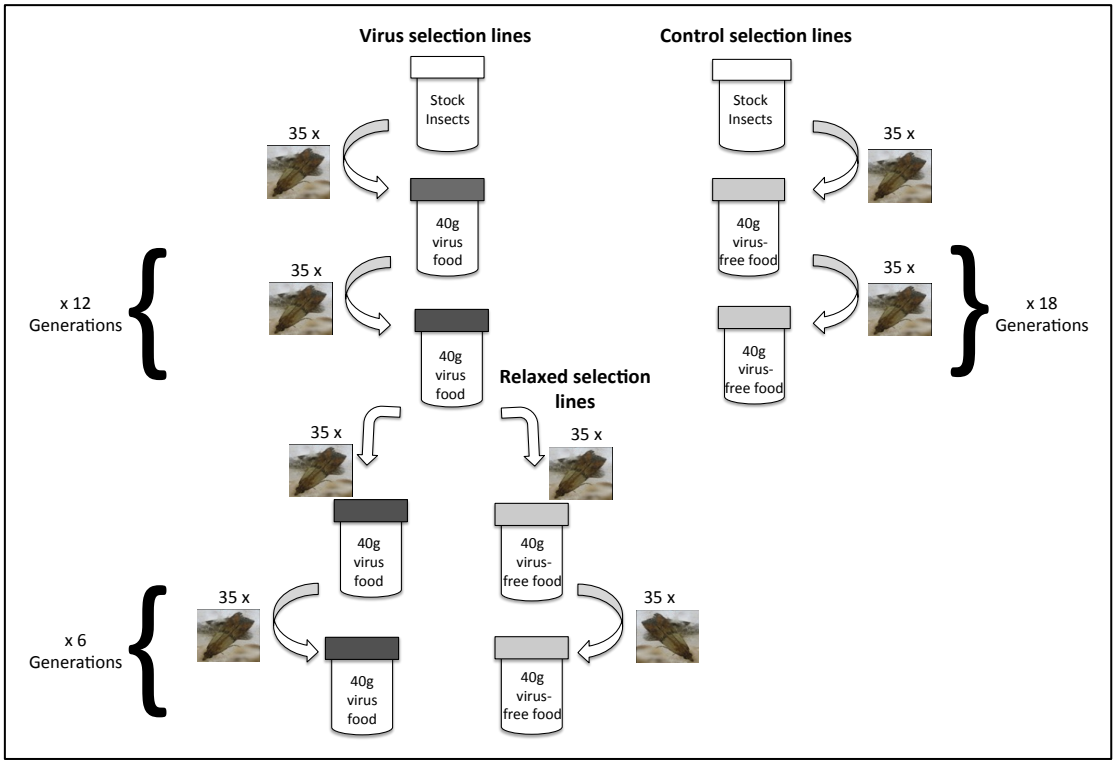


Figure 5.1. Protocol for artificial selection of antiviral resistance. To establish each virus selection line, 35 stock insects were placed on 40g food previously mixed with virus aliquoted from a bulk stock solution estimated to infect 50% of larvae. Lines were maintained by placing 35 adults onto 40g food mixed with virus aliquoted from the same bulk stock solution. Following 12 generations of selection with virus, each line was split into two sublines. One subline was maintained under continued selection pressure as previously for a further 6 generations. The other subline, the relaxed selection line, was relaxed from selection pressure and maintained on food containing no virus for 6 generations. Control selection lines were established by placing 35 stock insects onto 40g virus-free food. Control selection lines were maintained for 18 generations by transferring 35 adults from each generation onto 40g virus free food. This schematic represents the procedure for one virus selection line and one control line. The selection procedure was repeated over 3 experimental blocks set up at different time points with 2 virus selection lines and 1 control line per experimental block.

same way but on virus-free food) in each block. Insects from different selection line replicates were never mixed (figure 5.1).

5.2.3 Relaxation of selection pressure

After 12 generations, virus selection lines were split into 2 sub-lines. In one sub-line the selection pressure was continued as detailed above, whereas in the other sub-line the selection pressure was relaxed. Relaxed selection sub-lines were maintained in the same way as control selection lines, by placing 35 adults on 40g virus-free food for 24 hours to mate and lay eggs (see figure 5.1).

5.2.4 Determining antiviral resistance and its dose dependency

Following 12 generations of selection, insects were challenged with virus to determine the level of antiviral resistance. Experimental insects were established by placing 35 12^{th} generation selection line adults and 35 12^{th} generation control adults separately onto 40g virus-free food. F2 generation experimental insects were established in the same way. Excluding virus from food on which experimental insects were raised ensured that maternal effects were removed and infection following viral challenge could be attributed to previous long term selection. When F2 experimental insects produced from both virus selection lines and control selection lines reached third instar (11 days) they were removed from the food, kept separate and starved for two hours. Insects were orally inoculated with one of 11 virus doses: 0.001, 0.1, 0.5, 1, 2, 7, 10, 20, 40, 60 and 80% virus solution (diluted in blue food dye), using a pipette according to the standard oral bioassay procedure (Boots & Begon 1993, Appendix 1d). Approximately 25 selected and control larvae successfully inoculated with each virus dose were then transferred to separate cells of a 25-cell petri dish with abundant resource. Selected and control insects were also inoculated with control food dye

solution to ensure that there was no contamination and no vertical transmission of the virus in the virus selected insect lines. Insects were examined for viral symptoms 7-8 days following oral inoculation.

In addition, following 15 generations of selection (and 3 generations of relaxation) resistance in insects from continued virus selection, relaxed selection and control selection lines was assayed. Experimental insects were established by placing 35 adults from each selection, relaxed and control line separately onto 40g virus-free food. F2 generation experimental insects were established in the same way. Excluding virus from the F1 and F2 insect food ensured that maternal environment did not affect resitance. When F2 experimental insects from virus selection, relaxed and control lines reached third instar (11 days) they were removed from the food, kept separate and starved for two hours. Larvae were then orally inoculated, using the standard oral bioassay procedure, with virus solution of concentration estimated to infect 50% of stock larvae. Approximately 25 successfully inoculated larvae from each line were then transferred into single cells of a 25-cell petri dish with abundant food. 7-8 days following oral inoculation larvae were examined for the presence of viral infection symptoms. Following 18 generations of selection (and 6 generations of relaxation), exactly the same procedure was repeated.

5.2.5 Trade-offs associated with selection for antiviral resistance

Time to pupation and weight at pupation and the resulting growth rate was compared in insects subject to 18 generations of selection with virus and control insects. 35 18^{th} generation adults from each selection and control line were placed onto 40g virus-free food to mate and produce F1generation development assay insects. F2 generation assay insects were then established and raised on both high quality (as before) and low quality food comprising 50% methyl cellulose and 50% high quality food. On reaching the

fourth instar (14 days) these insects were transferred individually to 9ml screw top plastic tubes containing the same food quality as previously raised on. Approximately 25 insects per selection treatment (virus selection or control) per food quality (high quality or low quality) were transferred to these tubes. From day 17 onwards, tubes were checked daily for the presence of hardened pupae. Time to pupation and weight at pupation were recorded for each insect. Growth rate for each insect was then calculated by dividing pupal weight by time to pupation.

5.2.6 Statistical analysis

Evolution of antiviral resistance and its dose dependency

The relationship between virus dose and infection in selected insects and control insects was analysed using a generalized linear model (GLM) with binomial errors and a logit link function in R (version 2.14). The residual deviance of the model was reduced by log transforming the explanatory variable dose, and quasibinomial errors were used in the final model to account for the remaining overdispersion. Initially the main effect of virus dose, selection treatment (virus selection, control), experimental block and an interaction between virus dose and selection treatment was investigated. Experimental block was considered a fixed effect because there were only three and therefore it is hard to estimate its variance as a random term. There was no significant interaction between virus dose and selection treatment ($F_{1,83}$=1.28, p=0.26) and no difference in infection due to experimental block ($F_{2,84}$=1.09, p=0.34) . The final model therefore contained only the explanatory variables virus dose and host selection treatment.

Trade-offs associated with antiviral resistance

The effect of selection treatment (virus selection and control) and food quality (high quality and low quality) on age at pupation was investigated using a general linear

model in R (version 2.14). In addition, the effect of experimental block and an interaction between selection treatment and food quality was considered. However, the interaction between the selection treatment and food quality was removed from the final model as it was not significant ($F_{1,156}$=0.392, p=0.53).

Similarly, the effect of selection treatment and food quality on weight at pupation was determined using a general linear model. The effect of experimental block and an interaction between selection treatment and food quality was also investigated. However, experimental block ($F_{2,159}$=0.494, p=0.61) and an interaction between selection treatment and food quality ($F_{1,156}$=1.20, p=0.28) were removed from this model as they were non significant.

Finally the effect of selection treatment and food quality on growth rate was investigated. The effect of experimental block and an interaction between selection treatment and food quality on growth rate was also considered. However, experimental block ($F_{2,159}$=1.46, p=0.23) and the interaction between selection treatment and food quality ($F_{1,156}$=2.64, p=0.11) were both removed from the model as their effect on growth rate was non significant

Relaxation of selection pressure

To determine whether infection differed between insects maintained in continued selection pressure, insects relaxed from selection and controls insects data were fitted to a GLM with quasibinomial errors and a logit link function using R. An initial model incorporated the explanatory variables; experimental block, selection/relaxation time (15 generations of continued virus selection/12 generations of virus selection followed by 3 generations of relaxation and 18 generations of continued selection/ 12 generations of virus selection followed by 6 generations of relaxation from selection), selection treatment (continued selection, relaxed selection and control) and finally an interaction

between selection treatment and selection time. There was no significant interaction between selection treatment and selection time ($F_{1,22}$=0.671, p=0.52) so infection data from both selection times were left pooled for further analysis and the interaction term removed from further models. Experimental block ($F_{2,27}$=0.69, p=0.51), and selection time ($F_{1,26}$=0.011, p=0.92) were also removed from the model as they did not explain a significant amount of variation in the model, leaving only the main effect of selection treatment in the final model.

Selection treatment was found to have a significant effect on infection and to confirm whether insects relaxed from selection were significantly more susceptible to infection than insects maintained in selection pressure a separate GLM for these data only was analysed. In addition to selection treatment (continued selection and relaxation from selection pressure), the explanatory variables; experimental block ($F_{2,21}$=0.819, p=0.46), experimental line (which was nested with experimental block) ($F_{3,16}$=0.899, p=0.47) and selection time ($F_{1,20}$=0.170, p=0.68) were investigated. However, only the variable selection treatment remained in the final model as all other explanatory variables were non-significant.

Finally, infection in insects from control selection lines and insects from stock populations was compared using a GLM to determine whether the selection line procedure itself affected resistance. Again, in addition to host treatment (control selection and stock) the explanatory variables; experimental block ($F_{2,9}$=3.32, p=0.11), selection time and an interaction between selection time and host treatment ($F_{2,6}$=2.26, p=0.18), were incorporated into the initial model. However, removal of the non-significant interaction and explanatory variables left only host treatment and selection time in the final model.

5.3 Results

5.3.1 Virus dose response in insects selected for antiviral resistance

Infection in insects selected for antiviral resistance was significantly lower (~10%) than infection in control insects ($F_{1,87}$=402, p<0.001) (figure 5.2). Infection was also significantly affected by virus dose ($F_{1,86}$=38.6, p<0.001) and as there was no interaction between virus dose and host selection treatment this dose-dependency did not change in virus selected insects. I therefore show the selection with virus has resulted in the evolution of resistance rather than heterogeneity in the dose response.

5.3.2 Trade-offs associated with antiviral resistance

Food quality had a significant effect on time to pupation and weight at pupation. Insects raised on low quality food took significantly longer to reach pupation than insects raised on high quality food ($F_{1,156}$=376, p<0.001) and insects raised on low quality food were significantly lighter at pupation compared to insects raised on high quality food ($F_{1,157}$=54.9, p<0.001). Insects selected for antiviral resistance did not differ in their age at pupation compared to control insects ($F_{1,158}$=0.616, p=0.43). However, on high quality food there is a trend suggesting that insects selected for resistance may take longer to reach pupation (figure 5.3). This trend is absent in insects raised on low quality food. Experimental block had a significant effect on age at pupation ($F_{2,159}$=4.03, p=0.02). Insects selected for resistance to virus were significantly lighter than control insects ($F_{1,162}$=5.79, p=0.017). There was no significant interaction between food quality and selection treatment but the effect of selection treatment seems to be more

pronounced when insects are raised on high quality food rather than low quality food (figure 5.4).

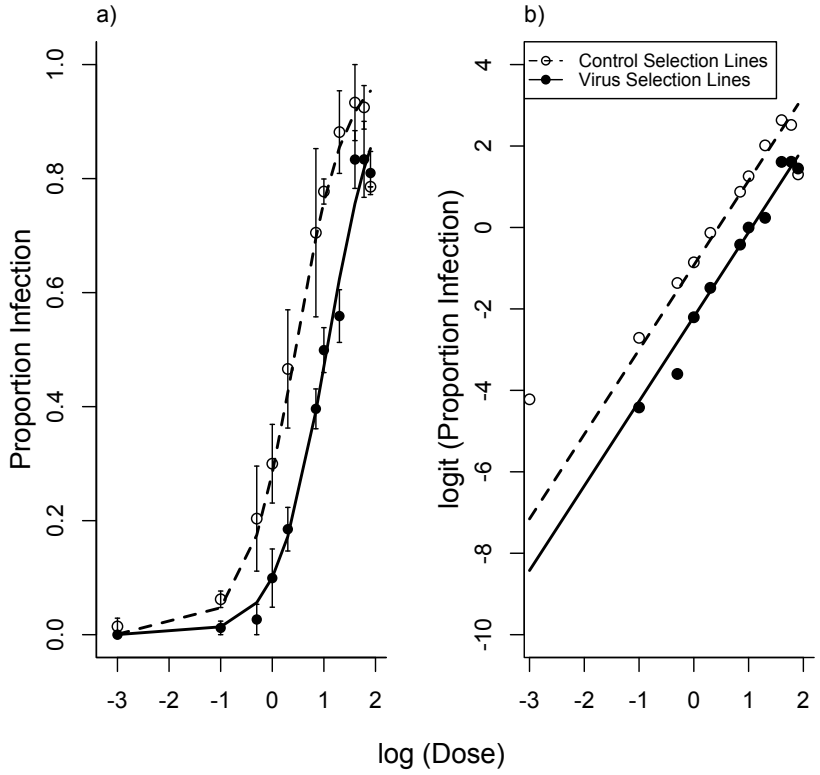


Figure 5.2. Dose response to orally incoculated PiGV in insects selected for antiviral resistance and control insects. a) Dose response relationship illustrated in sigmoidal form with proportion infection corresponding to logged virus dose. Points represent means ± s.e. and lines represent model predictions. b) Linearly transformed dose response relationship on which the logistic regression analysis was performed. Logit transformation involves taking the log of the infection odds so that logit(P) = log P/ (1-P), where P is proportion infection. Equations of the logistic regression curves produced by the model are y=2.0764x - 0.9273 for control insects and y=2.0764x - 2.19 for selected insects. Infection was dose dependent ($F_{1,86}$=38.6, p<0.001) and infection in selected insects was significantly lower than infection in control insects ($F_{1,87}$=402, p<0.001). However as indicated by the parallel lines, the dose dependency of infection did not differ between selected insects and control insects ($F_{1,83}$=1.28, p=0.26).

