The Impact of Nutrition on Within and Trans-generational Disease Resistance in the Cabbage Looper, *Trichoplusia ni*

by

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M.Sc., University of British Columbia, 2009
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Thesis Submitted in Partial Fulfillment of the

Requirements for the Degree of

Doctor of Philosophy

in the

Department of Biological Sciences

Faculty of Science

SIMON FRASER UNIVERSITY

Summer 2014

Approval

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Degree: **Doctor of Philosophy**

Title: **The Impact of Nutrition on Within and Trans-generational Disease Resistance in the Cabbage Looper, *Trichoplusia ni***

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Abstract

Parasites and pathogens are ubiquitous, and pose a threat to all living organisms. Investment in resistance mechanisms to fight parasite challenge can be costly, often resulting in trade-offs with other life-history traits. Host nutrition can alter the availability of resources to invest in resistance mechanisms and influence host-parasite interactions and their outcomes. I investigated the impact of nutrition on disease resistance in the cabbage looper, *Trichoplusia ni*. I assessed the role of dietary macronutrients on the expression of fitness costs exhibited by a *T. ni* strain that has evolved resistance to the bacterial pathogen, *Bacillus thuringiensis* (*Bt*). Reduced pupal weight and growth rate, which are fitness costs associated with *Bt*-resistance, resulted from reduced food intake rather than impaired macronutrient utilization. When given a choice, *Bt*-resistant *T. ni* self-composed a higher ratio of protein to carbohydrate (P:C ratio) than *Bt*-susceptible *T. ni*, allowing males to eliminate a fitness cost (reduced pupal weight), but not females. Next, I investigated the interaction between host nutrition and another key environmental factor, temperature, on the interaction between *T. ni* and two species of baculoviruses differing in host range (TnSNPV, narrow range; AcMNPV, broad range). Optimal performance of *T. ni* shifted to higher P:C ratios when challenged by either virus as survival increased with dietary protein content. This effect was strongly affected by temperature when challenged by AcMNPV but not TnSNPV. Virus performance was also differentially affected by the host's environmental condition, such that AcMNPV had a broader peak of optimal performance (combined measure of host mortality and virus production) across environmental conditions than TnSNPV. Lastly, I examined the impact of nutritional stress on the ability of *Bt*-challenged *T. ni* to prime the immune system of their offspring. If parental *T. ni* experienced only nutritional stress or *Bt*-challenge, they transferred nutritional stress tolerance or immune priming to their offspring respectively. However, as surviving each stressor is costly, when experienced simultaneously a trade-off was observed where only immune priming was transferred. This study highlights the important influence of host nutrition on host resistance to pathogens, costs associated with resistance, and pathogen virulence and growth.

Keywords: host-pathogen interaction; disease resistance; nutritional ecology; trans-generational immune priming; fitness costs

Acknowledgements

I have many people to thank for the completion of this thesis. My senior supervisor Dr. Jenny Cory for her patience, guidance and insightful comments that have helped me build my critical thinking, knowledge and confidence as a researcher. I would also like to thank my committee members Dr. Carl Lowenberger and Dr. Gerhard Gries for their valuable insights on my project. Thank you to all current and former post-docs, graduate students and lab technicians in the Cory Lab for their input on my research designs, results and presentations. I appreciated our lively scientific and non-scientific discussions. I could not have completed this project and other side-projects without the help of numerous undergraduate assistants and volunteers. I would especially like to thank Kevin Hua for his long-term volunteer commitment and Olivia Halpert-Scanderbeg and Miranda Oak for their hard work in helping me complete a part of this project.

I thank my former mentors, Dr. Murray Isman, Dr. Yasmin Akhtar and Dr. Judith Myers from my time as an undergraduate and Master's student. They have given me the foundation and passion to pursue science as a career.

Thanks to the Natural Sciences and Engineering Research Council of Canada, Simon Fraser University, Government of British Columbia, Mutual Fire Insurance Company and Sulzer Pumps Inc. for funding.

Last but not least, I would like to thank my lovely wife Ryoko for always encouraging me to pursue my interests. Thank you for labeling thousands of plastic cups for experiments that did not work, with minimal complaining!

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Chapter 1.

Introduction

Parasites are numerous and ubiquitous and affect all living organisms (Schmid-Hempel 2011). Parasites can range from highly virulent inflicting huge fitness costs on the host through losses in survival and reproduction to relatively benign and can clearly affect the ecological and evolutionary dynamics of natural host populations (Schmid-Hempel 2011). Understanding the factors that influence the susceptibility of organisms to parasites is an important goal if we are to understand and manage the spread and outcome of infectious disease. Relatively young disciplines such as ecological immunology and disease ecology are gaining in popularity, as they apply ecological and evolutionary principles to explain the underlying causes of variation in immunity to parasites between individuals or populations (ecological immunology) and the spread of parasites through populations and communities (disease ecology) (Hawley & Altizer 2011). These ecological and evolutionary approaches place considerable emphasis on the role of environmental factors in determining the outcome of host-parasite interactions (Hawley & Altizer 2011).

Parasitism by definition is an intimate and obligatory relationship between two different species in which one lives on, off, and at the expense of the other. Parasites exploit their host's resources by using the behavioral, physiological and energetic investment of the host. Parasites are enormously diverse, but they can usually be placed in six functional categories (summarized in Schmid-Hempel 2011):

Microparasites: usually refer to small parasites such as viruses, bacteria, fungi, and protozoa (up to a few hundreds of μm in size) that have short generation times and can multiply to large numbers within their host.

Macroparasites: usually large, multicellular parasites that include parasitic helminthes (nematodes, tapeworms, and flukes) and parasitic arthropods. They have slower generation times and are found in lower numbers in their hosts compared to microparasites.

Parasitoids: parasites that have a free-living stage, but live in or on a single host individual for most of their pre-adult lives. They ultimately either kill or sterilize the host prior to maturation. This category consists mostly of parasitic wasps or flies.

Endoparasites: parasites that live inside the host. Most parasitoids, parasitic nematodes and microparasites are endoparasites.

Ectoparasites: parasites that live on or attach to the host. These include mites, ticks, fleas and fungi.

Social parasites: parasites that do not directly feed on the tissue of their host, but instead gain other benefits from their host, such as consuming food collected by the host or seducing the host to raise the parasite's eggs. Social parasites are not included in the scope of this thesis.

Nutrient availability has received much attention in the last decade as a key environmental factor that influences the relationship between hosts and their parasites. Host nutrition is critical to the understanding of parasite-dependent population dynamics, because increasing population densities can both elevate the probability of parasite transmission and reduce the availability of nutritional resources (Gulland 1992; Knell, Begon & Thompson 1996; Ryder *et al.* 2005; Boots & Roberts 2012). Dietary nutrient availability of the infected host affects its ability to resist parasites because it alters the availability of key resources necessary for components of the host's resistance mechanisms. This is widely accepted for vertebrates, including humans, where nutrition is well known to play a key role in the optimal functioning of the immune system (for reviews see (Coop & Kyriazakis 1999, 2001; Hood & Skaar 2012). These studies are mainly driven by the objective of improving human and livestock health, and have demonstrated the importance of numerous nutrients including protein, essential amino

acids, linoleic acid (essential fatty acid), folic acid, β-carotene, vitamins A, B6, B12, C, E, and dietary elements Cu, Fe, Mn, Se, Zn (for reviews, see Scrimshaw & SanGiovanni 1997; Calder & Jackson 2000; Grimble 2007; Hood & Skaar 2012).

Although the resistance mechanisms of invertebrates against parasites are less studied, it is evident that diet can influence their effectiveness. Invertebrate studies have primarily focused on the effect of nutrient deprivation on parasite resistance, with the consensus being that reduced resources compromise resistance mechanisms and increase susceptibility (eg. Moret & Schmid-Hempel 2000; Brown, Loosli & Schmid-Hempel 2000; Lord 2010; Myers *et al.* 2011). For host resistance mechanisms involved in insect host-parasite interactions, most is known about the responses to pathogens, which by definition are parasites that cause disease, and parasitoids because of their use as biological control agents to manage insect pests. Most of the focus of this thesis will be on insect-pathogen interactions. However, the questions should be relevant to any insect parasite in any species.

1.1. General overview of resistance mechanisms

The majority of research on entomopathogens is related to groups used as biological control agents: our knowledge of the broader range of pathogens occurring in natural insect populations is considerably less. Microbial control mainly focuses on three groups: bacteria (mainly *Bacillus thuringiensis*), viruses (baculoviruses: nucleopolyhedroviruses and granuloviruses) and fungi (including Microsporidia) (Cory & Hoover 2006). The cuticle and midgut are the first and most effective lines of defense against pathogens in insects. The cuticle is the main barrier against the penetration of cutaneously entering pathogens such as fungi (Hajek & St. Leger 1994; Wilson *et al.* 2001). The gut has numerous barriers to pathogens, starting with the insect's salivary enzymes, such as glucose oxidase which produce hydrogen peroxide that will inactivate pathogens (Musser *et al.* 2005; Peiffer & Felton 2005). Some digestive enzymes such as a serine protease and a lipase in *Bombyx mori* larvae were found to have antiviral activity (Ponnuvel *et al.* 2003; Nakazawa *et al.* 2004). Lastly, the peritrophic membrane, which is a thin layer of extracellular matrix lining the midgut, acts as a barrier to prevent orally ingested pathogens such as baculoviruses from infecting the midgut (Peng, Zhong

& Granados 1999; Hoover, Washburn & Volkman 2000; Hoover *et al.* 2010; Plymale *et al.* 2008).

If a pathogen successfully circumvents the physical barriers, it will encounter a variety of immune defences including cellular responses such as phagocytosis, melanization, and encapsulation, and humoral responses such as the production of reactive oxygen species and antimicrobial peptides (Lemaitre & Hoffmann 2007; Ferrandon *et al.* 2007). Each group of pathogens have conserved surface characteristics that are not present in the host, called pathogen-associated molecular patterns (PAMPs). These include bacterial peptidoglycan (PGNs), lipopolysaccharides (LPS), and fungal polymeric β-(1,3)-glucans (Lemaitre & Hoffmann 2007; Ferrandon *et al.* 2007). PAMPs are recognized and bound by pathogen recognition receptors (PRRs) in the host insect, such as peptidoglycan recognition proteins (PGRPs) and Gram negative binding proteins (GNBPs) (Lemaitre & Hoffmann 2007; Ferrandon *et al.* 2007).

Recognition of foreign bodies by PRRs can trigger both the cellular and humoral immune responses. Haemocytes (cells found in the haemolymph) respond quickly by phagocytosing and melanizing pathogens, and encapsulating larger parasites. The prophenoloxidase activating cascade is the fastest of the humoral immune responses to act. Detection of PAMPs triggers a cascade of serine proteinases, called prophenoloxidase-activating proteinases, that cleaves zymogenic prophenoloxidase (pro-PO) into active phenoloxidase (PO). PO catalyzes the hydroxylation of monophenols to o-diphenols and oxidation of o-diphenols to quinones that then polymerize non-enzymatically to form melanin and subsequently collects directly around pathogens or on encapsulated objects or parasites, and wound sites. Free quinones and other reactive intermediates may contribute to the killing of foreign intruders trapped by melanin (Söderhäll & Cerenius 1998; Cerenius & Söderhäll 2004; Nappi & Christensen 2005). Interactions between PRRs and PAMPs also activate the expression of a suite of AMPs that clear infections. Different components of the immune responses work in a concerted effort to eliminate pathogens (Lemaitre & Hoffmann 2007; Ferrandon *et al.* 2007).

For RNA viruses, double stranded RNA (dsRNA) produced during viral RNA genome replication has been demonstrated as a major PAMP that triggers the RNA interference (RNAi) pathway (Kingsolver, Huang & Hardy 2013). dsRNA is recognized by Dicer proteins (PRRs), particularly Dicer-2 in *Drosophila* and mosquitoes, that cleaves dsRNA to produce small, interfering RNAs (siRNAs) that target viral RNA for degradation and consequently inhibit replication (Wang *et al.* 2006; Galiana-Arnoux *et al.* 2006). How the insect immune system recognizes lepidopteran baculoviruses is less well known. Baculoviruses are DNA viruses, and host immune defenses can involve encapsulation of infected haemocytes and melanization. Also, the expression of the AMP hemolin was induced in a Bombycidae host, *Antheraea pernyi*, in response to baculovirus infection (Hirai *et al.* 2004), but was not induced in some other species (Terenius, Popham & Shelby 2009). Recently, it was shown that the RNAi-based antiviral response can also contribute to the control of DNA virus infection (Bronkhorst *et al.* 2012), including baculovirus infection (Jayachandran, Hussain & Asgari 2012).

1.2. Primed resistance

Several studies have shown that previous exposure to parasites or an immune elicitor increases protection on subsequent challenge days, or even weeks, after the initial exposure (Moret & Siva-Jothy 2003; Sadd & Schmid-Hempel 2006; Pham *et al.* 2007; Roth *et al.* 2009; Tidbury, Pedersen & Boots 2011). This phenomenon has been termed immune priming (Schmid-Hempel 2005b). Its long-lasting effect might result from persistence of the induced defence molecules in the haemolymph or by an ongoing upregulation in the transcription of constitutive immune molecules (Schmid-Hempel 2005b). Transcription of AMPs in *Drosophila* typically starts within minutes after a challenge but moderates after 24–36 hours. These peptides can persist in the haemolymph for up to three weeks, as the compact three-dimensional structure of AMPs is believed to be quite resistant to cleavage by proteases under *in vivo* conditions (Uttenweiler-Joseph *et al.* 1998), although continuous low level transcription may also be involved. There is also evidence that immune priming can be specific to the same parasite species or strain (Sadd & Schmid-Hempel 2006; Pham *et al.* 2007; Roth *et al.* 2009). Moreover, heightened parasite resistance and immune activity in the offspring of

immune-primed parents has been found in a number of insect orders (e.g. Sadd and Schmid-Hempel 2007; Freitak, Heckel, and Vogel 2009; Moreau et al. 2012), a phenomenon termed trans-generational immune priming (TGIP; Little *et al.* 2003; Sadd *et al.* 2005; Moret 2006).

1.3. Costs associated with resistance

Activating an immune response may be energetically costly, thereby depleting limited resources required for other life-history traits (Schmid-Hempel 2005a). However, hosts often show no obvious costs associated with resisting pathogens, possibly because they may compensate for increased resource requirements by increasing resource intake. A classic study of costs associated with activating an immune response used starvation to restrict the availability of resources. This ensures that any resource allocation to immune defense reduces the pool of resources available for other bodily functions and eventually for survival. Activating the bumblebee (*Bombus terrestris*) immune system by injecting lipopolysaccharides and micro-latex beads, reduced the survival of starved individuals by 50-70% relative to starved controls (Moret & Schmid-Hempel 2000). Similarly, costs can be revealed indirectly by experimentally increasing resource allocation in another trait, and subsequently measuring decrease in the immune response or increase in susceptibility to pathogens. For example, selecting for increased sexual activity in male *Drosophila melanogaster* resulted in a decline in their immune activity (McKean & Nunney 2001, 2005, 2008). Although costs associated with an immune response are not always measureable, numerous examples have been found (Schwartz & Koella 2004; Jacot, Scheuber & Brinkhof 2004; Krams *et al.* 2013) including negative correlations between immunity and other life-history traits (Fellowes, Kraaijeveld & Godfray 1999; Hoang 2001).

Furthermore, life-history costs associated with trans-generational immune priming, such as increased susceptibility to an unrelated pathogen or reduced reproductive output, have been found in primed offspring (Sadd & Schmid-Hempel 2009; Roth *et al.* 2010; Zanchi *et al.* 2011; Trauer & Hilker 2013). Fitness costs also accompany evolved resistance. Genetic based trade-offs between resistance to parasites and life-history traits can be so costly in the absence of the parasite that

evolved resistance is often lost within a few generations (Boots and Begon 1993; Fuxa and Richter 1998; Janmaat and Myers 2003; 2006). Therefore, it could be the costs associated with maintaining elevated immune activity or the costs associated with genes that confer resistance that constrain the maintenance of resistance in populations.

1.4. Nutrient availability and disease resistance

Studies on the impact of nutrition on insect-parasite interactions have used various methods of nutrient manipulation to simulate the nutritional variability to which insects can be exposed. These studies have revealed that the changes in the quantity and quality of nutrients available to the host can alter both the physical barriers to infection and immune response, and consequently resistance to the pathogen. Methods of manipulating nutrients can include more crude approaches using starvation and food limitation, to varying food quality, such as by using different host plants, to investigating the specific effects of individual micro- and macronutrients.

1.4.1. Starvation and food limitation effects on resistance

Starvation and food limitation can happen to any insect, such as scarcity of prey for predators and hosts for parasitoids and blood sucking insects, as well as reduced availability of suitable host plants during high population densities for herbivores. Food deprivation may increase host susceptibility to parasites (Brown *et al.* 2000; Lord 2010), and is most often a consequence of reduced investment in resistance mechanisms (Feder *et al.* 1997; Siva-Jothy & Thompson 2002; Yang, Ruuhola & Rantala 2007; Myers *et al.* 2011). Protection incurred by physical barriers can also be indirectly reduced during starvation or food limitation. For example, as the rate of food passage decreases so does the need for peritrophic membrane production (Lehane 1997). The need to synthesize some digestive enzymes are also reduced during starvation (Terra & Ferreira 1981).

1.4.2. Host plant quality effects on resistance

While starvation and food shortage are often density-dependent, food quality can vary at any time. For herbivorous insects, nutritional quality is likely to vary between different plant species and families, and even between ages and genotypes within a species and different parts within a plant. For example, nine species of plants in the family Brassicacae, known to be hosts of insects such as the cabbage looper *Trichoplusia ni*, vary in nitrogen content from 1.9-5.9% dry weight and digestible carbohydrate from 11-60% dry weight (Morehouse & Rutowski 2010). Within the above ground portion of a cabbage plant, nitrogen content is usually highest in the inner leaves, followed by the outer leaves, and least in the stems (Hara & Sonoda 1981).

Environmental factors such as soil nutrients, light intensity, water availability, CO_2 concentration and temperature can also greatly alter the nutritional content of plants (Hara and Sonoda 1979; 1981; 1982; Bernays and Chapman 1994; Makino and Mae 1999; Hwang, Liu, and Shen 2008; Wang and Frei 2011). Supplementation of soil with nitrogen increases protein content in the leaves of cabbage plants by as much as two-fold (e.g. Hara and Sonoda 1982). Recent attention to the impacts of climate change have shown significant effects of increasing temperature and CO_2 concentration on the growth of plants. Temperature has strong effects on the production of plant secondary compounds such as phenolics and terpenes (Zvereva & Kozlov 2006), but can also alter nutrient content by changing leaf nitrogen, non-structural carbohydrate, starch and sugar contents, and the nitrogen to carbohydrate ratio of plants (Zvereva & Kozlov 2006; Wang *et al.* 2012; Dieleman *et al.* 2012). The combined effects of increased temperature and CO_2 concentration has a positive effect on plant growth such that increasing plant biomass results in diluted nitrogen content in the foliage (Dieleman *et al.* 2012).

Inter- and intraspecific variation in host plant composition significantly alters the resistance of host insects to parasites. For example, host plant species affected the resistance of the monarch butterfly *Danaus plexippus* to the protozoan parasite *Ophyrocystis elektroscirrha* (de Roode *et al.* 2008), while resistance of *Heliothis virescens* and *Helicoverpa zea* larvae to a nucleopolyhedrovirus was greater on reproductive tissue compared to vegetative tissue (Ali *et al.* 1998). Studying variability in

disease resistance with inter- and intraspecific differences in host plants is ideal since it is reflective of the diets that herbivorous insects would encounter in nature. However, from a nutritional quality perspective, host plants pose a problem because the effects of nutrients cannot be separated from the effects of plant defensive chemicals. These chemicals such as pyrrolizidine alkaloids, cardenolides, and glucosinolates are often toxic to insects, but can be used by specialized insects to defend against parasites and parasitoids (de Roode *et al.* 2008; Hopkins, van Dam & van Loon 2009; Singer, Mace & Bernays 2009; Lefèvre *et al.* 2010).

1.4.3. Nutrient effects on resistance

In order to isolate the effects of specific micro- and macronutrients on disease resistance, many studies on nutritional ecology and immunology use artificial diets. Optimal foraging theorists studying nutritional ecology have used an evolutionarily-inspired framework that focuses on a single 'currency', rate of energy gain, as a proxy for fitness (Raubenheimer, Simpson & Mayntz 2009). However, energy itself is not a nutrient. It is a property of the macronutrients protein, lipid and carbohydrate, and there is clear evidence that the composition of macronutrients in plants can vary substantially (Raubenheimer *et al.* 2009). Thus, without differentiating among these energetic components, measures of caloric intake aimed at maximizing one or more of these macronutrients, or optimizing their balance, is confounded with energy maximization (Raubenheimer *et al.* 2009). The Classical Insect Nutritional Ecology approach has been used to assess which factors (e.g. nutrients, plant defense chemicals, environmental factors) drive foraging decisions (Raubenheimer *et al.* 2009). It is a budgetary approach used for comparative purposes that quantifies the relationships between food intake and utilization as rates and efficiencies (Waldbauer & Friedman 1991). It has shown that insects, like other higher organisms, feed non-randomly on foods. Unfortunately, instead of revealing the relationships among factors, this approach obscures these relationships by compounding multiple factors into a single index (Raubenheimer *et al.* 2009).

The Geometric Framework for nutrition is used to integrate multivariate factors into a single schematic frame by assigning each factor their own axis and plotting one

factor against the other in bi-coordinate space (Raubenheimer and Simpson 1993, 1997, 1999; Simpson and Raubenheimer 1993a, 1995, 1999, 2000; Simpson et al. 1995). The nutritional state of the insect is represented as a point in the bi-coordinate space which indicates the amount of each nutrient consumed. This allows nutritional ecologists to simultaneously vary multiple nutrients and uncover important interactive effects (Simpson & Raubenheimer 1995). The most commonly studied nutrients are protein (source for nitrogen), the primary building block for growth, and digestible carbohydrate, the primary source of energy in herbivorous insects (Waldbauer & Friedman 1991; Simpson & Raubenheimer 1993). This method has revealed that most insects, when given a choice of multiple nutritionally unbalanced foods, will selectively feed to attain an ideal balance of protein and carbohydrate (Simpson & Raubenheimer 1993). The particular balance of protein and carbohydrate varies between species and can also vary depending on the physiological state of the insect (Behmer 2009). The Geometric Framework can also be applied to situations where the insect is restricted to a single unbalanced food. For example if the food is unbalanced in two nutrients, the insect has three options: (1) consume the diet until it meets its requirement for the first nutrient even though it consumes too little or too much of the other, (2) consume until it meets the requirement for the second nutrient while suffering an excess or deficit of the other, or (3) feed to an intermediate point where the excesses and deficits of both nutrients are less extreme (Behmer 2009). The use of the Geometric Framework under nutrient restriction can uncover trade-offs between excess and deficient nutrient intake that can be used to determine efficiencies and modes of nutrient utilization (Raubenheimer and Simpson 1992, 1994, 1995; Simpson and Raubenheimer 1995).