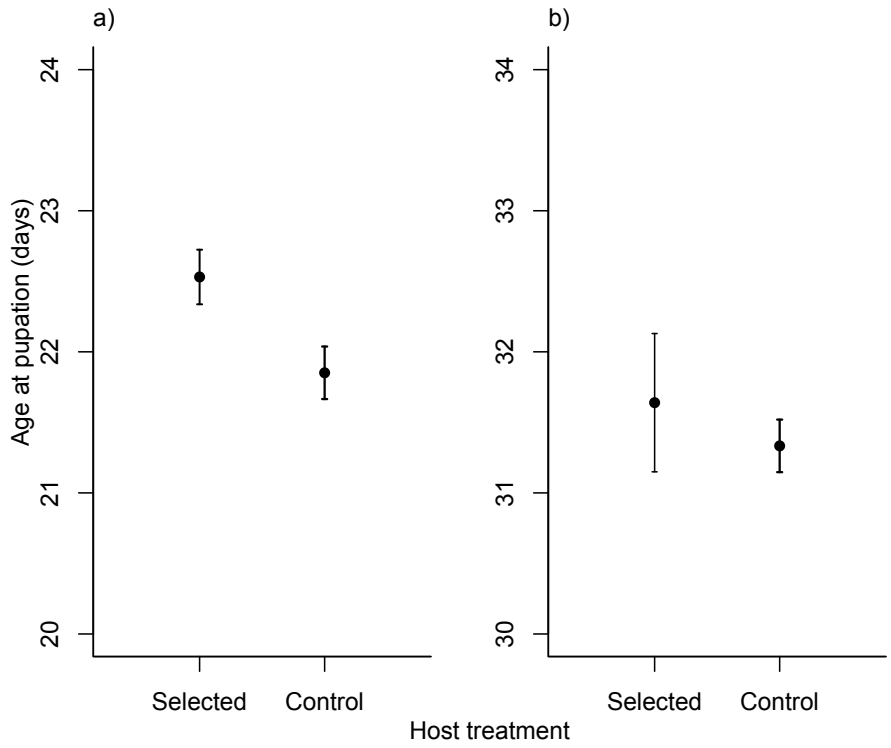


Figure 5.3. Age at pupation of selected and control insects raised on a) high quality food and b) low quality food. Insects raised on low quality food took significantly longer to reach pupation ($F_{1,156}$=376, p<0.001) than insects raised on high quality food. Selected and control insects did not differ significantly in their age at pupation ($F_{1,158}$=0.616, p=0.43). Filled circles represent means ± s.e.

Insects selected for resistance to virus had a significantly lower growth rate compared to control insects ($F_{1,163}$=7.20, p=0.008) and insects raised on low quality food had a significantly lower growth rate compared to insects raised on high quality food ($F_{1,162}$=187, p<0.001) (figure 5.5). The lack of selection treatment by food quality interaction ($F_{1,156}$=2.64, p=0.11), suggests that the effect of treatment did not differ significantly between insects raised on high quality food and insects raised on low quality food. However, the data suggests that the reduction in growth rate in selected insects compared to control insects was more pronounced on high quality food.

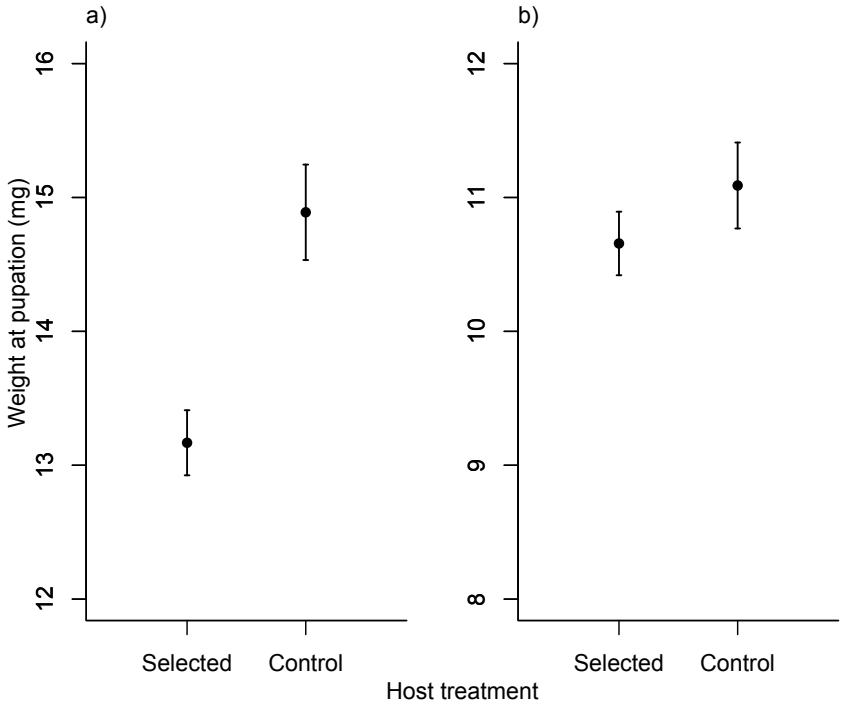


Figure 5.4. Weight at pupation of selected and control insects raised on a) high quality food and b) low quality food. Insects raised on low quality food were significantly lighter at pupation compared to insects raised on high quality food ($F_{1,157}$=54.9, p<0.001). Insects selected for resistance to virus were significantly lighter than control insects ($F_{1,162}$=5.79, p=0.017). Filled circles represent means ± s.e.

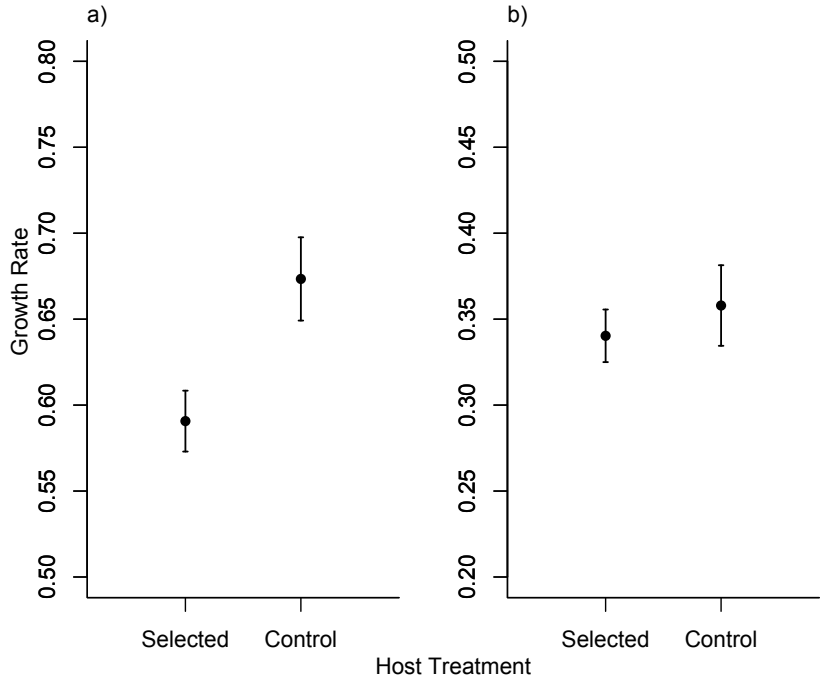


Figure 5.5. Growth rate of selected and control insects on a) high quality food and b) low quality food. Insects selected for resistance to virus had a significantly lower growth rate compared to control insects ($F_{1,163}$=7.20, p=0.008) and insects raised on low quality food had a significantly lower growth rate compared to insects raised on high quality food ($F_{1,162}$=187, p<0.001). Filled circles represent means ± s.e.

5.3.3 Relaxation of selection pressure

Infection was significantly different in insects from continued virus selection, relaxed selection and control selection lines ($F_{2,27}$=30.23, p<0.001) (figure 5.6). This trend was consistent for data taken at both selection (and relaxation) time points as indicated by the lack of interaction between selection time and treatment. Infection in insects relaxed from selection pressure was significantly different to infection in insects maintained under conditions of continued selection pressure ($F_{1,22}$=16.70, p<0.001). Specifically, relaxation of selection for 3 months resulted in a 16.5% increase in mean susceptibility and relaxation of selection for 6 months resulted in a 19% increase in mean

susceptibility compared to insects maintained in continued selection pressure. Infection in selection control insects did not differ significantly from infection in stock insects ($F_{1,9}$=1.94, p=0.20), but infection in stock and control insects was significantly different at the two selection times ($F_{1,10}$=6.13, p=0.035).

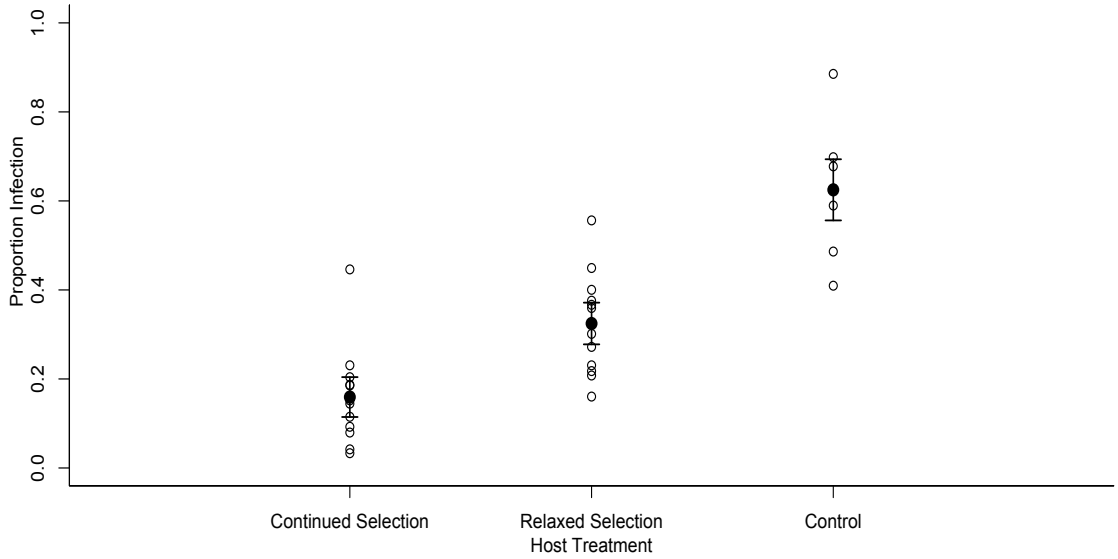


Figure 5.6. Infection following oral inoculation in insects from continued virus selection, relaxed selection and control selection lines. Data collected at both selection time points are pooled as analysis revealed no significant interaction between selection treatment and selection time point. Infection different significantly between insects maintained on each selection treatment ($F_{2,27}$=30.2, p<0.001). Specifically, infection in insects relaxed from selection pressure was significantly greater than infection in insects maintained in selection pressure ($F_{1,22}$=16.7, p<0.001). Open circles represent treatment replicates and filled circles represent means ± s.e.

5.4 Discussion

I show that resistance to PiGV in *P. interpunctella* can be selected for and therefore is underpinned by heritable genetic material. I also show that while selection increases

resistance, it does not change the dose dependency of the response. I find that insects selected for resistance are smaller and have a lower growth rate compared to controls. I show that the reduction in size and growth rate in selected insects may be more pronounced when insects are raised on high quality food rather than, as expected, on low quality food. In addition, I find that in the absence of selection pressure, susceptibility to PiGV increases significantly in three generations. This shows that the costs associated with resistance are strong enough to result in its loss in the absence of a parasite. This study therefore shows that resistance to virus can evolve in *P. interpunctella* but it may be constrained by trade-offs that are context dependent.

I find that infection in selected insects and control insect increases with virus dose but that infection in selected insects is consistently lower than infection in control insects. The relative increase in resistance seen in selected insects compared to control insects is therefore independent of virus dose. This is important because it shows that I have selected for resistance rather than heterogeneity in response to virus dose. Clearly within any artificial selection procedure there is a fairly constant dose of pathogen to which the host population is exposed over multiple generations. An increase in resistance to one virus dose may therefore represent a dose specific increase in resistance in response to a specific selection pressure. By assessing resistance over multiple doses I show that this is not the case. In addition, the results highlight that the increase in resistance following selection is consistent over a wide range of doses. It seems unlikely therefore that the mechanism of resistance is an incompatibility between host and virus. The peritrophic membrane which separates the gut lumen from the gut epithelial cells may provide a possible resistance mechanism in *P. interpunctella* and has been proposed as important in the resistance of Lepidopteron hosts to orally ingested virus (Levy et al. 2011). The quantitative nature of the response that we see is consistent with this type of mechanism.

The occurrence of genotypic trade-offs is central to life-history theory (Schmid-Hempel 2003; Sheldon & Verhulst 1996; Stearns 1989). These genetic based trade-offs result when genes linked to resistance traits have a negative effect on other host fitness traits. By showing that insects selected for resistance to virus are lighter than control insects and therefore when combined with development time, have a significantly reduced growth rate I provide evidence for a trade-off in *P. interpunctella* between resistance to virus and development size. As size is often closely correlated with fecundity(Calvo & Molina 2005; Honek 1993; Rasotto et al. 2010), this trade-off may have significant consequences for individual insect fitness and host population size. Previous studies on *P. interpunctella* have also indicated a trade-off between resistance to virus and developmental traits. However, in contrast to the present study, where no significant difference between age at pupation between selected and control insects was found, these studies found that resistant insects took significantly longer to reach pupation compared to controls (Boots 2011; Boots & Begon 1993). Age and weight at pupation were measured on the same individual in this study and age and weight at pupation are often correlated in insects. It is therefore useful to examine the individual effects of resistance on age at pupation and weight at pupation but also in addition, combine these measures into one value, growth rate, for each individual. It could be argued that growth rate is the best measure of cost not least because it is more robust statistically to examine the response of this univariate trait rather than the two correlated components of it. However it is also useful to examine the response of the underlying traits. Overall however all of the selection experiments that have demonstrated costs to resistance in *P. interpunctella* have shown that there is reduced growth rates in resistant lines. The strongest cause was size at pupation in this study but development time in previous studies (Boots & Begon 1993; Boots 2011).

Trade-offs between development and antiviral resistance are not ubiquitous in Lepidopteron insects. For example, *T. ni* selected for resistance to its baculovirus TnSNPV showed no reduction in fecundity or difference in development compared to controls (Milks et al. 2002). In addition to development, trade-offs may occur between parasite and parasitoid resistance and other traits including longevity, larval viability (Ye et al. 2009) and reproduction (Luong & Polak 2007) . Therefore a complete understanding of the extent of trade-offs associated with resistance in *P. interpunctella* will require the study of other fitness traits.