Recently, macronutrient restriction by modifying protein to carbohydrate ratios after pathogen challenge were found to affect insect resistance to nucleopolyhedroviruses and bacteria, such that higher levels of protein increased resistance (Lee *et al.* 2006b; Povey *et al.* 2009, 2014). It was hypothesized that the increased level of protein consumed was used to produce haemocytes and molecules involved in humoral immunity, as infection of *S. littoralis* by a nucleopolyhedrovirus resulted in reduced accumulation of nitrogen in the body for growth (Lee *et al.* 2006b). Moreover, consumption of a higher ratio of protein to carbohydrate increased the density of haemocytes, antibacterial and phenoloxidase activities, and protein concentration in

the haemolymph of two *Spodoptera* species (Lee *et al.* 2006b; Povey *et al.* 2014). When allowed to self-compose their own balance of protein and carbohydrate after pathogen challenge, pathogen challenged insects selected a higher ratio of protein to carbohydrate, exhibiting self-medicating behaviour (Lee *et al.* 2006b; Povey *et al.* 2009, 2014). Similarly, macronutrients may affect the physical barriers to infection. Feeding higher quality protein diet to the larvae of *Spodoptera littoralis* resulted in more heavily melanized cuticles (Lee, Simpson & Wilson 2008b), although whether this increases resistance to cuticular entering parasites was not tested.

1.4.4. Trans-generational effects of nutrients on offspring resistance

While reduced quantity or quality of resources is generally associated with reduced within-generation immune defense (Siva-Jothy & Thompson 2002; Myers et al. 2011; Triggs & Knell 2012), in some cases it has resulted in heightened disease resistance and immune activity in offspring (Myers *et al.* 2011; Boots & Roberts 2012). However, the results are mixed and may depend on the type of nutrient manipulation. Boots and Roberts (2012) reduced the nutrient quantity available to the Indian meal moth, *Plodia interpunctella* by diluting the standard diet with varying proportions of methylcellulose and found that the offspring of nutritionally stressed parents were more resistant to a baculovirus and had elevated phenoloxidase activity. In contrast, Triggs and Knell (2012) reduced the quality of their standard diet of *P. interpunctella* which consisted of 10:1:1 (wheat bran/brewer's yeast/glycerol) by doubling the amount of wheat bran (20:1:1). They found reduced haemocyte density and phenoloxidase activity in the offspring of nutritionally stressed parents which is contradictory to the findings by Boots and Roberts (2012). Whether the reduced immune activity of offspring in Triggs and Knell's study translated into increased susceptibility to parasites was not tested. Other methodological differences may have contributed to these contrasting results. For example, Boots and Roberts (2012) reared their parental and offspring generations in high density groups whereas Triggs and Knell (2012) reared theirs individually. Rearing density is known to have within generation effects on disease resistance (Goulson & Cory 1995; Wilson *et al.* 2003), and may also have trans-generational effects (Miller, Pell & Simpson 2009).

1.5. Host nutrition and parasite fitness

An important cause of parasite virulence is the competition between host and parasite for resources that would normally be allocated by the host for growth, maintenance and/or reproduction (Bedhomme *et al.* 2004). The quantity or quality of nutrients available to the host is particularly important for the parasite because it can influence both the supply of nutrients available to the parasite for growth and metabolism, as well as affect the availability of nutrients necessary for components of the host's immune system to mount a response (Smith, Jones II & Smith 2005). To balance between host nutrition that benefits parasite growth and host nutrition that improves host survival, some parasites can manipulate the nutritional intake of their host to optimize parasite fitness. For example, under diverse nutritional regimes, the parasitoid wasp *Cotesia congregata* manipulated its host *Manduca sexta* to consume nutrients in a way that achieves uniform host growth, thereby constraining haemolymph nutrient concentrations within limits suitable for parasite growth and development (Thompson & Redak 2008). Parasites can also manipulate the way its host allocates resources, such that the allocation of resources will be to an area where the parasite will benefit directly. Parasitic castration is an example of this type of manipulation, where the parasite decreases the proportion of resources allocated to host reproduction while increasing the allocation of resources that the parasites can access (Lafferty & Kuris 2009).

1.6. The study system

The overarching theme of this thesis is to explore the effects of nutritional quality on the interaction between the cabbage looper *Trichoplusia ni* and its pathogens. I focus on two pathogen types: alphabaculoviruses (nucleopolyhedroviruses; NPVs) and the bacterium *Bacillus thuringiensis* (*Bt*).

1.6.1. Pathogens: NPVs

Baculoviruses (family Baculoviridae) are classified as a group of arthropod-specific viruses with rod-shaped nucleocapsids of 30–60 nm×250–300 nm, isolated from

insects of the orders Lepidoptera, Hymenoptera, and Diptera (Jehle *et al.* 2006). There are four genera within the family Baculoviridae: Alphabaculovirus (lepidopteran-specific NPV), Betabaculovirus (lepidopteran-specific Granuloviruses), Gammabaculovirus (hymenopteran-specific NPV) and Deltabaculovirus (dipteran-specific NPV) (Jehle *et al.* 2006). The complete infection cycle of most baculoviruses includes two morphologically distinct, but genetically identical, viral phenotypes that perform separate roles during infection; one establishes initial infection and the second spreads infection within the host (Volkman 1997, 2007). The first phenotype is the occlusion-derived virus (ODV) which is protected from the environment within a crystalline matrix of protein (polyhedrin) called an occlusion body (OB), which is the transmission stage of the virus, responsible for host-to-host transmission. The infection process begins when a larval host ingests OBs which then dissolve in the alkaline juices of the host's midgut lumen releasing the ODVs. Envelope proteins on the ODV surface necessary to initiate infection (*per os* infectivity factors) become activated by insect midgut proteases, such as trypsin (Slack *et al.* 2008). ODVs also carry metalloproteases called enhancins that degrade the insect intestinal mucin allowing passage through the peritrophic membrane to initiate primary infections in the columnar epithelial cells of the midgut (Slavicek & Popham 2005; Hoover *et al.* 2010). The midgut cells infected by ODV then produce budded virus (BV) which cause secondary infections in the tracheolar cells servicing the infected midgut epithelium (Engelhard *et al.* 1994; Flipsen *et al.* 1995; Washburn, Kirkpatrick & Volkman 1995; Volkman 2007). Infected tracheolar cells produce more BV and act as conduits that spread the secondary infection to other tissues throughout the insect, eventually producing large numbers of OBs. The OBs produced in most insect species are released when tissues lyse, aided by chitinase and cathepsin genes, causing the infected host to liquefy (Hawtin *et al.* 1997).

Within the genus Alphabaculovirus, two species have been isolated from wild cabbage looper populations, *T. ni* single nucleopolyhedrovirus (TnSNPV) and *T. ni* multiple nucleopolyhedrovirus (TnMNPV) (Jaques 1970); the latter is a strain of *Autographa californica* multiple nucleopolyhedrovirus (AcMNPV) (Theilmann *et al.* 2005). AcMNPV and TnSNPV are positioned in two distinctly different and likely monophyletic clades within Alphabaculovirus (Herniou & Jehle 2007), and shown to belong on the same node (Harrison *et al.* 2012). One clear difference between the two viruses is in the

number of nucleocapsids packaged within a single ODV envelope. As their names suggest, TnSNPV ODVs contain one nucleocapsid and AcMNPV ODVs contain multiple nucleocapsids. Another key difference is their host range. AcMNPV has a broad host range and can infect species within at least 15 Lepidopteran families (Cory & Myers 2003), while TnSNPV is believed to be much more restricted in its host range as it was only able to infect *T. ni* larvae from among five species tested spanning five families (Del Rincón-Castro & Ibarra 1997). MNPVs tend to have wider host ranges (Cory & Myers 2003), likely because the M phenotype can establish irreversible systemic infections more quickly compared to the S phenotype (Washburn *et al.* 1999). Occlusion-derived virus (ODV) fractions enriched for the MNPV or SNPV phenotypes of AcMNPV revealed that MNPV infected tracheal cells more quickly and efficiently to initiate a systemic infection (Washburn *et al.* 1995). MNPV is able to repackage the extra parental ODV nucleocapsids as BV to facilitate early infection of the tracheolar cells before *de novo* virus replication within infected midgut cells (Washburn *et al.* 2003). There are likely to be many other genetic differences between TnSNPV and AcMNPV, since the TnSNPV genome is 30kb larger than AcMNPV (Fielding *et al.* 2002). For example, *T. ni* that were selected in the laboratory for evolved resistance to TnSNPV were 20-fold more resistant to TnSNPV than control *T. ni.* However, this highly TnSNPV-resistant *T. ni* strain showed little cross-resistance to AcMNPV (less than 2-fold compared to the control), suggesting significant genetic variation and different routes of infection between the two viruses (Milks & Myers 2003).

1.6.2. Pathogens: *Bt*

Bt is a gram-positive, spore-forming bacterium that is characterized by the production of crystal-like parasporal inclusions which contain proteins called δ-endotoxins (Aronson 2002). Most δ-endotoxins belong to the Cry (crystal) family of proteins and have insecticidal activity (Vachon, Laprade & Schwartz 2012). This insecticidal toxin allows *Bt* to kill more rapidly than many other insect pathogens, and combined with its ease of production, has made *Bt* the most successful commercial microbial insecticide (Bravo *et al.* 2011). Many crop plants have been genetically modified to express *Bt* toxins and have been planted in 66 million hectares worldwide (James 2011). *Bt* spores containing Cry protoxins need to be ingested to be effective as

the spores must dissolve and protoxins solubilize in the insect midgut, followed by activation of the protoxins by insect digestive proteases (Vachon *et al.* 2012). Some strains of *Bt* can also produce vegetative insecticidal proteins (Vip) which are secretable insecticidal toxins produced during the vegetative growth phase (Bravo *et al.* 2011; Palma *et al.* 2012). The active toxins infiltrate through the insect midgut peritrophic membrane and interact with specific binding sites at the midgut brush border membrane, forming pores. High concentrations of Cry toxins on their own can result in extensive damage to midgut epithelial tissue and cause the death of the insect (Bravo, Gill & Soberón 2007; Vachon *et al.* 2012). The formation of pores by the toxins also allows *Bt* spores to enter the insect haemolymph, which then germinate and cause septicaemia and death (Johnston & Crickmore 2009; Raymond *et al.* 2010).

The extensive and repeated use of *Bt* sprays and toxins for pest control has placed strong selective pressure on populations of pest insects (Heckel *et al.* 2007; Carrière, Crowder & Tabashnik 2010). To date, three insect pest species have developed resistance to *Bt* sprays and at least six species have been recorded as being resistant to *Bt* crops expressing single Cry toxins in the field, and numerous other species have shown the potential to develop resistance in laboratory selection experiments (Cory & Franklin 2012). Genetic resistance usually occurs when populations become less susceptible to Cry toxins. This most often happens when the binding of toxins to midgut targets is reduced due to the alteration or loss of midgut toxin-binding proteins (Bravo & Soberón 2008). Lower susceptibility to the toxin can also occur by sequestration of the toxin (Gunning *et al.* 2005; Ma *et al.* 2005) and reduced peritrophic membrane permeability to the toxin (Hayakawa *et al.* 2004). Moreover, heightened immune activity in offspring of *Bt*-challenged parents (trans-generational immune priming) has also been shown to reduce susceptibility to a *Bt* formulation of spores and toxins (Rahman *et al.* 2004). Genetic resistance to *Bt* can increase fitness in the presence of *Bt* but often inflict fitness costs in its absence (Gassmann, Carrière & Tabashnik 2009). Fitness costs associated with *Bt*-resistance relate to reductions in survival, mass at maturity, growth rate and fecundity in comparison to unselected susceptible insects (Groeters *et al.* 1994; Alyokhin & Ferro 1999; Oppert *et al.* 2000; Akhurst *et al.* 2003; Janmaat & Myers 2003; Carrière *et al.* 2004). The magnitude of these costs are known to be influenced by environmental factors such as host plant

species and cultivar, plant secondary compounds, intraspecific competition, and entomopathogenic viruses and nematodes (Gassmann *et al.* 2009).

1.6.3. Insect

The cabbage looper *Trichoplusia ni* (Hübner) belongs to the family Noctuidae of the order Lepidoptera. It is a generalist leaf feeder, attacking vegetable crops as diverse as tomato, cucumber, bean, lettuce and spinach, as well as some ornamental and weed species. However, *T. ni* has a particular preference for cruciferous vegetables and is considered a major pest wherever crucifers are grown. It is multivoltine with a short life-cycle, taking approximately 24-33 days from egg to adult. The larval stage consists of five larval instars followed by pupation and an income breeding adult stage (Cranshaw 2004).

To initiate my research colonies, I was provided with insects from two *T. ni* colonies, one that was genetically resistant to *Bt* and another that was susceptible, from Dr. Carl Lowenberger and Jerry Ericsson at Simon Fraser University. The *Bt*-resistant *T. ni* colony was originally collected from a commercial tomato greenhouse in British Columbia, Canada in 2001 by Dr. Alida Janmaat and Dr. Judith Myers from the University of British Columbia. These insects have since been maintained on a wheat-germ based diet at 25°C and 16:8 (L:D) photoperiod. The resistant *T. ni* colony was routinely selected with 40 KIU ml^{-1} diet *Bt* subsp. *kurstaki* (Dipel 2X DF, Valent Biosciences, Libertyville, IL, USA) every generation to maintain resistance. The *Bt*-susceptible colony is derived from the originally collected resistant line but was maintained without *Bt* exposure (Janmaat & Myers 2003). Both the *Bt*-resistant and susceptible colonies were used in Chapters 2 and 3 to compare differences in nutrient intake, utilization, and susceptibility to *Bt.* Only the susceptible colony was used in Chapters 4 and 5.

1.7. Thesis chapters

In Chapter 2, I focused on the fact that in most studies of fitness costs associated with genetic resistance to *Bt*, the insect's access to nutrients is constrained by the use of

a single artificial diet or host plant. In nature, most herbivorous insects optimize life-history traits by self-composing their diet from host plant species and plant parts that vary in nutritional quality (Raubenheimer *et al.* 2009; Behmer 2009). Therefore, I use the Geometric Framework for nutrition and provide *Bt*-resistant and susceptible *T. ni* larvae with a choice of two nutritionally insufficient but complementary diets to test my hypothesis that given an opportunity to self-compose their ideal blend of nutrients, *Bt* resistant *T. ni* would compose a different blend compared to susceptible *T. ni* that would allow them to compensate and eliminate fitness costs.

In Chapter 3, I used the Geometric Framework for nutrition but restrict *T. ni* larvae to single nutritionally inadequate diets to test my hypothesis that the fitness costs of *Bt*-resistant *T. ni* might result from impaired nutrient utilization, due to *Bt*-resistance mechanisms such as altered or lost midgut proteins that act as receptors for *Bt* toxins (Bravo & Soberón 2008). I also examined how nutrition impacts the susceptibility of *Bt*-resistant and susceptible *T. ni* to *Bt*-challenge.

The third research chapter (Chapter 4) examines the impact of environmental factors on the interactions between host, parasite, and host diet. In particular, I focus on temperature because it affects metabolic processes in insects and their parasites, and has been shown to affect not only host survival and parasite growth after infection (Inglis, Johnson & Goettel 1996; Arthurs & Thomas 2001; Olsen & Hoy 2002; Subramanian *et al.* 2006; Cevallos & Sarnow 2010), but also affects the ability of the host to mount an immune response (Blanford & Thomas 1999; Thomas & Blanford 2003; Linder, Owers & Promislow 2008). My first objective was to assess how temperature and dietary protein to carbohydrate ratios of *T. ni* interact to influence their ability to resist virus challenge, their life-history traits, and the degree of costs to life-history traits associated with resisting virus challenge. The second objective was to determine how the rearing environment of the host affects the virulence of two species of alphabaculoviruses, TnSNPV and AcMNPV.

In the final research chapter (Chapter 5), I examined how pathogen challenge and nutrient quantity in a parental generation of *T. ni* will interact to influence investment in their offspring. Separately, both pathogen challenge and nutritional stress are known

to induce trans-generational effects. While parental exposure to a pathogen acts as a cue to elevate offspring immune defenses (trans-generational immune priming; TGIP) (Little *et al.* 2003; Sadd *et al.* 2005; Moret 2006), parental nutritional stress acts as a cue to transfer nutritional stress tolerance to offspring (Plaistow, Lapsley & Benton 2006; Vijendravarma, Narasimha & Kawecki 2010; Hafer *et al.* 2011; Triggs & Knell 2012; Saastamoinen, Hirai & van Nouhuys 2013). Both of these trans-generational effects have been shown to be adaptive if the pathogen or nutritional environment experienced by the parents matches that of the offspring (e.g. Rahman *et al.* 2004; Roth *et al.* 2009, 2010; Vijendravarma *et al.* 2010; Tidbury *et al.* 2011; Triggs & Knell 2012). However, both mounting an immune response to a pathogen and developing under nutritional stress are costly. Furthermore, TGIP and the transfer of nutritional stress tolerance have associated life-history costs for both parents and offspring (Fox & Mousseau 1996; Sadd & Schmid-Hempel 2009; Vijendravarma *et al.* 2010; Trauer & Hilker 2013). Therefore, there are likely to be trade-offs between these two costly trans-generational effects if parents were subjected to the two environmental stressors simultaneously. The reason why I was interested in trade-offs associated with parental pathogen challenge and nutritional stress is because in nature, for one environmental factor to vary independently from others is unlikely. In particular, the two factors I tested were likely to be linked because as population densities increase, nutritional resources become depleted and the probability of pathogen transmission increases (Knell *et al.* 1996; Ryder *et al.* 2005; Boots & Roberts 2012). Therefore, I examined trade-offs between nutritional stress and *Bt* challenge on parentally induced phenotypic change in the offspring of *T. ni.* I hypothesized that parents would be able to transfer either immune priming (immunity to *Bt*) or nutritional stress tolerance to their offspring if each factor was encountered separately. However, if both factors were encountered simultaneously, there would be a trade-off between TGIP and the transfer of nutritional stress tolerance such that only one would be transferred.

Lastly, in Chapter 6, I summarize the key findings from each of my research chapters and discuss some questions that may encourage future studies.

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1.9. Connecting statement

In Chapter 1, I described the effects that nutritional variation can impose on host resistance mechanisms and disease resistance. I also described the costs associated with within-generation, trans-generational and evolved resistance to pathogens. In Chapter 2, I examine whether the observed costs associated with evolved resistance to *Bt* are due to experimental restriction of *Bt*-resistant insects on fixed nutrient diets or a single host plant. Therefore, I investigated whether *Bt*-resistant *T. ni* could compensate for fitness costs by altering their intake of nutrients.

Chapter 2.

Genetic resistance to *Bacillus thuringiensis* alters feeding behaviour in the cabbage looper, *Trichoplusia ni*

A modified version of this chapter has been published:
Shikano I. & Cory J.S. (2014) Genetic resistance to *Bacillus thuringiensis* alters feeding behaviour in the cabbage looper, *Trichoplusia ni*. *PLoS ONE*, 9, e85709.

2.1. Abstract

Evolved resistance to xenobiotics and parasites is often associated with fitness costs when the selection pressure is absent. Resistance to the widely used microbial insecticide *Bacillus thuringiensis* (*Bt*) has evolved in several insect species through the modification of insect midgut binding sites for *Bt* toxins, and reports of costs associated with *Bt* resistance are common. Studies on the costs of *Bt*-resistance restrict the insect to a single artificial diet or host-plant. However, it is well documented that insects can self-select appropriate proportions of multiple nutritionally unbalanced foods to optimize life-history traits. Therefore, I examined whether *Bt*-resistant and susceptible cabbage loopers *Trichoplusia ni* differed in their nutrient intake and fitness costs when they were allowed to compose their own protein:carbohydrate diet. I found that *Bt*-resistant *T. ni* composed a higher ratio of protein to carbohydrate than susceptible *T. ni.* *Bt*-resistant females showed a fitness cost of reduced pupal weight, while males did not. The

absence of reduced pupal weight in resistant males was associated with longer development time and increased carbohydrate consumption compared to females. I demonstrate a sex difference in fitness costs and a new behavioural outcome associated with *Bt* resistance.

2.2. Introduction

The evolution of resistance to pathogens, parasites and chemical insecticides is often accompanied by negative pleiotropic effects in the absence of the selection pressure (Kraaijeveld & Godfray 1997; Vijendravarma, Kraaijeveld & Godfray 2009; Martins *et al.* 2012). Repeated exposure of insect pests to the toxin-forming bacterium *Bacillus thuringiensis* (or its Cry toxins expressed in plants) has resulted in the evolution of resistance (Tabashnik, Van Rensburg & Carrière 2009; Cory & Franklin 2012). Fitness costs of insect resistance to *Bt* are well-documented and primarily relate to reductions in survival, fecundity, and mass and increases in development time in comparison to unselected susceptible insects (Groeters *et al.* 1994; Alyokhin & Ferro 1999; Oppert *et al.* 2000; Akhurst *et al.* 2003; Janmaat & Myers 2003; Carrière *et al.* 2004).

The most common genetic resistance mechanism is the modification or loss of midgut binding proteins (cadherin or aminopeptidase N) for *Bt* Cry toxins, necessary for the toxins to cause death (Pigott & Ellar 2007). Changes to midgut binding proteins are hypothesized to increase midgut membrane permeability to toxic phytochemicals and pathogens (cadherin), and interfere with protein digestion (aminopeptidases) (Gassmann *et al.* 2009). Thus the magnitude of any cost to *Bt* resistance is likely to be influenced by diet. This has been shown to be the case, with the costs of *Bt* resistance being exacerbated on lower quality or better-defended diets or host plants (Janmaat & Myers 2005; Williams *et al.* 2011; Raymond, Wright & Bonsall 2011).

A key tactic in managing the evolution of resistance to *Bt* relies on the use of non-*Bt* refuges (Carrière *et al.* 2005, 2010; Raymond *et al.* 2007a). If the factors that increase the costs of *Bt* resistance and their underlying mechanisms were better understood, this knowledge could potentially be used to enhance resistance

management strategies. Most studies of fitness costs associated with *Bt* resistance restrict the insect to a single artificial diet or host-plant. However, in nature, most herbivorous insects have access to foods that vary in nutritional quality, and they can compose their diet from multiple, nutritionally unbalanced foods to optimize life-history traits (Raubenheimer *et al.* 2009; Behmer 2009). I hypothesized that midgut-based resistance to *Bt* was likely to alter nutrient intake and that resistant insects may be able to compensate for fitness costs when given a choice of diets. I used the Geometric Framework for nutrition to test my hypotheses by comparing *Bt*-resistant and *Bt*-susceptible lines of cabbage loopers, *Trichoplusia ni* (Hübner). In this approach it is possible to quantify how an organism regulates the intake of two or more food components at the same time using a graphical model (Raubenheimer *et al.* 2009; Behmer 2009). An important component of this approach is that it incorporates the behaviour of the organism, allowing it to choose between diets or whether to stop or continue feeding. *T. ni* has developed resistance to *Bt* as a result of the frequent applications in greenhouses (Janmaat & Myers 2003; Wang *et al.* 2007). Resistance to the Cry1Ac toxin has recently been shown to be related to alterations in two midgut aminopeptidase Ns (Tiewsiri & Wang 2011; Zhang *et al.* 2012). In addition, *Bt*-resistant *T. ni* have well-documented, context-dependent costs, including reduced survival and pupal weight, and increased development time (Janmaat & Myers 2003, 2005).