The key result of this study is that the trade-off seen between resistance and weight at pupation, and therefore growth rate, is more pronounced on high quality food than low quality food. In contrast to the result of the present study, trade-offs between resistance and other fitness traits have been most evident in a lower quality environment (e.g. Boots 2011; McKean et al. 2008; Schwarzenbach & Ward 2006). However, I see the opposite trend. It is possible that while trade-offs between resistance and other fitness traits are not evident in high quality resources as the negative effect of resistance on other traits is buffered by resource, costs of resistance are not visible in low quality environment as they are clouded by the large costs associated with the poor environment itself. For example, in this study, growth rate is almost halved in control insects on low quality resource compared to insects on high quality resource. The potentially smaller reduction in growth rate due to selection may therefore not be seen on poor quality resource. Previous work on this system has shown that greater resistance evolves in hosts selected on high quality food compared to low quality food (Boots 2011) providing further support for the occurrence of costly resistance in this system but indicating that, in contrast to what I find, the costs are less on high quality food allowing more resistance to evolve. In addition, Boots (2011) showed that the costs of evolving resistance on low quality food were more pronounced when these

insects were raised for a generation on high quality food than when these insects were raised on the low quality food they were selected in. The explanation given for this was that insects had adapted to their poor environment however, in light the current study, it is possible that the reduced costs seen in insects raised on poor environments was a consequence of the poor environment itself limiting the detection of costs present. Overall the results of these studies show that costs to resistance may be resource dependent both in terms of the selection and the assay environment.

As important as the presence of trade-offs between life-history traits for predicting the evolution of traits, is the shape of the trade-off relationship (Boots & Haraguchi 1999; Hoyle et al. 2008). For example, using a theoretical approach, Boots and Haraguchi (1999) showed that intermediate resistance evolves when hosts suffer increasing costs as resistance increases, whereas if costs decrease with increasing resistance the hosts will evolve either high levels of resistance or minimal resistance. There is however, little empirical evidence for the shape of trade-off relationships. Despite evidence for a cost of resistance, with little information about the mechanisms behind antiviral resistance in *P. interpunctella*, predicting the shape of potential trade-offs between resistance and development and therefore the evolutionary trajectory of this trait is difficult. If a gut based barrier is the key to antiviral resistance in *P. interpunctella* it may be expected that the trade-off shape between resistance and development traits may be convex, so that increasing resistance is increasingly costly leading to the evolution of an intermediate level of resistance in the population. The fact that I find an intermediate level of resistance following selection in this study and Boots (2011) find that individuals selected for resistance on low resources show increased costs support the hypothesis that the shape of the trade-off between resistance and development is convex. However further study is clearly needed to identify the specific nature of the trade-offs we see in this system.

Baculoviruses have the potential to be used as biological control agents of Lepidopteron pest species. This study and related studies demonstrate that the evolution and implications of baculovirus resistance in these insects will be variable and influenced by environmental heterogeneity. Specifically, the findings from this study and a previous study by Boots (2011) indicate that in *P. interpunctella* feeding on high quality resource, viral infection may be less but costs of resistance may be greater, impacting host population sizes, whereas in insects feeding on low quality resource infection may be greater but lower costs of resistance may be incurred. Overall this study highlights that while resistance to virus can evolve in response to selection it may carry costs and these costs may depend on the resources available to the insect. I therefore demonstrate that the optimum level of resistance in *P. interpunctella* to PiGV may be determined by the complex interaction between selection pressure, costs and environment.

Chapter 6: The Specificity of Antiviral Resistance in a Lepidopteron Insect: Cross Resistance against Multiple Baculoviruses.

6.1 Abstract

The specificity of evolved resistance has important implications for disease emergence, the maintenance of genetic diversity and the epidemiology of disease. Here, I examine *Plodia interpunctella* hosts resistant to its natural viral parasite *Plodia interpunctella* Granulosis Virus (PiGV) (Baculovirus) for cross resistance to a different strain of PiGV and a different baculovirus *Ephestia cautella* Nucleopolyhedrosis virus (EcNPV). In addition I examine the effect of relaxing selection pressure on resistance to these three baculoviruses. I find no evidence for specificity in resistance with resistance to PiGV conveying resistance to a different strain of PiGV and EcNPV. Resistance to all three viruses is significantly reduced in insects relaxed from selection pressure for 6 generations. I therefore provide evidence that antiviral resistance in *Plodia interpunctella* is non-specific but costly.

6.2 Introduction

The persistent threat of parasite attack presents a strong selection pressure on hosts resulting in the evolution of a range of defence mechanisms (Schmid-Hempel 2012). Non-immunological defences include avoidance behaviour (Lefevre et al. 2012; Parker et al. 2010) physical barriers (Levy et al. 2011; Schmid-Hempel 2005a) and the antiparasitic action of bacterial symbionts (Hedges et al. 2008; Moreira et al. 2009). Hosts are of course also armed with an immune system, which often acts in combination with other defences preventing the invasion of parasites or limiting their

proliferation. Anti-parasite defences and immune components exhibited by vertebrates are well studied (Murphy et al. 2011). However, our understanding of the defences and immune function of invertebrates is less comprehensive and our knowledge of defences against viral parasites is especially limited (Kemp & Imler 2009).

A number of potential antiviral defences have been highlighted in insects. These include general non-immunological barriers such as the insect cuticle, the unfavourable conditions and digestive components of the gut (Keating et al. 1990; Nakazawa et al. 2004; Ponnuvel et al. 2003) and the peritrophic membrane which separates the gut lumen from the migut epithelial cells (Gullan & Cranston 2010; Levy et al. 2011). Cellular immunity, such as the encapsulation of virally infected cells by haemocytes (Begon et al. 1993; Trudeau et al. 2001) and components of the humoral immune response, such as phenoloxidase (PO) (Popham et al. 2004; Shelby & Popham 2006) have also been found to play a role in antiviral defence. In addition, intracellular processes such as apoptosis (Clarke & Clem 2003b) and RNA interference (RNAi) (Wang et al. 2006) may function to prevent and limit viral infection within insect hosts. While knowledge of the mechanistic basis of antiviral resistance and antiviral defence components in isolation is important, a greater understanding of host resistance, which is likely to be the result of combined action of these many defences, is fundamental in understanding and predicting host parasite dynamics.

Previous work in insects has highlighted that resistance to virus can be selected for and is therefore a heritable trait (e.g. Boots 2011; Boots & Begon 1993; Fuxa & Richter 1998; Milks & Myers 2000). Evidence also suggests that as with resistance to other parasites (Sheldon & Verhulst 1996), antiviral resistance in insects carries costs. For example, resistance of the Indian meal moth, *Plodia interpunctella*, to its natural viral pathogen *P. interpunctella* Granulosis Virus (PiGV) is traded-off with an increase in development time (Boots 2011; Boots & Begon 1993) and reduced pupal

weight (chapter 5). In addition, velvetbean caterpillar moths, *Anticarsia gemmatalis*, selected for resistance to *A. gemmatalis* Nucleopolyhedrosis Virus (AgNPV) showed reduced reproductive potential, reduced survival and reduced pupal weight compared to control insects (Fuxa & Richter 1998). However, little is known about the nature of resistance to virus and in particular, whether resistance is general or specific to individual virus strains or types.

The specificity of antiviral resistance has important implications. Recent work suggests that viruses are the most likely pathogens to emerge on a novel host, due both to their short generation times and high mutation rates (Cleaveland et al. 2001; Woolhouse et al. 2005). There are many hypotheses about the ecological and demographic factors that may influence the likelihood and sustainability of disease emergence on a new host (Daszak et al. 2000; Jones et al. 2008; Woolhouse et al. 2005). However, while factors such as increased contact rates, population density and changes in host geographical ranges are likely to affect the opportunity for pathogen transmission to a new host (Jones et al. 2008), host defence against a novel parasite will ultimately determine its success and sustainability. Whether antiviral resistance is specific or general and protective against multiple strains and types of parasites, will therefore affect the likelihood of disease emergence on a novel hosts and the long term dynamics of host-parasite populations. The nature of antiviral resistance also has implications for biological control strategies with, for example, the evolution of non specific antiviral resistance threatening the control of insect pest species using viruses (Asser-Kaiser et al. 2007). A greater understanding of the nature of resistance to viral control agents is necessary for the development of more effective insect pest control strategies and for predicting the wider impact of the use of viral biological control agents on natural host-parasite dynamics. In addition, insects vector many human viruses such as Dengue Virus and West Nile Virus (Hemingway & Ranson 2000;

Turell et al. 2005). The specificity of antiviral defence in insect vectors will have important implications for the epidemiology of these harmful human viruses.

In this study I use a tractable Lepidopteron insect model system, to determine the specificity of antiviral resistance. *P. interpunctella* was subjected to artificial selection for resistance to its natural obligate killing parasite PiGV1 (Baculovirus). I examined whether *P. interpunctella* selected for resistance to PiGV1 were also resistant to a different strain of PiGV, PiGV2 or a distinct Baculovirus *Ephestia cautella* NPV (EcNPV). The effect of relaxing the selection pressure on the specificity of antiviral resistance was also examined. *P. interpunctella* subject to artificial selection for resistance to PiGV1 showed cross resistance against PiGV2 and EcNPV and lost resistance to each virus at the same rate in the absence of the selection pressure.

6.3 Materials and methods

6.3.1 The insect-virus system

The Indian meal moth, *P. interpunctella,* is a pest of stored agricultural products and its natural environment can be accurately mimicked in the laboratory. Insects were reared in 1 litre Nalgene screw top tubs, on a cereal based diet consisting of 50% Ready Brek ©, 30% bran and 20% rice, with 20g yeast, 0.2g sorbic acid, 0.2g methyl paraben, 25ml honey and 25ml glycerol added to 100g of cereal mix and were kept at 27°C in a 16:8 h light dark regime. Two genetically distinct strains of the naturally occurring DNA baculovirus virus, PiGV, PiGV1 and PiGV2 (Rae 2008) were used in this experiment. In addition, the DNA baculovirus EcNPV, which infects *E. cautella*, a Lepidopteron host belonging to the same Family (Pyralidae) as *P. interpunctella* was used.

Both PiGV and EcNPV are transmitted through oral ingestion of virus particles. Virus occlusion bodies enter the midgut where their protein coat is dissolved, releasing virions into the midgut cavity which then enter the midgut epithelial cells. Once the virus has passed through the midgut, virus proliferation in secondary tissue such as the fat body occurs, leading to cell lysis, tissue destruction and eventual host death. *P. interpunctella* larvae infected with PiGV are white and *P.interpunctella* larvae infected with EcNPV are brown and therefore easily distinguishable from each other and healthy larvae. PiGV symptoms in *P. interpunctella* are present 7-8 days following oral challenge with virus at third instar and EcNPV symptoms in *P. interpunctella* are present 13-14 says following oral challenge at third instar. Once symptomatic, larvae die before pupation.

Purified virus solution was produced by centrifugation of a homogenate of infected individuals (Smith & Crook 1988, Appendix 1a, 1b). PiGV was extracted from PiGV infected *P. interpunctella* and EcNPV was extracted from EcNPV infected *E. cautella.*

6.3.2 Artificial selection for resistance to virus

Antiviral resistance was artificially selected for in *P. interpunctella* by raising larvae on food containing PiGV1, aliquoted from a bulk stock solution. Virus selection lines were created by placing 35 adults onto 40g food, previously mixed with PiGV 1 virus aliquoted from the same bulk stock solution, for 24 hours to mate and lay. The volume of PiGV1 virus solution added to food was previously estimated to result in infection of approximately 50% of larvae. Virus solution was added to the glycerol component of the food mix, which was then combined with the remaining insect food ingredients to ensure thorough mixing of virus through the food. Each generation, adults first to emerge (~10% of total) were discarded and 35 newly emerged insects were transferred

to 40g fresh food containing stock virus for 24 hours to mate and lay eggs. Selection lines were set up at 3 different time points (3 experimental blocks) with 2 virus selection line replicates and 1 control selection line replicate (maintained in the same way but on virus-free food) in each block. Insects from different selection line replicates were never mixed (figure 6.1).

6.3.3 Relaxation of selection pressure

After 12 generations, virus selection lines were split into 2 sub-lines. In one sub-line the selection pressure was continued as detailed above, whereas in the other sub-line the selection pressure was relaxed. Relaxed selection sub-lines were maintained in the same way as control selection lines, by placing 35 adults on 40g virus-free food for 24 hours to mate and lay eggs.

6.3.4 Specificity of evolved resistance to virus

At the start of generation 16, in addition to establishing the next generation of selection lines, an experimental population was set up for each virus selection subline and control line. At this time point, virus selection sub-lines had been subjected to 15 generations of selection for resistance against PiGV1 and relaxed selection sub-lines had been selected with PiGV1 for 12 generations and then been removed from antiviral resistance selection pressure for 3 generations. Experimental insects were established by placing 35 adults onto 40g virus-free food and F2 generation experimental insects were established in the same way. Excluding virus from food on which F1 generation experimental insects were raised ensured that infection following viral challenge could be attributed to previous long term evolution not maternal environment. When F2 experimental insects produced from all selection

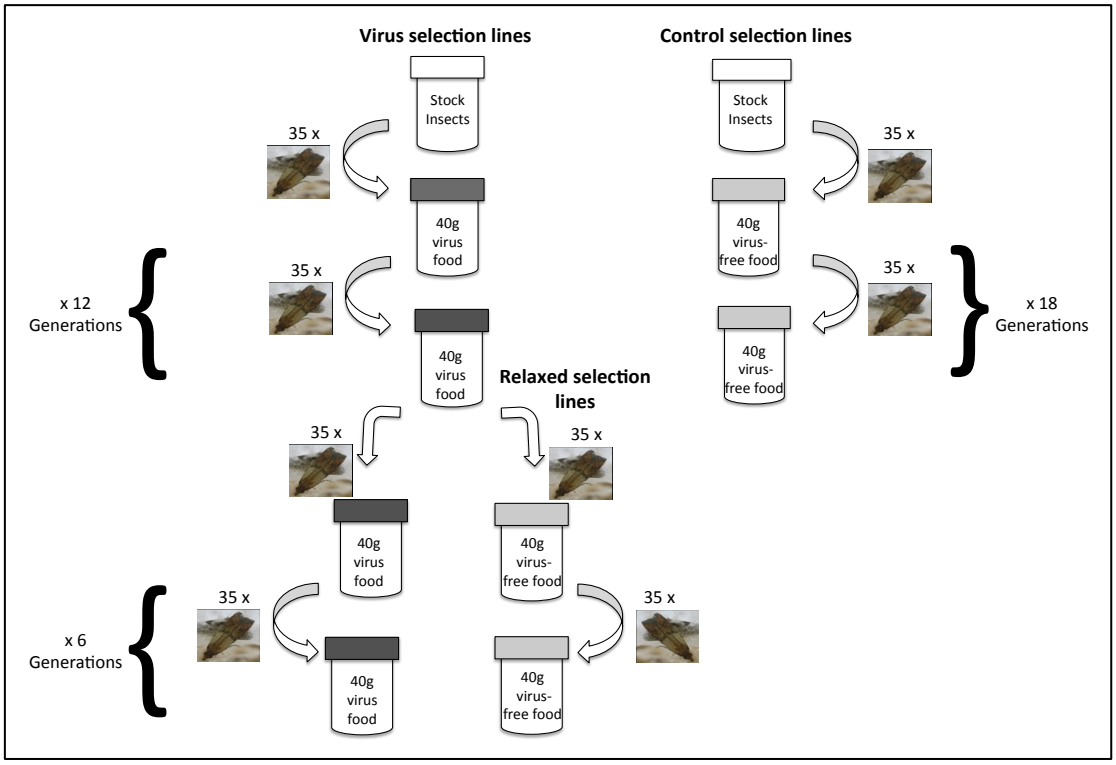


Figure 6.1. Protocol for artificial selection of antiviral resistance. To establish each virus selection line, 35 stock insects were placed on 40g food previously mixed with virus aliquoted from a bulk stock solution estimated to infect 50% of larvae. Lines were maintained by placing 35 adults onto 40g food containing virus aliquoted from the same bulk stock solution. Following 12 generations of selection with virus, each line was split into two sub-lines. One sub-line was maintained under continued selection pressure as previously for a further 6 generations. The other sub-line, the relaxed selection line, was relaxed from selection pressure and maintained on food containing no virus for 6 generations. Control selection lines were established by placing 35 stock insects onto 40g virus-free food. Control selection lines were maintained for 18 generations by transferring 35 adults from each generation onto 40g virus free food. This schematic represents the procedure for one virus selection line and one control line. The selection procedure was repeated over 3 experimental blocks set up at different time points with 2 virus selection lines and 1 control line per experimental block.

sublines and control selection lines reached third instar (11 days) they were removed from the food, kept separate and starved for two hours. Insects from each line were then split into three groups. One group was fed droplets of PiGV1 solution, using a pipette, following the standard oral bioassay procedure (Boots & Begon 1993, Appendix 1d). In addition, to assess the specificity of evolved resistance, the susceptibility of insects from each selection treatment were orally inoculated with a different strain of PiGV, PiGV 2 and a different baculovirus, EcNPV. 25-30 insects from each selection treatment, successfully dosed with each virus were then transferred to single cells of a 25-cell petri dish and examined for viral PiGV infection 7-8 days later and EcNPV infection 13-14 days later. Stock insects were also orally inoculated with either PiGV1, PiGV2 or EcNPV. Concentrations of each virus solution used were previously estimated to result in infection of approximately 50% of stock adults.