2.3. Materials and methods

2.3.1. Study animals

The *Bt*-resistant *T.ni* colony was originally collected from a commercial tomato greenhouse in British Columbia, Canada in 2001 (Janmaat & Myers 2003), and has since been maintained on a wheat-germ based diet at 25°C and 16:8 (L:D) photoperiod. The *Bt*-resistant strain used in the present study was collected during the same year and from a greenhouse close to the source of the Cry1Ac resistant strain used to determine alterations in aminopeptidase Ns (labeled T2c in Janmaat & Myers 2003). At the time of collection, both strains were highly resistant to DiPel, a formulation of *Bt* subsp. *kurstaki* containing Cry1Aa, Cry1Ab, Cry1Ac, and Cry2A (Janmaat & Myers 2003). The resistant

T. ni colony used in the present study is routinely selected with 40 KIU ml^{-1} diet *Bt* subsp. *kurstaki* (Dipel 2x DF, Valent Biosciences, Libertyville, IL, USA) every generation to maintain resistance. The *Bt*-susceptible colony is a revertant line obtained by not exposing the resistant line to *Bt* (Janmaat & Myers 2003). Inbreeding and genetic drift have been minimized by mass mating high numbers of moths (approximately 200 moths) each generation and periodic back-crossing between the resistant and susceptible colonies. The *Bt*-resistant insects used here were not selected with *Bt* for one generation (Bt-RU) to avoid possible transgenerational effects (Janmaat & Myers 2005). Bt-RU larvae were 55-fold more resistant to *Bt* than Bt-S at the time of the experiment.

2.3.2. Artificial diets

Experimental larvae were reared on the wheat-germ based diet (colony diet) from egg-hatch until exposure to the wheat-germ-free, nutritionally-defined treatment diets. 'Treatment diets' consisted of altering the ratios of protein (casein) and digestible carbohydrate (sucrose) that made up 60% of the dry weight in the following proportions (% protein: % carbohydrate): 50:10, 30:30, 20:40, 10:50. Other ingredients include Wesson's salt (5%), cholesterol (1.5%), ascorbic acid (1%), sorbic acid (0.5%), sodium alginate (2.5%), sucrose-free Vanderzant vitamin mix (3.5%), wheat-germ oil (1%) and cellulose (25%) (Bio-Serv, Frenchtown, NJ, USA). Diets were suspended in 1.35% agar solution in a 5:1 agar solution:dry diet ratio. The wheat-germ based 'colony diet' also consisted of approximately 60% protein and carbohydrate, and had an approximate protein to digestible carbohydrate ratio of 1 to 1.1.

2.3.3. Choice experiments

Freshly moulted final (fifth) instar Bt-S and Bt-RU larvae were weighed and individually provided with two pre-weighed nutritionally suboptimal but complementary diet blocks, and were allowed to self-compose their diet. Three choice experiments were performed to examine the consistency of self-selected macronutrient ratios, with a choice between the highest P:C ratio diet block (50:10) and one of the carbohydrate-biased or equal ratio diet blocks (i) 50:10 & 30:30, ii) 50:10 & 20:40, and iii) 50:10 &

10:50). A total of 72, 75, and 79 larvae were used for each choice experiment, respectively. The diet was replaced each day until pupation; any diet remaining was collected, dried at 50°C for 24 hrs until constant mass, and then weighed. Daily consumption was estimated by calculating the difference between the dry initial and final remaining mass of the diet blocks. The dry initial mass of the diet blocks was estimated by constructing a regression equation with pre-weighed diet blocks without larvae. All larvae successfully pupated, and the date of pupation was recorded. Three days after pupal initiation, pupae were sexed and then dried at 50°C for 48 hrs until constant mass and then weighed.

2.3.4. Statistical analyses

Differences in the cumulative bivariate consumption of protein and carbohydrate by day between Bt-RU and Bt-S were analysed by multiple analysis of covariance (MANCOVA) using Pillai's trace statistic. Sex and choice experiment were included as fixed effects and initial larval weight was included as a covariate. Univariate analyses of protein and carbohydrate intake were obtained from post hoc analyses as part of the bivariate MANCOVA. Analysis of covariance (ANCOVA) for pupal weight was performed separately. Pupal weight was squared before analysis to normalize the data. Tukey HSD comparison was used to compare means among treatments. Time to pupation was analyzed using accelerated failure-time analysis using a Weibull distribution. For all analyses, all factors and their interactions were fitted initially in the model and non-significant interactions were removed sequentially to produce the final minimal model. SAS 9.3 was used for all analyses (SAS Institute, 2010, Cary, NC, USA).

2.4. Results

When provided with two nutritionally suboptimal but complementary diet blocks, the intake target of Bt-S larvae was more carbohydrate-rich than Bt-RU larvae (Figure 2.1; Table 2.1). Both Bt-S and Bt-RU showed some flexibility in their final intake targets (Days 0-pupation) as there was a significant effect of choice and a marginally significant three-way interaction between colony, choice and sex. It appears males showed more

variation in their nutrient intake target than females. The daily variation in feeding between Bt-S and Bt-RU indicates that Bt-S consumed more food (protein and carbohydrate combined) each day than Bt-RU. The mean nutrient intake targets from the three choice experiments for female Bt-S and Bt-RU were 64.4p:48.4c and 65.6p:30.5c respectively, and 65.0p:48.9c and 68.2p:38.7c respectively for male Bt-S and Bt-RU. Thus, female Bt-RU consumed a higher ratio of protein to carbohydrate than male Bt-RU (2.1:1 and 1.8:1 respectively), while both sexes of Bt-S consumed the same P:C ratio (1.3:1).

Table 2.1. MANCOVA analyses for protein and carbohydrate intake by Bt-S and Bt-RU from the three choice experiments.

	Protein and carbohydrate intake								
	Day 0-1			**Days 0-2**			**Days 0-pupation**		
source	d.f. (Num, Den)	*F*-value	*P*-value	d.f. (Num, Den)	*F*-value	*P*-value	d.f. (Num, Den)	*F*-value	*P*-value
initial weight	2, 219	0.45	0.64	2, 218	**10.90**	**<0.0001**	2, 218	0.40	0.67
sex	2, 219	**5.54**	**<0.01**	2, 218	2.06	0.13	2, 218	**5.84**	**<0.01**
colony	2, 219	**41.33**	**<0.0001**	2, 218	**26.82**	**<0.0001**	2, 218	**40.73**	**<0.0001**
choice expt	4, 440	2.39	0.05	4, 438	**5.93**	**<0.0001**	4, 438	**2.68**	**0.03**
colony x choice expt	4, 436	0.67	0.62	4, 434	2.16	0.07	4, 434	2.05	0.09
colony x sex	2, 216	0.75	0.47	2, 218	**5.93**	**<0.01**	2, 218	**4.88**	**<0.01**
choice expt x sex	4, 430	1.70	0.15	4, 430	0.51	0.73	4, 430	0.69	0.60
colony x choice expt x sex	4, 426	1.43	0.22	4, 426	2.39	0.05	4, 426	2.36	0.05

Analyses were performed on each of the three cumulative intake points (days 0-1, 0-2, and 0-pupation).
Non-significant interactions ($p \geq 0.05$) were removed sequentially.
Significant results ($p < 0.05$) are in bold font.
Colony = susceptible vs. resistant

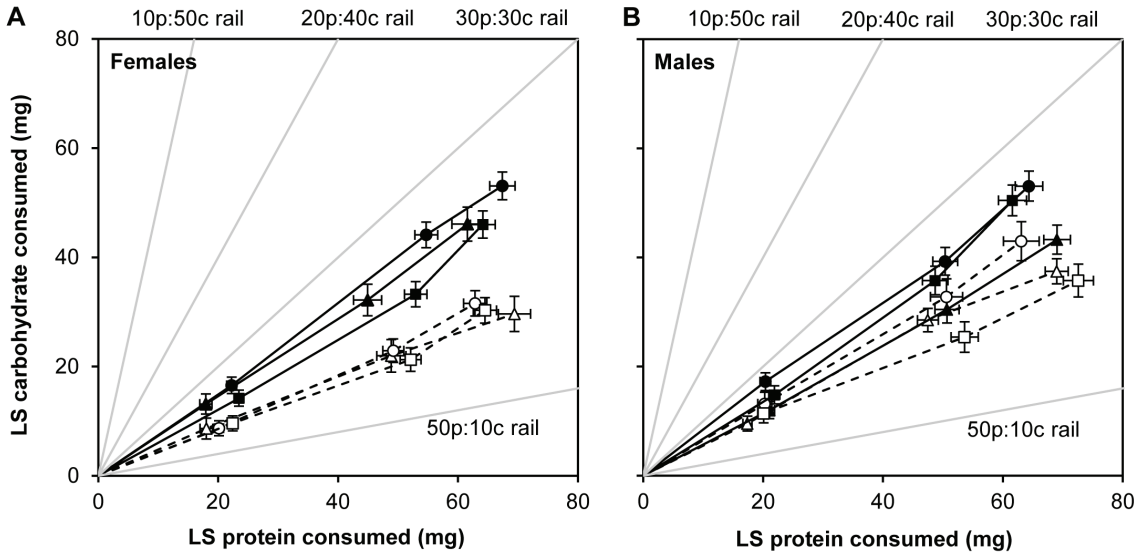

Figure 2.1. *Bt* resistance alters nutrient intake.
Bivariate least squares means (±SE) of protein and carbohydrate intake composed by (A) female and (B) male Bt-S (solid black symbols) and Bt-RU (open symbols). Points along each trajectory correspond to the cumulative intake of protein and carbohydrate over consecutive days. Intake points for day 0-1, days 0-2, and days 0-pupation are shown. The solid gray lines indicate the macronutrient 'rails' of each diet block provided. The choices were as follows: 50p:10c & 30p:30c (triangle); 50p:10c & 20p:40c (circle); 50p:10c & 10p:50c (square).

This prompted an examination of the univariate ANCOVA results for protein and carbohydrate intake separately. Protein consumption showed a significant three-way interaction that is likely due to differences in the direction of the effects among choice experiments. However, there were no significant differences when compared by Tukey HSD (Figure 2.2). There were no two-way interactions or main effects in the model either. In contrast, there was no three-way interaction for carbohydrate intake, but a strong effect of colony, sex and their interaction (Table 2.2). Bt-S larvae consumed more carbohydrate than Bt-RU larvae; however, whereas both Bt-S sexes consumed the same amount of carbohydrate, Bt-RU males consumed more carbohydrate than Bt-RU females (Figure 2.3). Therefore, the higher P:C ratio composed by Bt-RU resulted from similar protein intake as Bt-S, but reduced carbohydrate intake, and was more pronounced for females than males.

Table 2.2. ANCOVA results for protein and carbohydrate intake, and pupal weight.

source	protein			carbohydrate			pupal weight squared		
	d.f.	*F*-value	*P*-value	d.f.	*F*-value	*P*-value	d.f.	*F*-value	*P*-value
initial weight	1, 213	0.85	0.36	1, 219	1.6	0.20	1, 219	**16.94**	**<0.0001**
sex	1, 213	2.74	0.10	1, 219	**6.28**	**0.01**	1, 219	**9.59**	**<0.01**
colony	1, 213	1.62	0.20	1, 219	**90.23**	**<0.0001**	1, 219	**40.77**	**<0.0001**
choice expt	2, 213	1.27	0.28	2, 219	**4.25**	**0.02**	2, 219	**10.47**	**<0.0001**
colony x choice expt	2, 213	2.70	0.07	2, 217	1.07	0.35	2, 217	2.79	0.06
colony x sex	1, 213	1.45	0.23	1, 219	**7.05**	**<0.01**	1, 219	**14.11**	**<0.001**
choice expt x sex	2, 213	1.22	0.30	2, 215	0.30	0.74	2, 215	0.60	0.55
colony x choice expt x sex	2, 213	**4.09**	**0.02**	2, 213	1.12	0.33	2, 213	1.79	0.17

Non-significant interactions (p≥0.05) were removed sequentially.
Significant results (p < 0.05) are in bold font.
Colony = susceptible vs. resistant

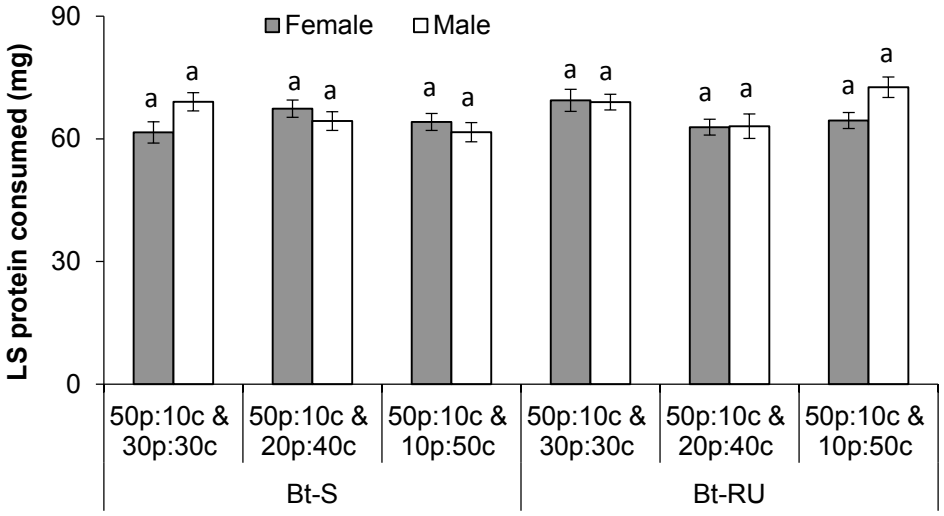

Figure 2.2. Variability of protein intake.
Least squares means of the three-way interaction between colony, sex, and choice experiment on Table 2.2. Same letters indicate no significant differences between means determined by Tukey HSD analysis (p>0.05).