This process was also repeated at the start of generation 19, when virus selection sub-lines had been subjected to 18 generations of selection for resistance against PiGV1 and relaxed selection sublines had been selected with PiGV1 for 12 generations and then been removed from antiviral resistance selection pressure for 6 generations.

6.3.5 Statistical analysis

Specificity of evolved resistance to virus

The specificity of host resistance to virus was assessed using a generalized linear model (GLM) in R (version 2.14). The effect of host selection treatment (selection with virus and control) on host resistance (proportion infection) to each virus type (PiGV1, PiGV2, EcNPV) and an interaction between host selection treatment and

virus type was examined. As the data were in the form of proportions, a binomial error structure was incorporated into the model. Overdispersion in the data was corrected for by using a quasibinomial error structure. In addition to host selection treatment and virus type, the effect of selection time (15 generations and 18 generations), experimental block (n=3) and an interaction between selection time and host selection treatment on host resistance was also assessed. However, the interaction between selection time and host selection treatment ($F_{1,46}$=1.50, p=0.23) and the explanatory terms, experimental block ($F_{2,51}$= 1.06, p=0.36) and selection time ($F_{1,50}$=2.30, p=0.137) were non significant and therefore removed from the final model.

Infection in control and stock insects was analysed using a GLM with binomial error structure. Firstly, infection in insects subject to 15 generations of control selection procedure and stock insects challenged at that time was analysed. The data showed overdispersion which was corrected with quasibinomial errors. In addition to host (control and stock) and virus type (PiGV1, PiGV2, EcNPV), the effect of experimental block and an interaction between virus type and host treatment was investigated. Experimental block ($F_{2,15}$=0.799, p=0.77) and an interaction between virus type and host selection treatment ($F_{2,10}$=0.0191, p=0.98) were subsequently removed from the model as they were non significant, leaving only the key variables; host treatment and virus type in the final model. Analysis of infection in insects subject to 18 generations of control selection procedure and stock insects challenged at that time was also carried out. In addition to host and virus type, the effect of experimental block and an interaction between virus type and host treatment was investigated. Experimental block (χ^2_2=1.98, p=0.37) and an interaction between virus type and host (χ^2_2=2.09, p=0.35) were subsequently removed from the model as they were non significant, leaving the key variables; host treatment and virus type in the final model.

Specificity in loss of resistance in absence of selection pressure

Further analysis was carried out to determine the effect of relaxing selection pressure on resistance to PiGV1, PiGV2 and EcNPV. Firstly, infection with all three viruses in insects subject to continued selection over 15 generations and insects selected over 12 generations but relaxed from selection pressure for 3 generations was analysed. The data were fitted to a GLM with quasibinomial error structure as the data were overdispersed. In addition to host selection treatment (continued selection and relaxed selection) and virus type (PiGV1, PiGV2 and EcNPV), the effect of experimental block, experimental line (which was nested within block) and an interaction between host selection treatment and virus type on host infection was investigated. However, experimental block ($F_{2,33}$=2.65, p=0.09), experimental line ($F_{3,27}$=0.865, p=0.47) and the interaction between virus and treatment ($F_{2,25}$=2.27, p=0.12) were non significant and therefore removed from the final model leaving only the key explanatory variables: host selection treatment and virus type in the final model.

The effect of a longer period of relaxation from selection on infection following challenge with all three viruses was also investigated. Infection following challenge with PiGV1, PiGV2 and EcNPV in insects selected for 12 generations and relaxed from selection pressure for 6 generations and insects subjected to continued selection pressure for 18 generations was analysed using a GLM. Again, the data was overdispersed and therefore a quasibinomial error structure was incorporated into the GLM. In addition to host selection treatment (continued selection and relaxed selection) and virus type (PiGV1, PiGV2 and EcNPV), the effect of experimental block, experimental line (which was nested within block) and an interaction between host selection treatment and virus type on infection was investigated. Experimental line ($F_{3,27}$=0.765, p=0.52) and the interaction between host selection treatment and

virus type ($F_{2,25}$=2.13, p=0.14) were removed from the final model as they did not explain a significant amount of the variation in proportion infection. Experimental block, and the key variables virus type and host selection treatment remained in the final model.

Finally, to determine whether insects relaxed from selection pressure for 6 generations lost all evolved resistance, infection in insects selected for 12 generations and relaxed from selection pressure for 6 generations was compared to infection in control insects was analysed using a GLM with quasibinomial errors. An interaction between host treatment (relaxation of selection pressure and control) and virus type (PiGV1, PIGV2, EcNPV) was initially investigated but was removed from the final model as it was found to be non significant ($F_{2,19}$=0.0716, p=0.93). Experimental block, virus type and host selection treatment were incorporated into the final model as explanatory variables.

6.4 Results

6.4.1 Specificity of evolved resistance to virus

Artificial selection of *P. interpunctella* for resistance to virus, by maintaining insects on food containing the virus PiGV1, resulted in the evolution of non-specific antiviral resistance. Infection in insects selected for antiviral resistance was significantly lower (mean infection in selected insects was ~40% lower than mean infection in control insects) than infection in control insects ($F_{1, 52}$=90.5, p<0.001). Infection in insects challenged with PiGV1, PiGV2 and EcNPV differed significantly ($F_{2,50}$=7.59, p=0.0013) (figure 6.2). The lack of significant interaction between virus type and host selection treatment highlighted in the initial analysis

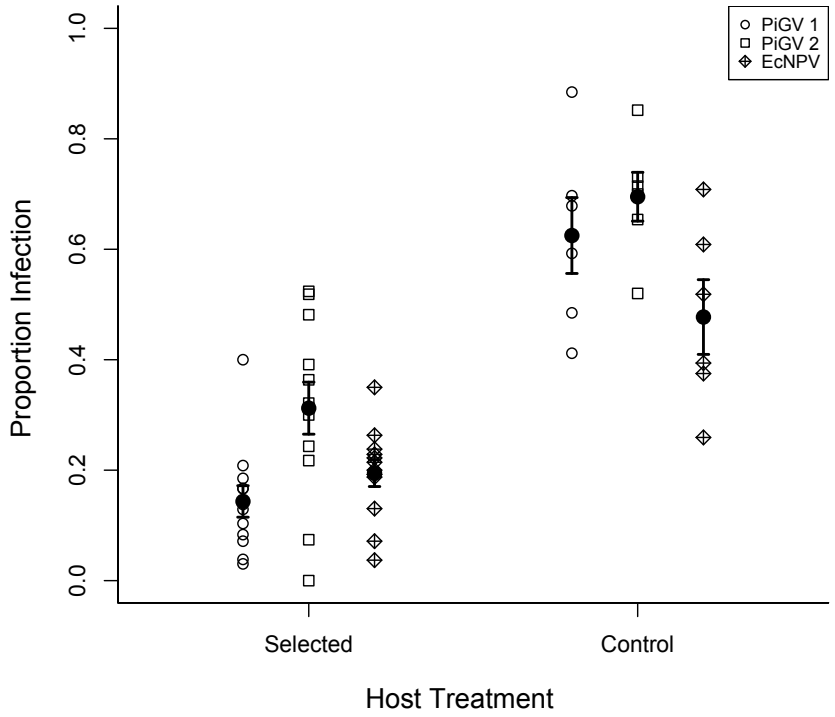


Figure 6.2. The specificity of antiviral resistance in *P. interpunctella*. Infection in insects selected for antiviral resistance was significantly lower (~40%) than infection in control insects ($F_{1, 52}$=90.5, p<0.001). Infection in insects challenged with PiGV1, PiGV2 and EcNPV differed significantly ($F_{2,50}$=7.59 p=0.0013). However, no significant interaction between virus type and host selection treatment was found ($F_{2,44}$=2.86, p=0.068), therefore indicating that the artificial selection carried out in this experiment resulted in the evolution of non-specific resistance against all three viruses. Points represent treatment replicates and filled circles represent means ± s.e.

($F_{2,44}$=2.86, p=0.068) suggests that selection carried out in this experiment resulted in the evolution of a non-specific resistance against all three viruses. However, although non-significant, the data indicate a trend that the increase in resistance to PiGV1 may be greater than the increase in resistance to PiGV2 or EcNPV in selected insects relative to control insects.

Initial analyses highlighted that infection in control insects, subjected to 15 generations of virus-free selection line procedure, and stock insects was not significantly different ($F_{1,14}$=0.155, p=0.70). Therefore, 15 generations of virus-free

selection procedure did not affect host resistance so that any change in resistance in virus selection line insects can be attributed to the presence of the virus alone. Infection resulting from challenge with each virus was significantly different ($F_{2,15}$=9.30, p=0.0027) but, as no interaction between host and virus type was found ($F_{2,10}$=0.0191, p=0.98), this was consistent across both control insects and stock insects. However, infection in insects subjected to 18 generations of virus-free selection line procedure was significantly higher (~18%) than in stock insects (χ^2_1=15.2, p<0.001). This indicates that increased susceptibility to virus may be a consequence of long term maintenance of insects using the selection procedure and highlights that comparison of virus selected insects to control selected insects rather than stock insects is necessary to distinguish the effect of virus from the effect of the selection procedure,. The level of infection resulting from challenge with each virus was significantly different (χ^2_2=17.6, p<0.001) but, as there was no interaction between host and virus type, this was consistent across both selection line control insects and stock insects.

6.4.2 Specificity in loss of resistance in absence of selection pressure

Infection with PiGV1, PiGV2 and EcNPV was also investigated in insects relaxed from selection pressure. Relaxing selection for a period of three months, did not result in a significant loss of resistance compared to insects maintained in continued selection ($F_{1,32}$=3.46, p=0.072), although a trend for a loss of resistance in insects relaxed from selection pressure is evident (figure 6.3a). However, relaxation for 6 generations resulted in a significant loss of resistance (~10%) compared to insects subjected to continued selection pressure ($F_{1,32}$=6.98 p=0.013) (figure 6.3b). Evolved resistance was not lost completely and infection in insects relaxed from selection pressure for 6 generations was significantly lower (~25%) than infection in control

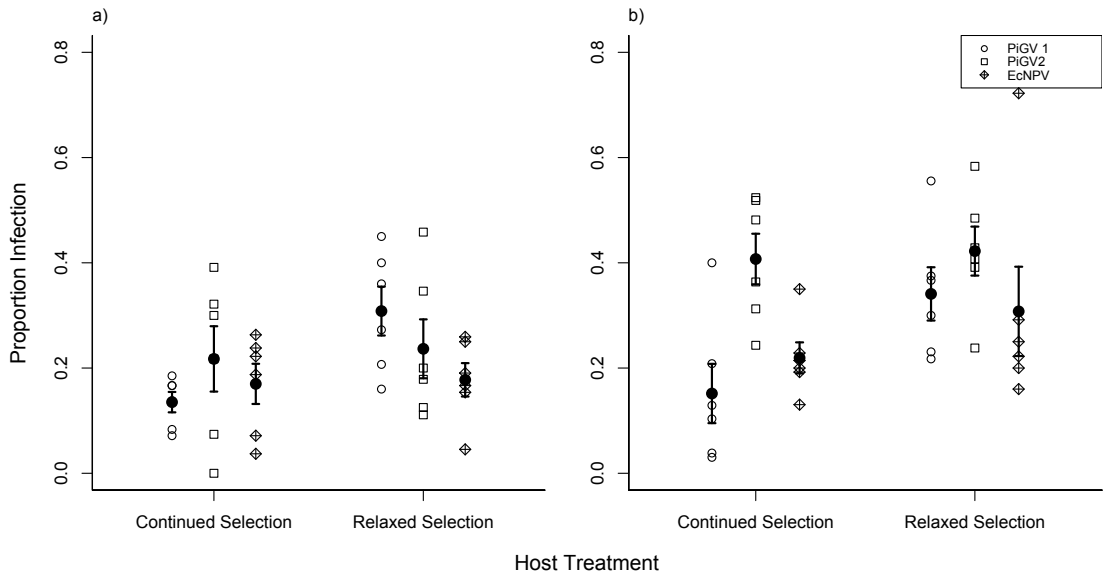


Figure 6.3. a) Relaxing selection for a period of three months, did not result in a significant loss of resistance compared to insects maintained in continued selection ($F_{1,32}$=3.46, p=0.072), although a trend for a loss of resistance in insects relaxed from selection pressure is evident. b) Relaxation for 6 generations resulted in a significant loss of resistance (~10%) compared to insects subjected to continued selection pressure ($F_{1,32}$=6.98 p=0.013).

insects across all three viruses ($F_{1,21}$=26.4, p<0.001*)*. Infection also differed significantly between experimental blocks across insects maintained under selection pressure and insects relaxed from selection for 6 generations ($F_{2,33}$=3.53, p=0.042) and infection with each virus was significantly different ($F_{2,30}$=7.68, p=0.002). No significant interaction between host selection treatment and virus type ($F_{2,25}$=2.13, p=0.14) was found suggesting that relaxation of selection pressure results in equal loss of resistance to all three viruses. However, the data indicate a trend that the loss of resistance to PiGV1 is greater than the loss of resistance to PiGV2 and EcNPV.

6.5 Discussion

This study shows that *P.interpunctella* insects selected for increased resistance to PiGV also show increased resistance to a genetically distinct strain of PiGV, PiGV2, and a different baculovirus, EcNPV. This cross resistance suggests that the mechanism underpinning resistance to all three viruses is likely to be similar. In addition, this study highlights that in the absence of selection pressure, resistance is lost and resistance to each of the three viruses is lost at the same rate. This suggests that there are costs to this general resistance and, in further support for the hypothesis that resistance to all three viruses is underpinned by the same mechanism, the costs of resistance to each virus are similar. I therefore provide evidence that antiviral resistance in *P. interpunctella* is non-specific and costly.

In concurrence with the present study, fall armyworms*, Spodoptera frugiperda,* resistant to *S. frugiperda* NPV (SfNPV) showed cross resistance to *S. frugiperda* GV (SfGV) and to *Autographa californica* NPV (AcMNPV) (Fuxa & Richter 1990). In addition, cabbage loopers, *Trichoplusia ni*, resistant to *T. ni* NPV (TnSNPV) showed cross resistance to *Pieris rapae* GV (PrGV) and *T. ni* GV (TnGV) (Milks & Myers 2003). However, the level of cross resistance to different baculoviruses and pathogens may be variable and in some instances marginal or absent (Milks & Myers 2003). The nature of host resistance is likely a consequence of the mechanism that underpins the resistance. Less is known about the defences present and employed to counter viral infection in insects and the nature of insect resistance to virus compared to insect responses to bacterial and fungal parasites (Imler & Elftherianos 2009). By providing evidence for non-specific resistance to virus in *P. interpunctella,* this study suggests that the mechanistic basis of antiviral resistance could be 1) the result of a non specific barrier protecting cells from parasite invasion, for example the peritrophic membrane

in the gut, 2) a non specific intracellular process which acts to clear virally infected cells irrespective of the strain or species of virus present, for example, by apoptosis, or 3) a constitutive immunological defence, whose action lacks capacity for specificity, for example the PO cascade. However, the lack of specificity in resistance found in this study, coupled with the lack of increased resistance to injected virus uncovered in this system previously (chapter 7), implies that a general gut based mechanism, such as the peritrophic membrane could be strongly associated with resistance in this insect. There is support for the role of the peritrophic membrane in antiviral resistance in other Lepidopteron insects. For example, the peritrophic membrane of resistant *A. gemmatalis* was found to be thicker and stronger compared to this membrane in susceptible strains of this *A.* gemmatalis (Levy et al. 2011). Detailed tracing of the baculovirus infection process in both selected and control *P. interpunctella* will be the next step in determining whether the peritrophic membrane of selected insects does act to block virus entering the midgut cells and upderpin antiviral resistance in this insect.

The non-specific nature of antiviral resistance indicated by this study has wider implications. Disease emergence events can result from parasites infecting novel hosts (Woolhouse et al. 2005), commonly phylogenetically close to their endemic hosts (Davies & Pedersen 2008; Longdon et al. 2011). This study assesses resistance to a genetically distinct strain of virus and a virus known to infect a closely related host species, both of which therefore represent a potential disease emergence threat in nature. The generality of antiviral resistance and its maintenance for multiple generations, even in the absence of selection pressure, found in this study suggests that the risk of widespread newly emerging viral infections in *P. interpunctella* may be reduced in populations already exposed to a viral pathogen. However, while non-specific resistance may be advantageous and reduce the negative impact of viruses on beneficial host populations, it clearly presents a disadvantage when considering the

use of baculoviruses as natural biological control agents of insect pest species such as *P. interpunctella*. The evolution of non specific resistance to a naturally occurring virus or an introduced viral biological control agent will clearly limit the effectiveness of future use of other viral biological control agents and the control of insect pest species by this method.

The connection between specificity of host resistance and maintenance of genetic diversity in host parasite defences has been the subject of extensive theoretical investigation (reviewed in Sadd & Schmid-Hempel 2009c) and recent empirical work (Berenos et al. 2011; Kerstes et al. 2012). Specific resistance maintains variation in coevolving host and parasite populations due to frequency dependent selection. Clearly, non-specific host resistance will eliminate the selective advantage of rare parasites, potentially resulting in a loss of genetic variation in host-parasite populations. However, it has been argued that if resistance carries costs, variation in host resistance traits will be maintained (Sheldon & Verhulst 1996). The loss of antiviral resistance in the absence of selection pressure found in this study is indicative that *P. interpucntella* showing increased resistance are at a selective disadvantage and incur a fitness cost. Costs associated with the evolution of resistance in *P. interpunctella* are discussed in more detail in chapter 5.

Non specific resistance to parasites may also result in the evolution of more virulent parasites (Gandon et al. 2001; Mackinnon et al. 2008). A large proportion of parasite resistant hosts in a population may drive the evolution of parasites capable of challenging host defences and also result in an increased likelihood of co-infection in susceptible hosts resulting in within host parasite competition, a potential driver of parasite virulence (Alizon & van Baalen 2008; May & Nowak 1995), however this is not ubiquitously supported by experimental studies (Milks & Myers 2000).

In this study, artificial selection of *P. interpunctella* with PiGV1 was used to generate resistant insects from which the specificity of resistance could be assessed. Coevolution of PiGV1 was prevented and therefore this study is only the first step in understanding the nature of resistance of *P. interpunctella* to virus. While viruses are considered able to evolve rapidly given their short generation and high mutation potential during replication (Cleaveland et al. 2001; Woolhouse et al. 2005) the lack of increase in virulence in response to increased resistance in a Lepidopteron host to virus (Milks & Myers 2000) suggests that, at least in some instances, baculoviruses may be slow to evolve counter measures against resistant hosts. Examination of the nature of resistance of *P. interpunctella* to virus which is allowed to coevolve will further our knowledge of antiviral resistance and on the capacity of baculoviruses to counter host defences. In nature, hosts are rarely challenged by a single parasite in isolation. The importance of parasite heterogeneity was recently highlighted in a study which examined the evolution of bacterial host resistance and its associated costs in an environment containing single or multiple phage strains (Koskella et al. 2012). Koskella et al. (2012) demonstrated that while resistance following selection with both single and multiple phage strains showed specificity, the costs of resistance in the form of reduced phage growth was greater in bacterial hosts selected against multiple phage strains. A greater understanding of the evolution of resistance in *P. interpunctella* and the mechanisms controlling the maintenance of genetic diversity in this host will therefore require consideration of multiple parasite attack.

The generality of antiviral resistance in terms of its protection against multiple viruses has been investigated in this study. However the extent of the generality of antiviral resistance and whether resistance to virus also conveys resistance to bacterial and fungal pathogens is not clear. Further examination of the response of viral resistant hosts to a broad range of different parasites will shed light on the extent of the

generality of insect resistance thereby indicating the potential overlap in defence mechanisms to different parasites species. However, increasing the range of parasites against which the response of selected hosts are assessed, often blurs results regarding host defence specificity and raises questions about when the resistance seen stops being the consequence of a specific host defence and becomes the result of incompatibility between host and parasite, described by non-host resistance theory (Heath 1981).

In summary, this study shows that in *P. interpunctella* antiviral resistance is not specific. Little is known about the mechanisms that underpin antiviral resistance in insects but its lack of specificity enables speculation on the role of a general physical barrier such as the peritrophic membrane in the gut. Although further work is needed to clarify whether non-specific antiviral resistance is a feature of this study system only or common to many insect taxa, a lack of specificity in parasite resistance will have a pronounced impact on host-parasite populations including the threat of disease emergence.

Chapter 7: Using an Artificial Evolution Experiment to Examine the Relative Importance of Prehaemocoelic and Haemocoelic Insect Antiviral Defences for Resistance

7.1 Abstract

The insect immune system is increasingly widely studied with immune responses to bacterial and fungal pathogens well characterized. However, our understanding of the insect immune response to virus is less comprehensive and in particular we know little about the relative role of gut and haemocoelic defenses. Here I use *Plodia interpunctella* (Lepidoptera) previously selected for resistance to its natural viral parasite; *Plodia interpunctella* Granulosis Virus (PiGV) (baculovirus) for 18 generations, to determine the relative importance of prehaemocoelic and haemocoelic defenses for antiviral resistance. Specifically, I compare viral infection following either oral inoculation or intrahaemocoelic injection of PiGV in larvae previously selected for resistance to PiGV. I find that insects selected for resistance are more resistant to oral inoculation of PiGV compared to control insects but do not differ in their susceptibility to PiGV injected into the haemocoel. This differential response to oral inoculation of virus and injection of virus suggests that the gut is central to antiviral resistance and provides focus for study of antiviral resistance mechanisms.

7.2 Introduction

The study of the invertebrate immune system, and in particular the insect immune system, has increased substantially over recent years. It is now clear that insects can mount a complex and dynamic immune response integrating numerous different components (Rolff & Reynolds 2009; Schmid-Hempel 2005a; Siva-Jothy et al. 2005). However, while the response of insects to bacterial and fungal pathogens are well

understood, our knowledge of insect antiviral defence is less comprehensive (Imler & Elftherianos 2009; Strand 2008). Understanding insect-virus interactions is important given the negative effect that viruses can have on insect population dynamics (Cox-Foster et al. 2007; Ilyinykh 2011) the role of insect vectors in the transmission of human and wildlife viruses (Turell et al. 2005) and the potential for using viruses as biological control agents to combat insect pest species (Alexandre et al. 2010).

Lepidopteron insects, in combination with their naturally occurring baculovirus parasites, are commonly used as model systems to investigate the ecology (Reeson et al. 1998; Triggs & Knell 2012; Wilson & Reeson 1998) and evolution (Boots 2011; Boots & Begon 1993; Fuxa & Richter 1998) of insect-virus interactions. Baculoviruses are host-specific, obligate killing DNA viruses (Blissard & Rohrmann 1990). They have two phenotypes; the occluded virus, which consists of virions encased within a protein coat, and the budded virus, which lacks the protein coat. As with many insect parasites, natural transmission of baculoviruses occurs when virus occlusion bodies are ingested by larvae. The initial site of virus exposure in the insect is therefore the gut, or more specifically, the midgut. Here the alkaline conditions destroy the occlusion body resulting in the release of infectious virions. These virions may cross the peritrophic membrane, into the epithelial cells and systemic infection is established when progeny BVs pass across the basal lamina into the haemocoel and infect secondary tissue such as the tracheal system and the fat body (Blissard & Rohrmann 1990). While this infection process is well documented it remains unclear as to where, and therefore at what stage of the infection process, resistance to infection occurs.

While Lepidopteron-baculovirus systems are ideal models for looking at insect-virus interactions due to the success and ease of maintenance of Lepidopteron hosts in the lab, the motivation for the use of these systems is also driven by the potential to use baculoviruses as control agents of Lepidopteron insect pests (Alexandre et al. 2010;

Tinsley 1977) and because of the impact that baculoviruses have on insect population dynamics (Goulson & Cory 1995). A greater understanding of Lepidopteron-baculovirus interactions, in particular the evolution of resistance in Lepidoptera, will aid the development and success of baculoviruses in pest control and the potential to reduce the negative effect of these viruses, in addition to increasing our understanding of insect-virus interactions generally.

Antiviral defences have been found in both the gut and haemocoel of many insects including Lepidoptera. In the midgut, defences include unfavorable pH (Keating et al. 1990) the presence of digestive enzymes (Ponnuvel et al. 2003) and physical barriers including the the peritrophic membrane located between the gut lumen and the gut epithelial cells (Asser-Kaiser et al. 2011; Levy et al. 2011) and the basal lamina located on the basal side of the gut epithelial cells (Engelhard et al. 1994). For example, the peritrophic membrane may be key in resistance of velvetbean caterpillars, *Anticarsia gemmatalis*, to *A. gemmatalis* Nuclelpolyhedrosis Virus (AgMNPV). Compared to resistant larvae, the peritrophic membrane of susceptible larvae was weaker with a lower chitin content and provided a less efficient barrier against the virus (Levy et al. 2011). Virally infected midgut cells may also be cleared through the action of cell sloughing. In support of the role of cell sloughing, greater numbers of rejected cells were found in the cotton bollworm, *Helicoverpa armigera*, orally challenged with *H. armigera* Stunt Virus compared to controls (Brooks et al. 2002).

Defences are also present in the haemocoel to combat systemic infection. In a recent study it was found that infection in the Indian meal moth, *Plodia interpunctella*, following both oral inoculation with *P. interpunctella* Granulosis Virus (PiGV) and intrahaemoclic injection of PiGV was dose dependent (Saejeng et al. 2011), therefore highlighting the presence of antiviral resistance mechanisms in the haemocoel.

Haemocoelic defences may include cellular responses such as those involving haemocytes. Haemocytes were found to contribute to the resistance of the corn earworm, *Helicoverpa zea,* to orally administered *Autographa californica* multiple NPV (AcMNPV), clearing melanised, virally infected tracheae from the haemocoel by encapsulation (Trudeau et al. 2001). Haemocytes have also been implicated in the resistance of *P. interpunctella* to its granulosis virus (Begon et al. 1993) and defence of the African cottom leafworm, *Spodoptera littoralis*, following injection with budded AcMNPV (Rivkin et al. 2006). Humoral responses such as the action of phenolxidase (PO) which is involved in the melanisation process may also play a role in antiviral defence in some Lepidopteron species (Popham et al. 2004), although its role is not ubiquitous (Saejeng et al. 2010). Intracellular antiviral immune defences may also occur. For example, the role of apoptosis in defence against baculoviruses is supported by the presence of apoptosis inhibiting genes in baculoviruses (Clarke & Clem 2003b; Feng et al. 2007) and the reduction in infectiviity shown by baculovirus mutants lacking the apoptosis inhibiting gene (Salvesen & Duckett 2002). Subcellular inhibition of viral replication by RNA interference (RNAi) (Kemp & Imler 2009; Saleh et al. 2009; Wang et al. 2006) has been proposed as a key mechanism of defense against RNA viruses in *Drosophila* with mutants lacking components necessary for the RNAi process such as the enzyme Dicer-2 showing increased susceptibility to virus (Galiana-Arnoux et al. 2006; Zambon et al. 2005). While it is possible that RNAi plays a role in the defence of insects, including Lepidoptera, against DNA viruses there is no specific evidence to support this hypothesis. Intracellular mechanisms such as apoptosis, may play a role in limiting systemic infection, as well as acting in midgut cells, with for example, evidence of apoptosis of virally infected haemocytes documented (da Silveira et al. 2005; Zhang et al. 2002).

So while it is clear that antiviral defence mechanisms are present in both the gut and the haemocoel, their relative contribution to antiviral resistance remains unclear. In this study I use *P. interpunctella* previously selected for resistance to its natural viral parasite PiGV (Baculovirus) over 18 generations to determine the relative importance of the gut and the haemocoel in antiviral resistance. I compare viral infection following oral inoculation of PiGV or intrahaemocoelic injection of PiGV in *P. interpunctella* previously selected for resistance to PiGV and control insects. I find that insects previously selected for resistance are more resistant to oral inoculation of PiGV compared to controls but do not differ in their resistance to injected PiGV compared to controls. This differential response to oral inoculation of virus and injection of virus suggests that the gut may be central to antiviral resistance in *P. interpunctella*.