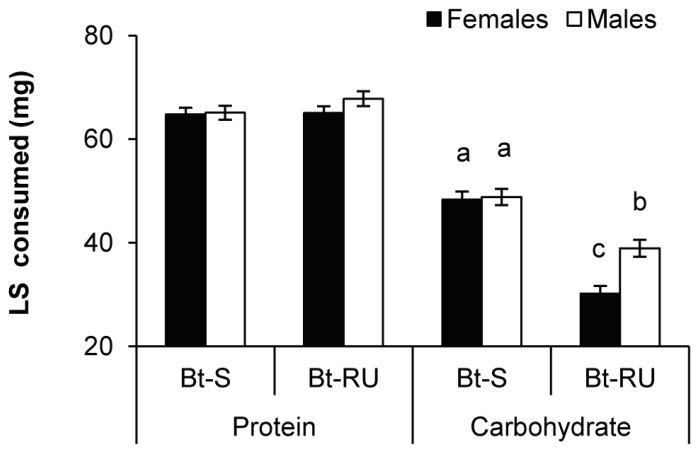

Figure 2.3. Sex differences in compensatory feeding.
Least squares means (±SE) of total protein and carbohydrate consumption for female and male Bt-S and Bt-RU. Different letters indicate significant differences from Tukey HSD comparison ($p<0.05$).

Higher carbohydrate consumption by Bt-RU males compared to Bt-RU females was associated with heavier pupal mass, such that they weighed the same as Bt-S pupae (colony by sex interaction, Figure 2.4A, Table 2.2). Pupal weight of Bt-S and Bt-RU were also significantly affected by choice experiment such that pupal weights decreased as the distance in nutritional space between the protein-biased diet (50p:10c) and the carbohydrate-biased diet (30p:30c, 20p:40c and 10p:50c) became greater. I examined this effect further by performing a Tukey HSD comparison on the marginally significant interaction between choice experiment and colony. The analysis revealed that Bt-S maintained heavy pupal weights on the two choice experiments that were closer together in nutritional space (50p:10c & 30p:30c and 50p:10c & 20p:40c), while Bt-RU had high pupal weight only when the nutritional space between the two choices was closest (50p:10c & 30p:30c; Figure 2.4B). Differences in pupal weight could be explained by a significant three-way interaction for the number of days taken to initiate pupation (Table 2.3). Male Bt-RU achieved the same pupal weight as Bt-S, but consistently fed for a longer period of time to reach pupation (Figure 2.5). On the other hand, female Bt-RU did not take longer to develop except on the most skewed diet choice (50p:10c & 10p:50c).

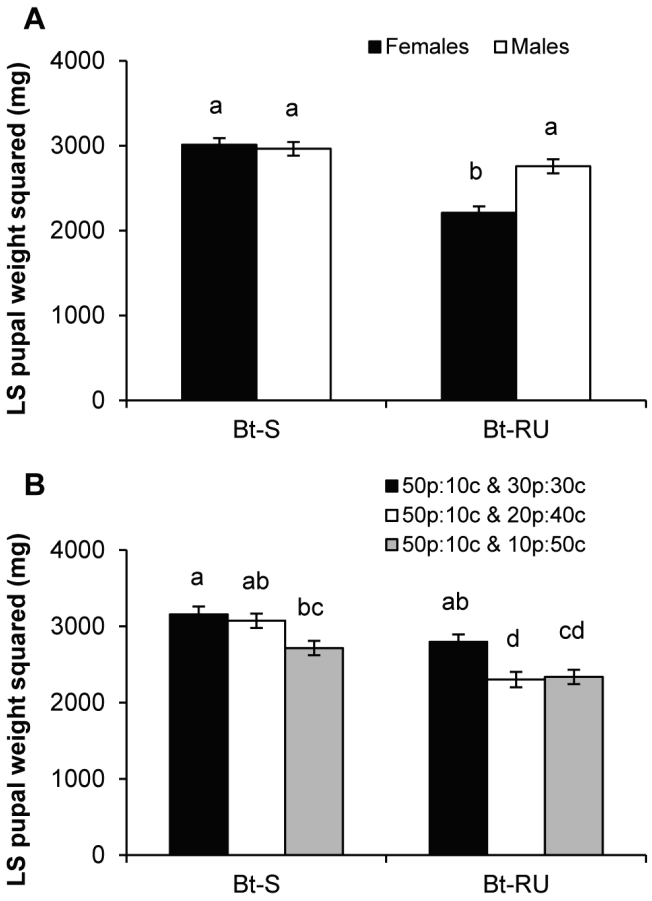

Figure 2.4. Potential for compensatory feeding that reduces fitness cost.
(A) least squares means (±SE) of square-transformed dry pupal weight for female and male Bt-S and Bt-RU. (B) Least squares means (±SE) of square-transformed dry pupal weight for Bt-S and Bt-RU for each choice experiment. Different letters indicate significant differences from Tukey HSD comparison ($p<0.05$).

Table 2.3. Accelerated failure-time analysis for days to pupation.

source	days to pupation		
	d.f.	X^2	*P*-value
initial weight	1	0.01	0.90
sex	1	**4.57**	**0.03**
colony	1	**22.10**	**<0.0001**
choice expt	2	**9.08**	**0.01**
colony x choice expt	2	0.70	0.70
colony x sex	1	**3.99**	**0.05**
choice expt x sex	2	2.89	0.24
colony x choice expt x sex	2	**8.14**	**0.02**

Colony = susceptible vs. resistant
Significant results ($p < 0.05$) are in bold font.

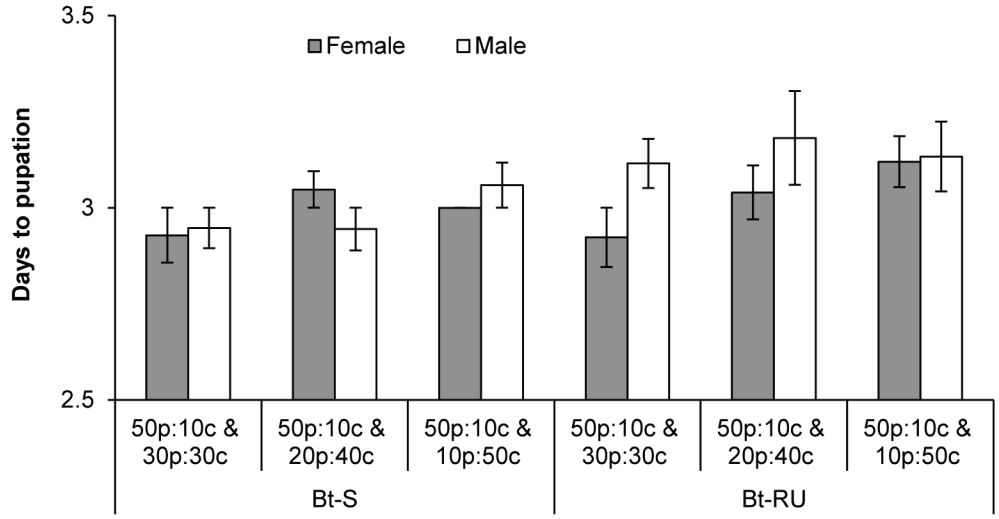

Figure 2.5. Delayed development accompanies compensatory feeding.
Mean number of days (±SE) to initiate pupation in the final instar by Bt-S and Bt-RU.

2.5. Discussion

Allowing susceptible and resistant *T. ni* larvae to self-compose their diets in the final instar revealed that while both Bt-S and Bt-RU larvae regulate protein and carbohydrate intake, they differed in their nutrient intake target ratio. Bt-RU larvae ate less carbohydrate than Bt-S but similar quantities of protein. Bt-S composed a more balanced diet while Bt-RU larvae composed a P:C ratio that was considerably higher in

protein (1.3p:1c and 2p:1c respectively). Broadly, this is in line with other Lepidoptera, in that protein intake is either equal to or greater than carbohydrate ingestion (Behmer 2009). The reduced carbohydrate intake was primarily accounted for by resistant females eating less carbohydrate than the males. Reduced food intake by Bt-RU that resulted from reduced carbohydrate intake suggests that the two colonies may be adopting different lifestyles, such that one feeds more and grows faster while the other feeds more conservatively and grows slower. Such differences in lifestyles are often associated with different costs. For example, higher foraging rates can be associated with higher predation rates (Werner & Anholt 1993).

Bt-RU female pupae weighed less than the susceptible pupae of both sexes, whereas resistant male pupae weighed the same. However, male Bt-RU achieved a larger pupal weight at the cost of longer development time. Male pupal weight is weakly heritable and positively correlated with *Bt* susceptibility in *Bt*-resistant *T. ni* (Janmaat & Myers 2011). *T. ni* males mate multiple times and it is hypothesized that increased weight improves mating success and frequency (Janmaat & Myers 2011). Female pupal weight is generally correlated with fecundity; however, no relationship with *Bt* susceptibility has been found in *T. ni* (Janmaat & Myers 2011). The reduction in female pupal weight found here is thus harder to explain but does suggest an inability to compensate for fecundity loss. One hypothesis is that since feeding on a diet with a higher P:C ratio increases immune activity and survival after pathogen challenge in another Lepidopteran species (Lee *et al.* 2006b), selection pressure may be higher for female Bt-RU to survive *Bt*-challenge, and thus ingest more protein, whereas males are selected for mating success and frequency. Virus-challenged caterpillars have shown similar behaviour to improve their likelihood of surviving infection by reducing carbohydrate consumption while maintaining protein consumption (Povey *et al.* 2014). Achieving higher immune activity through higher P:C consumption may also allow female Bt-RU to have enhanced immunity in the next generation, as induced *Bt* tolerance has been shown to be transferred to the next generation by maternal effects (Rahman *et al.* 2004).

Given that Bt-RU and Bt-S are back-crossed to maintain genetic similarity and have been continuously reared on the same diet, these differences in feeding behaviour

are consistent with an association with resistance to *Bt*. Studies with more lines of Bt-resistant *T.ni* and other species are needed to confirm the generality of this finding. Investigation of precise mechanisms are beyond the scope of this paper but altered nutrient intake might result from changes in the feedback mechanism that provides information about the nutrient content of food and chemical composition of the haemolymph (Thompson & Redak 2000), or from other, as yet unidentified, mechanisms resulting from the modification of midgut binding proteins (Wang *et al.* 2007; Tiewsiri & Wang 2011). It is important to mention that the use of casein as the only protein source (from an animal) and sucrose as the only carbohydrate source are not representative of the natural dietary nutrients for *T. ni*. Whether the results from this study hold in situations where other protein and carbohydrate sources are available or on plants differing in nutritional quality warrant further investigation.