7.3 Materials and methods

7.3.1 Insect-virus system

The Indian meal moth, *P. interpunctella,* is a pest of stored agricultural products and therefore has a natural environment that is easy to mimic in the laboratory. Insect food consists of 50% Ready Brek ©, 30% bran and 20% rice, with 20g yeast, 0.2g sorbic acid, 0.2g methyl paraben, 25ml honey and 25ml glycerol added to 100g of cereal mix and insects were kept at 27°C in a 16:8 h light dark regime. We used the naturally occurring baculovirus, PiGV. Typical of baculoviruses, natural transmission of PiGV is through ingestion of virus particles by larvae. The virus can also be artificially transmitted through injection directly into the haemocoel of larvae (Saejeng et al. 2010). Infected larvae are a characteristic opaque white colour and therefore easily distinguishable from healthy individuals. Once symptomatic, larvae die before pupation. The purified stock virus solution, used in the artificial selection procedure and the oral

inoculations, was produced by centrifugation of a homogenate of infected insects cadavers (Smith & Crook 1988, Appendix 1a, 1b).

7.3.2 Artificial selection for resistance to virus

Resistance to PiGV was artificially selected for in *P. interpunctella* by raising larvae on food containing this virus. Virus selection lines were created by placing 35 adults onto 40g food, previously mixed with virus aliquoted from a bulk stock solution, for 24 hours to mate and lay eggs. The volume of virus solution added to food was previously estimated to result in infection of approximately 50% of larvae. To enable thorough mixing of virus through the food the virus solution was added to the glycerol component of the food mix, which was then combined with the remaining insect food ingredients. Each generation, adults first to emerge (~10% of total) were discarded and then 35 newly emerged adult insects were transferred to 40g fresh food containing virus aliquoted from the same bulk stock for 24 hours to mate and lay eggs. Selection lines were set up at 3 different time points (3 experimental blocks) with 2 virus selection lines and 1 control selection line (maintained in the same way but on virus-free food) in each block.

At generation 19, in addition to establishing the next generation of the selection lines, an experimental population was set up for each virus selection line and each control, virus-free selection line. Experimental lines were established by adding 35 adults to 40g virus-free food for 24 hours to mate and lay eggs. This was to ensure that the experimental insects had the same maternal environment and that their response to viral challenge could be attributed to multiple generations of selection rather than the consequence of parental virus exposure or environment. Early emerging insects were discarded from experimental populations and to establish insect populations for assay

(F2 generation experimental insects) 35 adults were placed on 40g virus free food for 24 hours and left to mate and lay eggs.

7.3.3 Oral inoculation

Stock virus solution was diluted in blue food dye solution (75% blue food dye in double distilled water with 5% sucrose) to a concentration previously calculated to be LD_{50} in stock *P. interpunctella*. Fifty third instar (11days) assay insects were removed from their food and starved for two hours. Droplets of the virus/dye solution were then orally administered to the larvae using a pipette following the standard droplet feeding method (Boots & Begon 1993, Appendix 1d). Following successful inoculation (indicated by the presence of dye in half of the length of the gut), insects were placed in individual cells of a 25 cell petri dish with abundant food. Between 25 and 30 insects from each line were orally inoculated. Virus selection line insects and control line insects were kept separate throughout and checked for symptoms of viral infection 7-8 days post oral inoculation.

7.3.4. Intrahaemocoelic injection

The intrahaemocoelic injection protocol was adapted from Saejeng et al. (2010).Virus was extracted from donor infected insects orally inoculated with LD_{80} virus solution 7 days previously. Donor insects were immobilized on ice for 15 minutes and transferred to an upturned petri dish where they were covered with cling film to restrict movement and increase haemolymph pressure. The final proleg (furthest from head) was then punctured with a stainless steel 'extra fine' entomological pin allowing a small droplet (~1.5µl) of haemolymph, containing virus, to escape. 0.5µl distilled water was added to this droplet of haemolymph using a pipette to slow evaporation. Using a sanded 1mm glass capillary (Narishige, Tokyo) attached to a pneumatic picopump (WPI, model PV280) this solution was then transferred to an eppendorf on ice containing 10µl

distilled water. This procedure was repeated for 50 donor insects. This virus solution (total volume ~110µl) was then centrifuged at 1000g for 30 seconds to remove any insect debris present. The supernatant containing the virus was then transferred to a fresh eppendorf containing 10µl dye solution (used in the oral inoculations) and kept on ice ready to be injected. The supernatent contained mainly budded virus as it is the budded virus which is present in the haemolymph at this stage. Some occluded virus will be present in small quantities 7 days post oral inoculation and increases up to 14 days post inoculation (Begon et al. 1993). The solution was loaded into a calibrated finely sharpened 1mm glass capillary needle and 1ul injected using a picopump into the second proleg of fourth instar (15 days) assay insects previously removed from their food and chilled on ice for 15 minutes. 40 insects from each virus selection line and control selection line were injected. Injected insects were monitored for 30 mins and insects showing injury from injection (<10%) were discarded. 25 successfully injected insects from each virus and control selection line were transferred to a single cell of a 25 cell petri dishes and given excess food. Symptoms of viral infection were then assessed 7-8 days following injection. 25 stock, 25 virus selection line and 25 control insects from each experimental block were injected with a dye /water solution as a control for the injection procedure.

7.3.5. Statistical analysis

The effect of selection treatment (virus selection and control selection) on susceptibility to viral infection (proportion infection) following intrahaemocolic virus injection and oral inoculation of virus was analysed separately using generalized linear models (GLMs) in R, version 2.14. The data was proportion infection so binomial errors were incorporated into the models. In addition to the effect of selection treatment, the variation in proportion infection following oral inoculation and injection between the

three experimental blocks was investigated. However, block was found to be non significant for oral inoculation data ($F_{2,6}$=0.58, p=0.59) and was therefore removed from the model.

7.4 Results

Virus selection insects were significantly less susceptible to orally administered virus compared to control insects ($F_{1,7}$=5.75, p=0.0054) (figure 7.1). Mortalities in insects orally inoculated with virus were very low (<4%). However, I found no difference between infection in virus selection line insects control line insects following

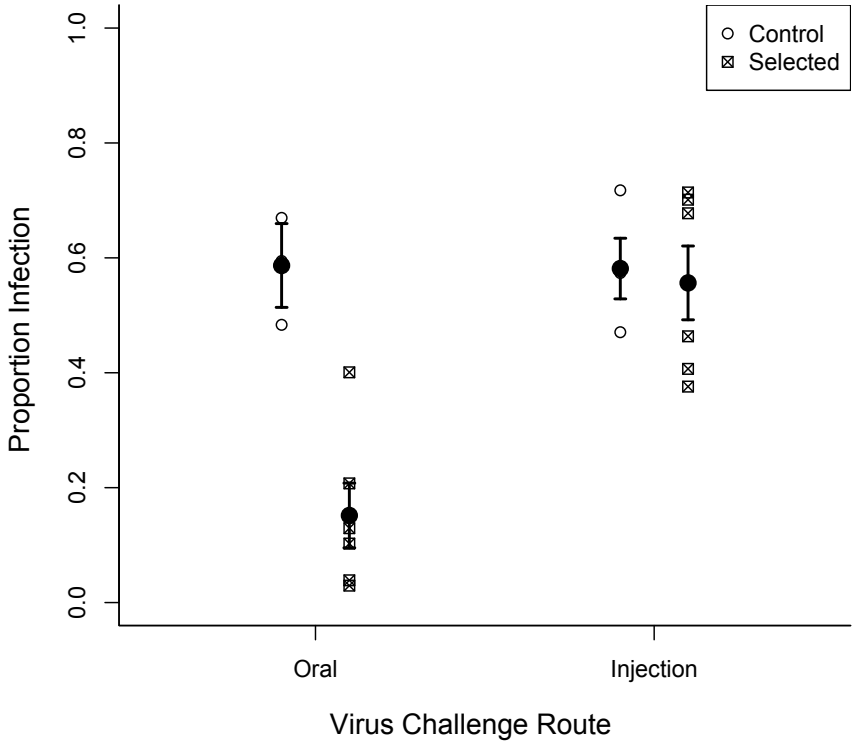


Figure 7.1. Infection in insects previously selected for resistance and control insects following oral inoculation with virus and intrahaemocoelic injection of virus. Selection for resistance reduces susceptibility to oral inoculation ($F_{1,7}$=5.75, p=0.0054) but does not reduce susceptibility to intrahaemocoelic injection compared to controls (χ^2_1=0.210, p=0.646). Open circles represent control insect block and open diamonds represent

resistant insect block. Points represent treatment replicates and filled circles represent treatment means ± s.e.

injection of PiGV directly into the haemocoel (χ^2_1=0.210, p=0.646) (figure 7.1). Infection resulting from injection differed between experimental blocks (χ^2_1= 13.72, p<0.001). As stated previously, larvae showing severe injury 30 minutes following injection were discarded. The number showing severe injuring was <10% across all treatments (including injection controls). Mortality in injected insects used to determine susceptibility was very low (<8%) across all treatments (including injection and covert infection controls).

7.5 Discussion

I find that *P. interpunctella* selected for resistance to PiGV show reduced susceptibility to oral inoculation of PiGV compared to controls but do not differ in their susceptibility to intrahaemocoelic injection of PiGV compared to controls. Resistance to PiGV, acquired following artificial selection, is therefore dependent on a prehaemocoelic mechanism.

The results of this study suggest that the mechanistic basis of increased resistance in *P. interpunctella* to PiGV is located in the gut. Gut defenses include a wide variety of factors present in the gut lumen such as proteases (Nakazawa et al. 2004), lipases (Ponnuvel et al. 2003) and more generally the pH of the gut which may be hostile for invading viruses and other pathogens (Keating et al. 1990). Another potentially crucial antiviral defense in the gut is the major physical barrier, the peritrophic membrane, which separates the gut contents from the midgut epithelial cells (Levy et al. 2011). A role for the peritrophic membrane, has been previously highlighted through the comparison of naturally resistant and susceptible strains of

Lepidopteron hosts. For example, in *A. gemmatalis*, the peritrophic membrane was implicated as a key component of resistance as a thicker, stronger membrane was found in strains resistant to AgMNPV compared to more susceptible strains (Levy et al. 2011). Further evidence for the crucial role of the peritrophic membrane in Lepidopteron antiviral resistance comes from the presence of proteases called enhancins produced by baculoviruses which degrade the proteins present in this matrix enhancing viral infectivity (Derksen & Granados 1988; Slavicek & Popham 2005). While these defenses prevent virus particles entering gut epithelial cells, resistance could also result from rapid termination of viral replication within cells or destruction and clearance of infected host cells in the midgut. Resistant strains of the silkworm moth, *Bombyx moryx*, inhibited proliferation of BmNPV in the midgut and the trachea (Bao et al. 2009) rather than preventing virus entry into cells. Although the specific mechanism by which the proliferation was inhibited is not certain, Boa et al. (2009) show an increase in expression of genes including a gene encoding serpin-5, a serine protease involved in the phenoloxidase cascade, in resistant larvae. A well studied intracellular antiviral defence in Lepidoptera is apoptosis, a type of programmed cell death. The role of apoptosis in antiviral defense against baculoviruses was initially highlighted in the 90s by the discovery of genes present in baculoviruses that function to inhibit apoptosis in Lepidoptera (Clem et al. 1996). Since then, evidence for the function of apoptosis in antiviral defence in the midgut includes increased destruction and clearing of midgut epithelial cells infected with baculovirus by apoptosis and sloughing (Chikhalya et al. 2009). The basal lamina, the final barrier between the gut and the haemoceol, may also be key to resistance to oral inoculation with virus. Baculoviruses are known to pass through the basal lamina directly at sites of membrane weakness or damage (Granados & Lawler 1981) or bypass the membrane using the host tracheal system as a conduit (Engelhard et al. 1994). Resistance may therefore be a consequence of selection for

more robust basal laminae or blockage of virus entering the tracheal system. In *S. littoralis*, high level of resistance are seen following oral inoculation with AcMNPV, whereas insects are highly susceptible to injection with a budded form of this virus (Rivkin et al. 2006). A study using a GFP expressing recombinant of AcMNPV showed that infection in *S. littloralis* following oral inoculation of the virus was limited by immune defenses in the midgut and surrounding tracheal cells which lead to encapsulation and melanzation of virally infected cells (Rivkin et al. 2006). However, while the role of the gut in resistance in *S. littoralis* against AcMNPV was further supported by a later study, encapsulation and melanzation of midgut associated tracheal cells was not evident as a resistance mechanism (Haas-Stapleton et al. 2003) highlighting the potential complexity and diversity in antiviral resistance within a single species of Lepidopteron insect.

Although there is considerable support that antiviral resistance occurs prior to virus entering the haemocoel, there is evidence that systemic resistance mechanisms are present. Asser-Kaiser et al. (2011) found that resistant strains of the codling moth, *Cydia pamonella*, were fully resistant to both oral inoculation with occluded *C. pomonella* GV (CpGV) and intrahaemocoelic injection with the budded phenotype of this virus. Furthermore, increased resistance of *H. zea* to AcMNPV compared to *Heliothis virescens* (tobacco budworm) to this virus was found to be a consequence of the action of haemocytes in the haemocoel rather than defenses in the midgut or tracheal epidermis (Trudeau et al. 2001). In addition, in *P. interpunctella,* a systemic response to injected PiGV does occur. The fact that infection following injection of virus has been previously found to depend on dose (Saejeng et al. 2011) and in this study injection of virus did not result in 100% infection is suggestive that in *P. interpunctella* a systemic antiviral response does occur. However, while it is clear that antiviral resistance mechanisms are present in the haemocoel of Lepidoptera insects including *P.*

interpunctella in this study, I show that these defences are not responsible for the increase in resistance seen in selected insects.

Here there was no coevolution of PiGV, with only selection for *P. interpunctella* resistance against a stock virus. Systemic responses to baculovirus challenge found in Lepidopteron hosts could be a consequence of counteradaptation in baculoviruses to the evolution of gut defenses in hosts. Further investigation of resistance of *P. interpucntella* to coevolving PiGV will shed light on the implications of virus coevolution on the location of host resistance.

Budded virus solution used for intrahaemocoelic injection was extracted from donor *P. interpunctella* larvae and may therefore have contained components present in the haemocoel of infected insects including immune components. While this could have affected the outcome of viral injection, given that the focus of my finding was the comparison of infection between control and resistant insects, potential contamination of the virus solution being injected does not impact my result. Orally inoculated insects were 11 days old and injected insects were 14 days old. The timings of oral inoculation and intrahaemocoelic injection used in this study where chosen as a compromise between the level of developmental resistance and mortality risk with handling involved in each experimental procedure. In some insects, aging is correlated with a reduction in parasite resistance (Adamo et al. 2001; Doums et al. 2002). However, in Lepidoptera, resistance to oral inoculated virus (e.g. Engelhard & Volkman 1995; Sait et al. 1994) and injected baculovirus (e.g. Hoover et al. 2002) increases with developmental instar. The difference in the age of the two methods of inoculation may therefore have had an effect, but the comparison was between controls and resistant lines of the same age and furthermore the older intrahaemocoelic injection larvae would be expected to have more resistance not less.

It is likely that gut and systemic antiviral responses are variable and context dependent. A central theory in explaining variation in fitness traits is the presence of costs (Boots 2011; Boots & Begon 1993; Boots & Haraguchi 1999; Schmid-Hempel 2003; Sheldon & Verhulst 1996). Localization of the immune response to the gut could act to reduce direct costs of immune activity, such as damage to host tissue (Sadd & Siva-Jothy 2006). However, the systemic, non-localized response found in other studies suggests that midgut responses may, in some cases, be more costly to activate or endure than haemocoelic responses. In support of this, in *P. interpunctella*, developmental costs were incurred in larvae resisting infection following oral inoculation (Boots & Begon 1993; Saejeng et al. 2011) but were not detectable after intrahaemocoelic injection (Saejeng et al. 2011).

Although most evidence points to the conclusion that resistance is dependent on defences present in the gut, it is possible that viral exposure via the midgut could be essential in triggering antiviral responses in the haemocoel that lead to resistance in *P. interpunctella*. In theory, passage of virus from the gut to the haemocoel via the basal lamina or tracheal system could be essential in activating haemocoelic based responses. In addition, the occlusion body, a component of the orally administered virus only could be an important trigger for viral resistance. Although the occlusion body is lost quickly in the alkaline conditions of the midgut (Blissard & Rohrmann 1990) and despite the fact that we only have a limited knowledge of the mechanism of virus recognition in insects, it is possible that resistance relies on recognition of the occlusion body in the gut. The baculovirus infection process is well established with a lag of two days between oral exposure and the entrance of the virus into the haemocoel (Begon et al. 1993; Blissard & Rohrmann 1990). It is possible that resistance in *P. interpunctella* insects occurs in the haemocoel, but critically, relies on the time lag between oral exposure and the virus entering the haemocoel. By bypassing the oral exposure route

and injecting straight into the haemocoel, the time needed to activate an efficient systemic response may be lost, explaining a lack of increase in resistance to injected virus seen in the present study. Distinction between the gut being the location of defense and oral inoculation of virus being essential for the trigger of the resistance mechanism in *P. interpunctella* will require tracing virus infection through the midgut and into the haemocoel in both resistant and control larvae.

The best characterized insect antiviral response is that of the fruit fly, *Drosophila melanogaster*. However, studies investigating antiviral defence in *Drosophila* often use non pathogenic virus and bypass the natural oral transmission route, directly injecting virus into the haemocoel (e.g. Dostert et al. 2005; Zambon et al. 2005). The present study highlights that insects respond differently to orally inoculated virus compared to injected virus with levels of infection resulting from oral inoculation giving no insight into levels of infection which may result from injection of virus. It is clear therefore that injecting virus straight into the haemocoel may not provide accurate information about natural insect-virus interactions and antiviral responses, raising questions about how much we really know about natural antiviral responses in insects.

A greater understanding of Lepidopteron host responses to baculoviruses will aid the use of baculovirus in the biological control of many lepidopteran insect pest species. *P. interpucntella* is a natural pest of stored grains and causes extensive economic damage worldwide. The present study highlights that a key to biological control of this insect using baculovirus could be in the manipulation of the virus transmission route. Alternation between oral transmission and intrahaemocoelic transmission could prevent the evolution of baculovirus resistant insects with the development of a method of intrahoecolic transmission, for example by a vector, presenting a potential avenue of future research.

In summary, this study highlights that insects with increased resistance to oral inoculation of virus do not show increased resistance to virus directly injected into the haemacoel. This highlights the role of the gut in antiviral defence and given the lack of information about antiviral defense in insects, provides a focus for the study of potential antiviral mechanisms. It also suggests that investigating antiviral response and the ecology of insect-virus interactions by bypassing natural infection routes could give misleading results.

Chapter 8: General Discussion

In this thesis I have examined antiviral resistance in the Lepidopteron host *Plodia interpunctella* from an evolutionary and ecological perspective. The aim of this thesis was to investigate two form of antiviral resistance in insects: (a) the upregulation of an individual host's (or their offspring's) defences following previous exposure to a parasite, referred to as immune priming and (b) host resistance following long term selection pressure from a parasite, referred to as evolved resistance. The nature of these forms of resistance, in particular the costs and the specificity of the protection which they provide, was focused on throughout. Our knowledge of insect antiviral resistance is less comprehensive than our knowledge of insect resistance against bacterial and fungal parasites (Imler & Elftherianos 2009; Kemp & Imler 2009). The importance of gaining further knowledge of insect-virus interactions and antiviral resistance in insects is crucial given the role of insects as vectors of harmful human viruses such as Dengue Virus (Hemingway & Ranson 2000; Turell et al. 2005), the devastating impacts which viruses may have on beneficial insects (Cox-Foster et al. 2007; Ponnuvel et al. 2003) and the potential use of insect viruses in the biological control of insect pest species (Alexandre et al. 2010; Tinsley 1977).

8.1 Immune Priming

Invertebrates were traditionally thought to lack any capacity for memory in their defence against parasites due to the absence of immune cells necessary for the

vertebrate adaptive or acquired immune response (Hauton & Smith 2007; Kurtz 2005). However, this view is being challenged with examples of increased protection to a parasite upon secondary challenge within an individual or increased protection in offspring from parents exposed to a parasite documented (e.g. Little et al. 2003; Moret & Siva-Jothy 2003; Pham et al. 2007; Sadd et al. 2005; Schmid-Hempel 2005b). Due to its functional distinction from vertebrate adaptive immunity this process has been termed 'immune priming' in invertebrates. While a number of studies have been carried out to investigate immune priming to bacteria in invertebrate hosts (e.g. Moret 2006; Roth et al. 2010; Sadd et al. 2005), namely insects, the only investigation of immune priming to virus has been conducted using crustaceans (Witteveldt et al. 2004; Wu et al. 2002). My aim was to investigate immune priming to virus in a natural insect-virus combination. I show for the first time that immune priming to virus can occur in an insect. *P. interpunctella* previously exposed to *P. interpunctella* Granulosis Virus (PiGV) were more resistant to infection with this virus when subsequently exposed later in life compared to control insects (chapter 2). In addition, *P. interpunctella* offspring from parents primed with PiGV were more resistant to infection with PiGV (chapter 2). Some previous studies investigate immune priming use either heat killed parasites (e.g. Roth et al. 2009) of immune elicitors (e.g. Moret & Siva-Jothy 2003) to prime defences. While these studies provide valuable information on immune priming in insects, by priming the antiviral defences of *P. interpunctella* with a very low dose of its natural viral parasite I show that immune priming to virus may occur in natural systems.

Costs

Costs shape the optimal investment in a given trait (Schmid-Hempel 2003; Sheldon & Verhulst 1996; Stearns 1989). As such, if immune priming caries costs, it will only be seen when its benefits outweigh its costs. Understanding the costs associated with

immune priming is therefore fundamental to its occurrence and its implications. Despite their importance, costs associated with immune priming are not well studied. However, costs have been highlighted in the form of slower development in the mealworm beetle *Tenebrio molitor* (Moret 2006) and increased susceptibility to bacterial parasites in offspring against which their parents were not primed in the bumble bee, *Bombus terrestris (Sadd & Schmid-Hempel 2009b)*. Costs associated with immune priming to virus in insects have not been previously documented. I show that *P. interpunctella* primed with PiGV take a significantly longer time to reach pupation than control insects (chapter 3).

Specificity

We often think of specificity in terms of antigen specific binding by antibodies, a feature of the vertebrate immune system. However, specificity may also be considered in the form of the effectiveness of host resistance to one parasite against other parasite strains or types. Specificity in immune priming to bacteria has been documented in the red flour beetle, *Tribolium casteneum* (Roth et al. 2009) and *B. terrestris* (Sadd & Schmid-Hempel 2006). However, specificity in immune priming protection to virus in insects has not yet been explored. In this thesis I aimed to investigate the specificity of protection in immune primed insects to virus. I challenged insects previously primed with PiGV and offspring of insects primed with PiGV with *Ephestia cautella* Nucleopolyhedorsis Virus (EcNPV) in addition to PiGV (chapter 3). However, I found no increase in resistance to virus in immune primed individuals and therefore gained no insight into the specificity of immune priming protection to virus. Although immune priming has been investigated and not found in a number of systems (Gonzalez-Tokman et al. 2010; Voordouw et al. 2008) a lack of repeatability in immune priming has not been documented. The results from chapter 3 clearly raise questions about the

variability in immune priming to virus in *P. interpunctella* and in insects generally and the validity of the immune priming experimental protocol. The development costs associated with immune priming may explain the variability in immune priming. However, variation in proportion infection is often found in *P. interpunctella* following oral exposure to the same virus aliquot or the same percentage dose of virus. This may result from heterogeneity in baseline resistance to virus in stock insects or perhaps more likely, could reflect inconsistencies in the number of virus particles ingested by insects. Despite sonicating and vortexing the priming virus solution prior to administration the lack of priming response in chapter 3 could be the consequence of insects failing to ingest enough virus particles to prime the antiviral response. Developing a protocol by which virus particles within droplets of solution can be counted prior to ingestion by larvae may enable the relationship between the number of virus particles ingested and the priming response to be quantified.

Population level implications of immune priming using mathematical modeling

Mathematical modeling is a key tool in capturing and predicting host parasite population dynamics. While the effect of acquired immunity, where following recovery from infection hosts become immune for life (Anderson & May 1981), on host parasite population dynamics have been examined, the implications of immune priming have not previously been investigated. The simple and general mathematical model developed and presented in chapter 4 highlights that immune priming may allow a parasite to persist below the critical value of R_0=1 and have a pronounced effect on the stability of host parasite populations with limit cycles produced when priming rate is high and resistance in primed insects is reduced.

8.2 Evolved Resistance

In addition to adapting their defences according to their current or parental environment hosts may also evolve resistance in response to selection by parasites over multiple generations. Although previous work has documented evolution of resistance to virus in Lepidopteron hosts (Fuxa et al. 1988; Fuxa & Richter 1998; Milks & Myers 2000) and specifically in *P. interpunctella* (Boots 2011; Boots & Begon 1993), little is known about the nature of evolved resistance to virus. In concurrence with previous work, in this thesis I show that *P. interpunctella* populations maintained with virus for 12 generations are more resistant over a range of virus doses compared to populations maintained without virus (chapter 5,6 and 7). This indicates therefore that *P. interpunctella* can evolve resistance to PiGV in response to selection pressure by this virus.

Costs

Costs associated with evolved resistance are likely to constrain its evolution to fixation and affect its stability in the absence of the selection pressure (Schmid-Hempel 2003; Sheldon & Verhulst 1996; Stearns 1989). Although costs can manifest themselves in a number of ways, in this thesis, I examine developmental traits of insects selected for resistance to virus in the absence of virus. I therefore examine genetic based trade-offs associated with the evolution of increased resistance. In addition, to investigate the potential implications of any trade-offs, I assessed the stability of antiviral resitance in the absence of selection pressure. I find evidence for a trade-off between evolved resistance and development weight and therefore growth rate and show that in the absence of the virus resistance is lost (chapter 5). These results suggest that the evolution of resistance may be constrained by genetic based trade-offs between

resistance and development and these trade-offs are significant enough to reduce the fitness of resistant hosts in the absence of the parasite.

Resource quality and quantity have been shown to affect the detection of physiological trade-offs between fitness traits including resistance. When resources are low physiological costs may be incurred, the rational being that the resources used up through the activation of an immune response cannot be replenished therefore limiting the execution of other energy demanding traits (Moret & Schmid-Hempel 2000). In addition to physiological trade-offs, the influence of resource quality on genetic based trade-offs has also been documented. For example, in the fruit fly, *Drosophila melanogaster*, the genetic based trade-off between fecundity and resistance was only detected in insects on poorer resources (McKean et al. 2008). In this thesis I also investigate the effect of food quality on the genetic based trade-off between resistance to PiGV in *P. interpunctella* and growth rate. However, contrary to predictions and previous findings in *D. melanogaster* (McKean et al. 2008) and in this system (Boots 2011), I see a more pronounced trade-off between antiviral resistance and insect size on high quality food rather than low quality food (chapter 5). This study therefore highlights that the effect of resource quality on genetic based trade-offs may be complex in this system.

Specificity

Specificity in evolved resistance, for example to parasitoids and virus, in insects has been previously examined by looking at cross resistance to a number of different strains and types of parasites in previously selected insects (Fellowes et al. 1999b; Fuxa & Richter 1990; Milks & Myers 2003). However, the level of specificity seems to be highly variable. In this thesis I find evidence that resistance to PiGV in *P. interpunctella*

is not specific and offers protection against another strain of PiGV and a different virus, EcNPV (chapter 6).

Site of resistance

A common route of virus entry into insect hosts is through oral ingestion of virus particles. So, while a dose response to virus injected straight into the haemocoel has been documented in *P. interpunctella*, thereby providing evidence for systemic antiviral defenses (Saejeng et al. 2011), midgut defences are likely to play a significant role in antiviral resistance. However, the relative role of the gut defences and haemocoel defences in antiviral resistance are not well explored. By examining infection following injection of virus aswell as oral inoculation of virus in *P. interpunctella* selected for resistance in chapter 7 I address this issue. I find that insects showing increased resistance to oral inoculation of virus following selection do not show increased resistance to injected virus. This result thereby indicates that the gut based defences play a more crucial role in antiviral resistance compared to haemocoel based defences.

8.3 Implications

In the context of viral disease emergence my results highlight that while immune priming to virus may reduce the number of virally infected individuals and therefore the prevalence of virus in a population, it may lead to unstable population dynamics which in theory could lead to host extinction events. The presence of virus may also result in host life history changes. For example, individuals showing antiviral resistance following immune priming may take longer to develop and the evolution of genetic based resitance following long term selection is traded-off with insect size. Antiviral

resistance may therefore have a knock on effect on host population size with a slower development extending the time taken for insects to produce their first offspring and a smaller size potentially correlated with reduced fecundity (e.g. Calvo & Molina 2005; Honek 1993).

The affect of immune priming on parasite persistence and the dynamics of host-parasite populations is distinct from that of acquired immunity. My results show that immune priming may lead to the persistence of a parasite when its critical reproductive number is below 1 and whereas acquired immunity leads to damped oscillatory populations dynamics on the approach to a stable endemic equilibrium, immune priming results in prolonged cycling. The key aspect of immune priming is that, when exposed to a parasite, hosts may quickly become immune and, in contrast to acquired immunity, without ever having become infectious. While my focus has been on immune priming in invertebrates this form of immunity and acquired immunity, following infectiousness, are not necessarily mutually exclusive. In theory it is therefore possible that immune priming is a feature of host-parasite interactions across a wider rande of taxa, including humans, with for example, the potential for antibodies to be present in hosts which have never been infectious. Given that immune priming may change the criteria for the eradiation of disease and have consequences for host-parasite population stability, the presence of immune priming in vertebrate systems warrants future investigation.

The potential for antiviral resistance to evolve in this host to selection pressure from the virus means that the parasite is less likely to drive host extinction but also means that the use of baculoviruses for insect pest control may be limited. In addition, the fact that resistance to one virus may confer resistance to other viruses further limits the potential success of these viruses in the biological control insect pests. Hosts showing resistance to oral inoculation do not show resistance to injection of virus.

Therefore, one possible route to overcoming resistance to baculovirus in pest insects may be the manipulation of virus transmission to bypass the oral route of infection and thereby the evolved resistance mechanism.

Host resistance may have important implications for parasite traits such as virulence (Gandon et al. 2001; Mackinnon et al. 2008). It is possible that an increase in the proportion of resistant hosts in a population may result in selection for parasites more able to counter host resistance mechanisms. In addition, increased competition within the remaining susceptible hosts in the population may also select for parasites with increased virulence (Alizon & van Baalen 2008; May & Nowak 1995). The consequence of *P. interpunctella* resistance to PiGV for the virulence of PiGV is not known but should be the focus of future study.