Herbivorous insects have access to a variety of plants and plant parts that vary in nutritional content. My experiments demonstrate that *T. ni* larvae will compose a specific balance of nutrients, and that the evolution of pathogen resistance can change feeding behaviour to alter the nutrient intake target. Consumption of a higher ratio of protein by Bt-RU suggests that they may seek out plant parts higher in protein, adding to their destructive potential as pests by preferentially consuming the reproductive and younger parts of plants that are protein rich. Alternatively, such behaviour might enhance refuge-based resistance management strategies if these plant parts contain higher levels of defensive phytochemicals. Understanding the behavioural and physiological outcomes of resistance selection is important in predicting the destructive potential of resistant insects and the success of resistance management strategies.

2.6. Acknowledgements

I gratefully acknowledge the technical assistance of Rafel Sulaiman. I also thank Yves Carrière for useful discussion of the data, Kwang P. Lee for statistical advice, Carl Lowenberger and Jerry Ericsson for providing the insect colonies, Michelle Franklin and Michelle Tseng for their comments on an earlier version of the manuscript, and several anonymous reviewers for improving the quality of the manuscript. Financial support was provided by the Natural Sciences and Engineering Research Council of Canada

(NSERC) Discovery Grant to J.S.C. and an NSERC Alexander Graham Bell Canada Graduate Scholarship-Doctoral, the Pacific Century Graduate Entrance Scholarship from the government of BC and a Targeted Special Graduate Entrance Scholarship from SFU to I.S.

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2.8. Connecting statement

In Chapter 2, I demonstrated that *Bt*-resistant *T. ni* will compose a different P:C ratio than susceptible *T. ni.* This altered nutrient intake was associated with the elimination of a fitness cost (reduced pupal weight) in *Bt*-resistant males. In Chapter 3, I investigate whether the fitness costs of evolved *Bt*-resistance in *T. ni* on fixed nutrient diets is due to impaired nutrient utilization, possibly associated with gut-based resistance mechanisms. I also examine how dietary nutrients prior to *Bt*-challenge affect the survival of *Bt*-resistant and susceptible *T. ni.*

Chapter 3.

Dietary mechanism behind the costs associated with resistance to *Bacillus thuringiensis* in the cabbage looper, *Trichoplusia ni*

A modified version of this chapter has been accepted for publication:
Shikano, I. & Cory, J.S. (In Press) Dietary mechanism behind the costs associated with resistance to *Bacillus thuringiensis* in the cabbage looper, *Trichoplusia ni. PLoS ONE.*

3.1. Abstract

Beneficial alleles that spread rapidly as an adaptation to a new environment are often associated with costs that reduce the fitness of the population in the original environment. Several species of insect pests have evolved resistance to *Bacillus thuringiensis* (*Bt*) toxins in the field, jeopardizing its future use. This has most commonly occurred through the alteration of insect midgut binding sites specific for *Bt* toxins. While fitness costs related to *Bt* resistance alleles have often been recorded, the mechanisms behind them have remained obscure. As resistance to *Bt* in most lepidopteran species is associated with altered expression of midgut digestive proteins, I asked whether evolved resistance to *Bt* alters dietary nutrient intake, and if reduced efficiency of converting ingested nutrients to body growth are associated with fitness costs and variation in susceptibility to *Bt*. I fed the cabbage looper *Trichoplusia ni* artificial diets differing in levels of dietary imbalance in two major macronutrients, protein and

digestible carbohydrate. By comparing a *Bt*-resistant *T. ni* strain with a susceptible strain I found that the mechanism behind reduced pupal weights and growth rates associated with *Bt*-resistance in *T. ni* was reduced consumption rather than impaired conversion of ingested nutrients to growth. In fact, *Bt*-resistant *T. ni* showed more efficient conversion of nutrients than the susceptible strain under certain dietary conditions. Although increasing levels of dietary protein prior to *Bt* challenge had a positive effect on larval survival, the LC_{50} of the resistant strain decreased when fed high levels of excess protein, whereas the LC_{50} of the susceptible strain continued to rise. My study demonstrates that examining the nutritional basis of fitness costs may help elucidate the mechanisms underpinning them.

3.2. Introduction

Repeated use of chemical insecticides, as well as several microbial insecticides, has resulted in the evolution of resistance in numerous insect species (Hemingway & Ranson 2000; Eberle *et al.* 2008; Bravo & Soberón 2008; Carrière *et al.* 2010; Cory & Franklin 2012). However, the mutations that confer resistance often reduce fitness in the absence of the insecticide (Carrière *et al.* 1994; Kraaijeveld & Godfray 1997; Vijendravarma *et al.* 2009; Duncan, Fellous & Kaltz 2011; Martins *et al.* 2012). Hence, the evolution and stability of resistance to microbial and chemical insecticides is believed to be strongly influenced by fitness costs (Boots & Begon 1993; McKenzie 1996).

The toxin-producing bacterium *Bacillus thuringiensis* (*Bt*), is the most commercially successful microbial insecticide, and crop plants genetically modified to express *Bt* toxins have been planted in 66 million hectares worldwide (James 2011). Repeated exposure to *Bt* has placed strong selection pressure on its target herbivores, resulting in some instances in the evolution of resistance (Heckel *et al.* 2007; Bravo & Soberón 2008; Carrière *et al.* 2010). Resistance in most species decreases rapidly in the absence of *Bt* exposure, suggesting a trade-off in which alleles for *Bt* resistance increase fitness in the presence of *Bt* but inflict fitness costs in its absence (Gassmann *et al.* 2009). Fitness costs associated with resistance to *Bt* sprays or *Bt* toxins have been found in representatives from one family of Coleoptera (Chrysomelidae (Alyokhin & Ferro 1999)) and Diptera (Culicidae (Paris, David & Despres 2011)), and four families of

Lepidoptera (Noctuidae (Akhurst *et al.* 2003; Janmaat & Myers 2003), Plutellidae (Groeters *et al.* 1994; Raymond, Sayyed & Wright 2007b), Pyralidae (Oppert *et al.* 2000), and Gelechiidae (Carrière *et al.* 2004)).

Mortality from *Bt* occurs when *Bt* Cry toxins are ingested, either contained within *Bt* spores or expressed in transgenic crops, and interact with specific binding sites at the midgut brush border membrane, forming pores that result in cell lysis and septicemia, causing death of the insect (Bravo *et al.* 2007; Vachon *et al.* 2012). The evolution of *Bt* resistance is typically defined as a genetically based reduction in the susceptibility of a population to *Bt* Cry toxins (Bravo & Soberón 2008), and most commonly involves reduced binding of the toxins to midgut targets through the alteration or loss of midgut toxin-binding proteins (Bravo & Soberón 2008). Several other toxin-based resistance mechanisms have been found including sequestration of the toxin by lipophorin (Ma *et al.* 2005) or esterases (Gunning *et al.* 2005), and retention of the toxin by binding sites on the peritrophic membrane which prevent the toxin from reaching midgut targets (Hayakawa *et al.* 2004). However, resistance has also been shown to occur through elevated immune responses to formulations containing *Bt* spores (Rahman *et al.* 2004; Hernández-Martínez *et al.* 2010), which invade the haemocoel after the toxins breach the intestinal epithelium (Johnston & Crickmore 2009; Raymond *et al.* 2010). The *Bt*-resistant strain of *Trichoplusia ni* (Hübner), used in the present study was originally collected from a vegetable greenhouse and found to be highly resistant to DiPel (Janmaat & Myers 2003), a formulation of spores and toxins of *B. thuringiensis* subsp. *kurstaki* containing Cry1Aa, Cry1Ab, Cry1Ac, and Cry2. It has since been routinely selected for resistance to DiPel, but the mechanism of resistance in this strain is unknown.

Fitness costs associated with *Bt*-resistance are strongly influenced by ecological variation, such as the plants the insects feed on (Shirai *et al.* 1998; Carrière *et al.* 2005; Janmaat & Myers 2005; Bird & Akhurst 2007; Raymond *et al.* 2007b, 2011; Williams *et al.* 2011), and are magnified by defensive phytochemicals that reduce feeding performance through direct toxic effects or reduced availability of nutrients (Gassmann *et al.* 2009). In my *Bt*-resistant strain of *T. ni*, the degree of resistance-associated fitness costs (lower pupal weight and slower development rate) increased with declining host

plant suitability (Janmaat & Myers 2005, 2006). I hypothesized that these costs could be caused by an impaired ability to convert ingested nutrients into body growth, which might result from resistance mechanisms such as lost or altered midgut proteins. I have also recently shown that the *Bt*-resistant strain selects a higher ratio of protein to carbohydrate than the susceptible strain when they are allowed to compose their own protein to carbohydrate diet (Shikano & Cory 2014). The intake of more protein could indicate compensatory feeding to overcome impaired conversion of ingested protein into bodily nitrogen or increased protein requirements to maintain an elevated immune response (Lee *et al.* 2006b; Povey *et al.* 2014).

Nutritional studies have shown that insects regulate their nutrient intake to optimize performance (Waldbauer & Friedman 1991; Raubenheimer & Simpson 1997). In order to examine the mechanisms behind the fitness costs associated with resistance to *Bt*, I used a well-established Geometric Framework from nutritional ecology (Raubenheimer & Simpson 1993; Simpson & Raubenheimer 1993; Raubenheimer *et al.* 2009) to compare *Bt*-resistant and *Bt*-susceptible lines of cabbage loopers, *Trichoplusia ni*. This approach quantifies how insects regulate the intake of two or more food components at the same time (Raubenheimer *et al.* 2009; Behmer 2009). I restricted *Bt*-resistant and *Bt*-susceptible lines of *T. ni* to artificial diets differing in levels of dietary imbalance in two major macronutrients, protein and digestible carbohydrate to answer the following questions: (i) Do changes in nutrient availability affect the presence or level of costs associated with *Bt* resistance? (ii) Does the evolution of resistance to *Bt* affect nutrient intake? (iii) Is there evidence for impaired nutrient use in resistant insects? (iv) Do the observed fitness costs result from reduced nutrient intake or conversion efficiency? Lastly, (v) How does nutrient availability affect *Bt*-resistance? As reductions in pupal mass and growth rate, and delayed time to pupation have been shown to be fitness costs in *Bt*-resistant *T. ni* and other *Bt*-resistant lepidopterans (Gassmann *et al.* 2009), I chose these as my metrics to assess how nutrient availability affects fitness costs.

3.3. Materials and methods

3.3.1. Study Animals

The *Bt*-resistant *T. ni* colony has been maintained at 25°C and 16:8 (L:D) photoperiod on a standard wheat-germ based diet, since its original collection from a commercial tomato greenhouse in British Columbia, Canada in 2001 (labeled T2c in Janmaat & Myers 2003). Resistance to *Bt* was maintained by exposing larvae to 40 KIU ml^{-1} diet *Bt* subsp. *kurstaki* (DiPel 2x DF, Valent Biosciences, Libertyville, IL, USA) every generation. The *Bt*-susceptible colony derives from the *Bt*-resistant line but was reared without any *Btk* exposure (Janmaat & Myers 2003). Approximately 200 moths were mated each generation to minimize inbreeding. Back-crosses between the resistant and susceptible colonies were performed to homogenize their genetic background, and then re-selected with *Bt* so that the major genetic difference between the two colonies was likely to be *Bt* resistance (Janmaat & Myers 2007). For the purpose of this study, I used larvae from the *Bt*-susceptible line (Bt-S), and larvae from the *Bt*-resistant line that were not selected with *Bt* for one generation (Bt-RU) prior to the experiments to reduce trans-generational effects from the *Bt* selections (Janmaat & Myers 2005).