Optimal investment in antiviral resistance will be determined by both its benefit but also importantly, its costs (Boots 2011; Boots & Haraguchi 1999). Without knowledge of the costs associated with resistance it is therefore difficult to predict the extent to which hosts will invest in fitness traits such as resistance. By providing evidence that antiviral resistance in *P. interpunctella* is costly my work enables the more accurate prediction of a hosts investment in immune priming and the response of host populations to selection pressure. However, while I have highlighted that antiviral resistance in *P. interpunctella* may be associated with developmental costs it is possible that other forms of costs exist in this system, something that requires further study.

The nature of antiviral resistance may also influence the degree of variation which is maintained in this trait in natural populations (Schmid-Hempel 2003), something which is essential for future adaptation. By providing evidence for non-specific antiviral resistance in *P. interpunctella* my results suggest that variation in resistance may be reduced in this insect. Cross resistance against multiple viruses will reduce the selective advantage of rare parasites and variation in parasite genetics and

therefore in turn will reduce the variation in host resistance traits. My results also highlight that resistance may be costly, which in contrast to a non-specific resistance, is associated with the maintenance of variation. The implications of antiviral resistance may therefore be complex with its individual characteristics resulting in contrasting outcomes. Variation in host resistance in natural populations of Lepidoptera has been found, however, examination of direct link between variation and specificity and costs of resistance will aid a greater understanding of the causes of variation in natural populations.

8.4 Towards a Better Mechanistic Understanding of Antiviral Resistance

So in this thesis I have addressed questions regarding the evolution and ecology of insect antiviral defence. However in order to fully understand antiviral resistance, further investigation of antiviral resistance mechanisms in addition to an evolutionary and ecological approach is needed. The understanding of mechanisms of antiviral defence and the specific contributions of individual mechanisms to resistance in invertebrates is limited (Imler & Elftherianos 2009). However, the results from this thesis provide focus and insight for future mechanistic studies. By showing that evolved resistance to PiGV in *P. interpunctella* protects against a different strain of PiGV and a different baculovirus I highlight that the antiviral resistance mechanism in this insect lacks specificity in parasite recognition or action. Further, I demonstrate that evolved resistance does not protect insects against injected virus. The most likely reason for this is that selection has acted on a gut based mechanism. These results therefore highlight that barriers in the midgut, namely the peritrophic membrane, may be a good starting

point for the future investigation of antiviral resistance mechanisms in *P. interpunctella* and other insects.

Most mechanistic studies of antiviral resistance in insects focus on the responses of *Drosophila* hosts to injected virus (e.g. Dostert et al. 2005; Zambon et al. 2005). While any knowledge of responses following exposure to virus help us build a picture of antiviral defence in insects, in light of the findings of chapter 7, perhaps a more beneficial avenue would be to study the responses of *Drosopila* and other insects following the natural route of virus exposure.

RNAi has been proposed as a fundamental mechanism in insect antiviral defence in *Drosophila* (Galiana-Arnoux et al. 2006; van Rij et al. 2006; Wang et al. 2006). Studies highlighting the role of RNAi as an antiviral defence component focus on the response of *Drosophila* to RNA viruses. However, in *Drosophila* and other insect species the diversity of viruses with which they are infected is likely to be large with a DNA virus infecting *Drosophila innubila* recently discovered (Unckless 2011). Despite this and the large number of DNA baculoviruses which infect Lepidopteron insects little is known about the role of RNAi against DNA viruses. The RNAi process targets double stranded RNA (dsRNA), present in RNA virus genomes or replication intermediates. However, it is also possible that insect DNA viruses such as baculoviruses can produce dsRNA necessary to activate the RNAi process as plant DNA viruses do (Ding & Voinnet 2007; Obbard et al. 2009). The role of RNAi in the denfence of insects against DNA viruses therefore provides a very interesting avenue for future research.

The sequencing of the *D. melanogaster* genome (Adams et al. 2000) has clearly been a turning point for the study of antiparasitic defence in this insect at the molecular level. The molecular approach to the study of antiviral mechanisms in *P. interpunctella* is more difficult as there its genome is not yet sequenced. However, development of

techniques such as De Novo Transcriptome Assemby (Martin & Wang 2011, Appendix 3), provide a method by which changes in gene expression in manipulated insects can be assessed and insight into mechanisms gained. These methods therefore provide exciting new avenues for further investigation of antiviral defence mechanism at the molecular level.

8.5 Overview

In this thesis, using a combination of empirical and theoretical work I have shed light on the resistance of *P. interpunctella* to its natural viral parasite PiGV. By examining immune priming and evolved resistance to virus, I have gained insight into the role of an individuals environment and the influence of long term selection pressure on antiviral resistance. Throughout my results highlight the complex nature of the antiviral defences in insects. More generally this highlights the usefulness of insect pathogen model systems in the laboratory in improving our understanding of defence to infectious disease.

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Appendix 1: Empirical Techniques

a) Virus extraction

Purified virus solution was produced by centrifugation of a homogenate of infected insect cadavers (Smith & Crook 1988). *Plodia interpunctella* Granulosis Virus (PiGV) was extracted from PiGV infected *P. interpunctella* cadavers and EcNPV was extracted from EcNPV infected *Ephestia cautella* cadavers. Approximately 20 infected cadavers were placed in Eppendorf tubes and homogenised in 500µl distilled water (dH_20). These tubes were then centrifuged at 1000g for 1 minute to pellet out the insect debris, while leaving the virus particles in the supernatant. The supernatant containing the virus was transferred to a clean Eppendorf, taking care not to disturb the pellet. This virus containing solution was then centrifuged at 1900g for 3 minutes to pellet out the PiGV occlusion particles. For EcNPV extraction, the solution was centrifuged at 4000g for 3 minutes. EcNPV was not purified using sucrose gradients (see below) so the above centrifuge step was repeated three times. The supernatant was then discarded leaving the pellet containing the occluded virus particles undisturbed. The virus pellet was then resuspended in 500µl dH_20.

b) Virus purification

The extracted suspension of PiGV was added to ultracentrifuge tubes containing a gradient of sucrose solutions ranging from 60% sucrose at the bottom to 40% sucrose at the top. These were spun using an ultracentrifuge (Beckman, Rotor type-SW41) at 6700g for 1 hour at 20°C. The spin was conducted in a vacuum and the brake was left off to ensure that the tight band of virus occlusion bodies formed within the sucrose gradients during this spin was not disturbed and remained intact. Sucrose solution and small amounts of debris present above the virus band were removed and discarded. The

virus band was then carefully transferred to a fresh ultracentrifuge tube and diluted in dH_20 so that the tube was full to 5mm from the top. To pellet out the purified occlusion bodies this solution was then spun in the ultracentrifuge at 3800g for 20 minutes at 20°C. The spin was conducted in a vacuum with the brake on. The supernatant was removed and discarded and the pellet suspended in 1ml dH_20 to make up the concentrated, purified bulk virus solution. This solution was then aliquoted into Eppendorfs and frozen at -20°C until required.

c) Examination for covert infection using PCR

Covert, sublethal baculovirus infection has been found in Lepidopteron hosts and specifically, PiGV has been found to covertly infect *P. interpunctella* (Burden et al. 2002). To ensure that stock populations of *P. interpunctella* were not covertly infected with PiGV, DNA was extracted and amplified from stock insects and analysed for the presence of PiGV. 20mg/ml proteinase K was added to 'Squishing Buffer' (1ml 1M TrisHCL pH 8.2, 0.5ml 0.2M EDTA, 0.5ml 5M NaCl and 98ml dH_20 in 100ml Squishing Buffer). Each insect was homogenised in 50µl of squishing buffer mixed with proteinase K, heated to 55°C for 1 hour then boiled for 2 minutes. 2µl of this solution was then added to 9ul standard PCR master mix (1µl 10xReaction buffer, 4.55µl dH_20, 0.4µl 50mM $MgCl_2$, 1µl 2mM dNTPs, 0.05µl Taq (thermorprime plus), 1µl forward PiGV priming and 1µl backward PiGV primer per 1 sample's quantity of master mix) and the PCR run. PCR products were run on a 1.5% agarose gel. Positive controls of infected insects and virus solution were used to confirm the protocol had worked. Granulin DNA from PiGV was not found in any stock insects.

In further support for the lack of contamination of stock insects and the lack of covert, sublethal infection in experimental insects, virus-free control treatment groups were included in all experimental designs.

d) Oral inoculation of virus

Oral inoculations were conducted following a standard droplet feeding technique (Boots & Begon 1993). Bulk virus was diluted in 75% blue food dye (Dr. Oetker) in double dH_20 with 5% sucrose to the required viral concentration. Droplets of virus/dye solution were then orally administered to the larvae, which had been previously removed from their food and starved for 2 hours, using a pipette. Droplet volume was adjusted depending on the development stage of the larvae being inoculated. Second instar (8 days) larvae were given 0.5µl droplets, third instar (11 days) larvae were given 1µl drops and fourth instar (14 days) larvae were given 1.5µl drops. Successful oral inoculation of insects using this method was determined by the presence of virus solution (indicated by the blue food colouring visible through the transparent epidermis) in half of the length of the gut. The same inoculation procedure was used for control larvae but using only the dye solution. Once successfully inoculated, insects were transferred to a single cell of a 25 cell petri dish and provided with excess food. 7- 8 days following oral inoculation insects were examined for viral symptoms and infected and healthy insects counted and recorded. Insect mortality due to causes other than viral infection was very rare but recorded. Prior to experiments a number of dose response assays were carried out from which the desired dose of virus solution was determined.

e) Intrahaemocoelic injections

The budded virus extraction and intrahaemocoelic injection protocol were adapted from Saejeng et al. (2010). Third instar larvae were infected with PiGV, following the oral

inoculation procedure outlined above, to produce virus donors. 7 days after oral inoculation virus was extracted from donor insects. Donor insects were immobilized on ice for 15 minutes and transferred to an upturned petri dish where they were covered with cling film to restrict movement and increase haemolymph pressure. The final proleg (furthest from head) was then punctured with a stainless steel 'extra fine' entomological pin allowing a small droplet (~1.5μl) of haemolymph containing virus to escape. 0.5μl dH_20 was added to this droplet of haemolymph. Using a sanded 1mm glass capillary (Narishige, Tokyo) attached to a pneumatic picopump (WPI, model PV280), this solution was then transferred to an Eppendorf on ice containing 10μl dH_20. This procedure was repeated for 50 insects. This solution (total volume ~110μl) was then centrifuged at 1000g for 30 seconds to remove any insect debris present. The supernatant containing the budded virus was then transferred to a fresh Eppendorf containing 10μl dye solution (used for oral inoculations) and kept on ice ready to be injected using the pneumatic picopump. The solution contains mainly budded virus as it is the budded virus which is present in the haemolymph at his stage. Occluded virus will be present in small quantities at this time point and increases up to 14 days post inoculation (Begon et al. 1993). The solution was loaded into a calibrated, finely sharpened 1mm glass capillary needle and 1ul of solution was injected into the second proleg of 15 day old recipient larvae. Injection control insects were injected with food dye diluted with dH_20. Injected insects were monitored for 30 minutes and insects showing injury from injection were discarded. Successfully injected insects were transferred to single cells of 25 cell petri dishes and given excess food. Symptoms of viral infection were then assessed 7-8 days following injection. Insect mortality resulting from causes other than viral infection was rare but recorded.

Appendix 2: Gene Expression Profiling of *Plodia interpunctella* using De Novo Transcriptome Assembly. Extension work from chapter 2.

One approach to examine mechanisms that underpin the phenomenon of immune priming that I have examined in my thesis is gene-expression profiling using transcriptome assembly. Transcriptome assembly refers to the construction of entire transcriptomes from many short sequence reads produced using next generation sequencing technology. Transcriptome assembly may be aided by the presence of a reference genome sequence but can also be performed without a reference genome, a process referred to as de novo transcriptome assembly (reviewed in: Martin & Wang 2011). De novo transcriptome assembly therefore enables gene expression analysis for a large range of organisms which do not have a sequenced genome.

Prior to transcriptome assembly, high quality sequence data needs to be generated. In brief this involves the extraction and fragmentation of RNA which is then reverse transcriptased into a library of copy DNA (cDNA). This cDNA library is then sequenced using next generation sequencing technology producing many short sections of cDNA sequence or 'reads'. A sequencing platform such as Illumina is used to sequence these short reads. The assembly of these short reads to form larger transcripts and eventually a transcriptome, requires an assembly program such as Velvet. With the absence of a genome to map the transcripts onto, de novo transcriptome assembly relies on the presence of overlap between the reads to deduce the correct sequence and determine gene expression levels. Subsequent annotation of the assembled transcripts by blasting transcripts against the nucleotide database allows the function of the genes expressed to be determined.

Although mRNA levels may not always translate into protein expression

because of the action of post-transcriptional gene regulation processes such as RNA interference (RNAi), de novo transcriptome assembly is a highly valuable technique for exploring the link between physiological changes and gene expression in important organisms which lack a reference genome.

In order to use these approaches to examine the mechanisms underpinning immune priming I collaborated with Dr. Seanna Mc Taggart (University of Edinburgh). I carried out a priming experiment equivalent to that described in chapter 2 to provide the material for the analysis. Gene expression profiles of the immune primed insects are currently being investigated using the de novo transcriptome assembly technique. In particular the gene expression profiles are being assembled for:

a) 14 day old insects primed with virus at age 7 days,

b) 14 day old insects primed with control solution at age 7 days,

c) 15 day old insects challenged with virus at 14 days old and primed with virus at 7 days old and

d) 15 day old insects challenged with virus at 14 days and primed with control solution at 7 days old.

By creating gene expression profiles for virus primed and control insects both before and after subsequent challenge with virus the aim of this work is to gain insight into the molecular levels changes priming induces and the potential mechanism of immune priming.

Appendix 3: The Evolution of Immune priming in Invertebrate Hosts. Extension work from chapter 4.

In chapter 4 I presented theoretical work investigating the implications of immune priming for the ecology of host-parasite interactions. Using the same theoretical framework (the SIR-type SPI model), the evolution of immune priming in invertebrate hosts has also been examined. This work has been lead by Dr. Alex Best and involves the use of adaptive dynamics theory (Geritz et al. 1998). This technique is used to examine the evolution of a particular phenotypic trait by determining the fitness of a mutant individual which differs slightly in this trait and allows ecological processes such as immune priming to be examined. My major role as a collaborator has been in the design of the model framework and the biological interpretation of the results.

In brief, the fitness of an introduced mutant individual into the population, which differs in its priming rate relative to the resident population, was assessed and as a result, the long term evolutionary trajectory of immune priming was determined. The main findings of this work are that 1) increased parasite pathogenicity (parasite virulence or parasite sterility) in the host and a high immune protection gained following immune priming results in selection for and therefore the evolution of increased immune priming rate in the population, 2) increased pathogenicity and high immune priming protection also results in evolutionary branching, so that heterogeneity in immune priming evolves and 3) the evolution of priming rate towards a stable equilibrium may result in disruptive changes in population dynamics such as the occurrence of population cycles referred to as limit cycles.

Appendix 4: Within and transgenerational immune priming in an insect to a DNA virus

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