3.3.2. Artificial diets

Insects were routinely reared on the wheat-germ based diet ('colony diet') which they were originally established on. For the experiments, larvae were reared on the colony diet from egg-hatch until exposure to the wheat-germ free, nutritionally-defined treatment diets. The colony diet consisted of 60% protein and digestible carbohydrate, and had an approximate protein to digestible carbohydrate ratio of 1 to 1.1 (Bio-Serv, Frenchtown, NJ, USA). 'Treatment diets' were prepared according to Shikano and Cory (2014). They contained no wheat germ and consisted of dietary macronutrient ratios manipulated by altering the ratios of protein (casein) and digestible carbohydrate (sucrose) that made up 60% of the dry weight. The ratios were as follows (% protein: % carbohydrate): 50:10, 40:20, 30:30, 20:40, 10:50. They were selected to encompass the wide range of protein and carbohydrate contents found in *T. ni* host plants. For example, between-species variation in protein and digestible carbohydrate content in

nine Brassicacae species (preferred host plants of *T. ni*) ranged from 12-37% dry weight in protein (% nitrogen multiplied by conversion factor of 6.25; Morehouse & Rutowski 2010) and 11-60% dry weight in digestible carbohydrate (Morehouse & Rutowski 2010). Protein content can likely reach up to 50% as supplementation of soil with nitrogen is known to increase protein content in a Brassicacae specie (e.g. Hara & Sonoda 1982). Other components of the dry diet included Wesson's salt (5%), cholesterol (1.5%), ascorbic acid (1%), sorbic acid (0.5%), sodium alginate (2.5%), sucrose-free Vanderzant vitamin mix (3.5%), wheat-germ oil (1%) and cellulose (25%). The diets were provided to larvae in 1.35% agar solution in a 5:1 agar solution:dry diet ratio.

3.3.3. Feeding experiment

I followed the protocol of (Lee *et al.* 2002) with some minor modifications. Freshly moulted final (fifth) instar larvae (100 *Bt*-RU and 100 *Bt*-S) were weighed and individually fed a single pre-weighed block of one of five treatment diets in 30 ml plastic cups at 25°C and 16:8 (L:D) photoperiod. Uneaten diet was replaced with fresh diet blocks daily and frass was removed every day until pupation. Each day, the uneaten diet was dried to constant mass in a desiccating oven at 50°C for 24 hrs, then weighed to the nearest 0.1 mg. Pre-weighed diet blocks without larvae were run at the same time to construct a regression equation that was used to back calculate the initial dry mass of the diet blocks. Daily consumption was estimated by calculating the difference between the dry initial and final mass of the diet blocks. Only five Bt-S and one Bt-RU larvae either rejected the diet or failed to pupate and were removed from analyses. All pupae were weighed three days after pupal initiation then dried in a desiccating oven at 50°C for 48 hrs until constant mass. Dry initial larval mass, used to calculate relative growth rate (RGR), was estimated by using a regression equation constructed from weighing 20 live final instar larvae, freezing them at -20°C for 30min, then re-weighing after drying in a desiccating oven at 50°C for 24 hrs. RGR = [ln(dry pupal mass) – ln(dry initial larval mass)] / days to pupation (Gotthard, Nylin & Wiklund 1994).

Lipid and nitrogen content of pupae were measured to determine the differences in nutrient use associated with *Bt* resistance. To measure pupal lipid content, dried pupae were lipid-extracted in three changes of chloroform every 24 hrs, then re-dried

and re-weighed. Only subsets of the lipid-free pupae were analyzed for nitrogen content due to logistical constraints. A randomly selected subsample of ten lipid-free dry pupae from each diet treatment per colony was individually ground for 10 sec into a homogenous powder using a Mini-BeadBeater (BioSpec Products, Bartlesville, OK, USA). An approximately 2 mg powdered sample of each pupa was weighed to the nearest 0.001 mg and loaded onto an elemental analyzer (Vario Micro Cube CHNS Analyzer, Elementar Americas Inc.). Nitrogen content of each sample determined by the elemental analyzer was used to back-calculate and estimate nitrogen content in each pupa.

3.3.4. *Bt*-challenge experiment

Freshly moulted fourth instar larvae were fed one of five diet treatments individually in cells of 48-well tissue culture plates until moulting to the fifth instar. Freshly moulted fifth instars were transferred to individual cells of 12-well tissue culture plates containing colony diet treated with one of five concentrations of *Bt* (DiPel) (0, 0.25, 0.5, 1, 2, 4 KIU ml^{-1} diet for Bt-S; 0, 10, 20, 40, 80, 160 KIU ml^{-1} diet for Bt-RU). Larvae were challenged with *Bt* on colony diet to equalize the *Bt* exposure between diet treatments. There were 48 larvae per concentration of *Bt* per treatment diet per colony. Larval mortality was recorded after 3 days on *Bt*-treated diet.

3.3.5. Statistical analyses

Measures of diet consumption, pupal weight, relative growth rate, and pupal nitrogen and lipid content were analyzed using analysis of covariance (ANCOVA). Although female pupae tend to weigh less than male pupae, sex did not interact with P:C ratio and colony. Therefore, sex was used as a covariate along with initial larval mass. Diet consumed was log transformed and relative growth rate was squared to meet the underlying assumptions of ANCOVA. Tukey HSD comparisons were performed when significant differences among treatments were detected. Time to pupation was analyzed using Cox proportional hazards regression model. For all analyses, all factors and their interactions were fitted initially in the model and non-significant terms were removed sequentially to produce the final minimal model. All figures are presented with the least

squares means adjusted for sex and initial larval mass, and back transformed least squares means where transformations were used. Mortality data for the *Bt* assay were analysed using generalized linear models (GLM), using a binomial error structure and a logit link function. Chi-square pairwise contrasts were used to determine significant differences in mortality between diet treatments. As median lethal doses or concentrations are frequently used for comparison in the insect pathology literature, I also estimated the LC_{50} (concentration of *Bt* (DiPel) that killed 50% of exposed larvae plus its 95% confidence intervals) from the final, minimal GLM using the inverse prediction option in JMP. JMP (version 10, SAS Institute Inc., Cary, NC) was used for all statistical analyses.

3.4. Results

3.4.1. Performance

Bt-RU individuals had significantly lower pupal mass than Bt-S insects; however, this varied with the dietary P:C ratio, with the Bt-RU line being less affected by nutrient ratio (Figure 3.1A; Table 3.1). Bt-S pupae weighed more than Bt-RU on the three most balanced dietary P:C ratios, but the mass of Bt-S pupae decreased on the two extreme P:C ratio diets, resulting in similar masses for both lines. The relative growth rate (RGR) of Bt-S was also significantly faster than Bt-RU but this also varied with diet and only reached significance on the balanced diet (30p:30c) (Figure 3.1B; Table 3.1). For Bt-RU, RGR was significantly lower on the most carbohydrate-rich diet relative to the other diets. For Bt-S, RGR was significantly lower on both the extreme diets, compared to the three most balanced diets. The two *T. ni* strains took the same time to reach the pupal stage (Figure 3.1C; Table 3.1) and time to pupation was only delayed on the most carbohydrate-rich diet for both strains.

Table 3.1. Analyses for pupal weight, larval growth rate, and days to pupation for *Bt*-resistant and susceptible *T. ni*.

	[a]Pupal weight (n=194)			[a]Relative growth rate (n=194)			*Days to pupation (n=194)		
Source	DF	*F*	*p*	DF	*F*	*p*	DF	X^2	*p*
Initial mass	1,182	26.42	**<0.001**	1,182	84.17	**<0.001**	1	3.64	0.06
Sex	1,182	13.81	**<0.001**	1,182	0.011	0.92	1	1.17	0.28
Colony	1,182	7.02	**<0.01**	1,182	7.88	**<0.01**	1	0.025	0.87
P:C ratio	4,182	14.36	**<0.001**	4,182	35.21	**<0.001**	4	10.31	**0.04**
Colony x P:C ratio	4,182	5.45	**<0.001**	4,182	4.86	**<0.01**	4	0.77	0.94

[a]Analyses of Covariance; * Cox proportional hazards regression model
Colony = Bt-S vs Bt-RU
Values in boldface are significant at $p < 0.05$.

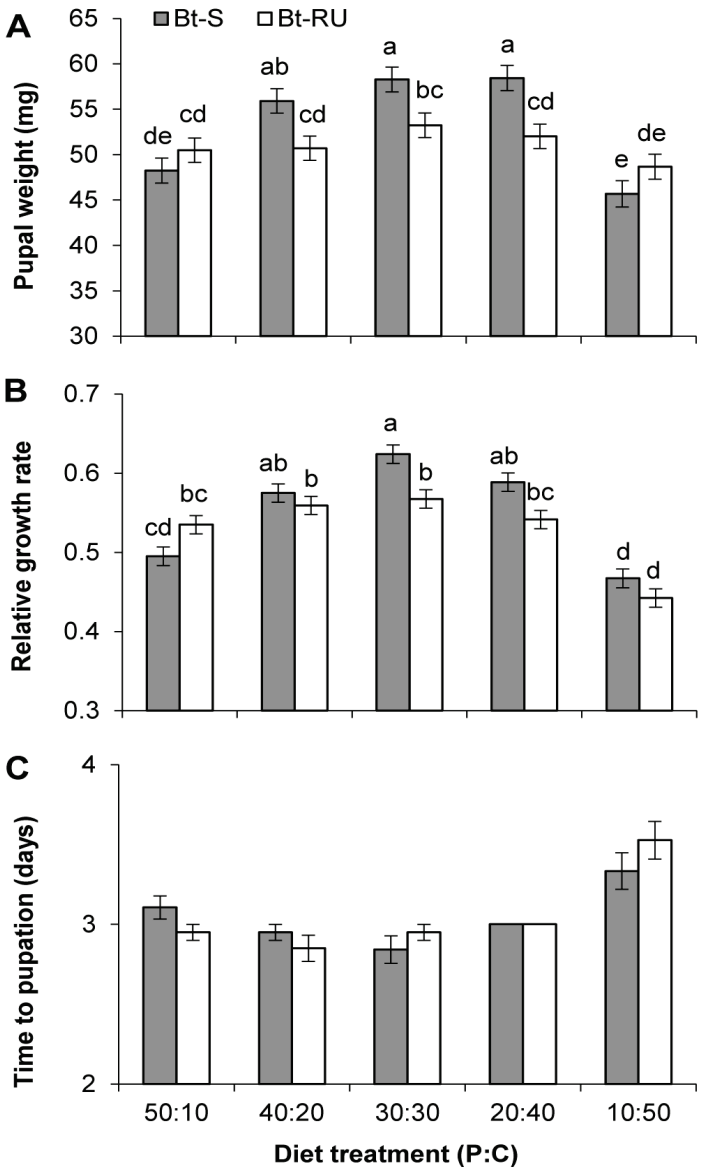

Figure 3.1. Fitness costs associated with *Bt* resistance in *T. ni*.
(A) Pupal dry weight and (B) relative growth rate (RGR) for final instar Bt-S and Bt-RU larvae across the five P:C ratio diets, presented as least squares means (±SE) adjusted for initial larval weight and sex. (C) Mean (±SE) number of days to pupation. RGR = [ln(dry pupal mass) – ln(dry initial larval mass)] / days to pupation (Gotthard *et al.* 1994). Different letters indicate significant differences (Tukey HSD comparison).

3.4.2. Nutrient intake and efficiency of conversion into body mass

Bt-RU consumed significantly less than Bt-S (Colony, $F_{1,182}$=33.84 p<0.0001; Figure 3.2) and consumption increased as the P:C ratio of the diet became increasingly carbohydrate-rich (P:C ratio of diet, $F_{4,182}$=160.21, p<0.001). The two insect lines

responded differently to changing P:C ratio and the reduced consumption of the resistant insects was primarily due to eating significantly less of the 30p:30c and 20p:40c diets (P:C ratio by Colony, $F_{4,182}$=3.54, p=0.008). Initial mass of larvae had no effect on diet consumption (Initial mass, $F_{1,182}$=0.14, p=0.71), while males consumed more diet than females (Sex, $F_{1,182}$=14.85, p=0.0002).

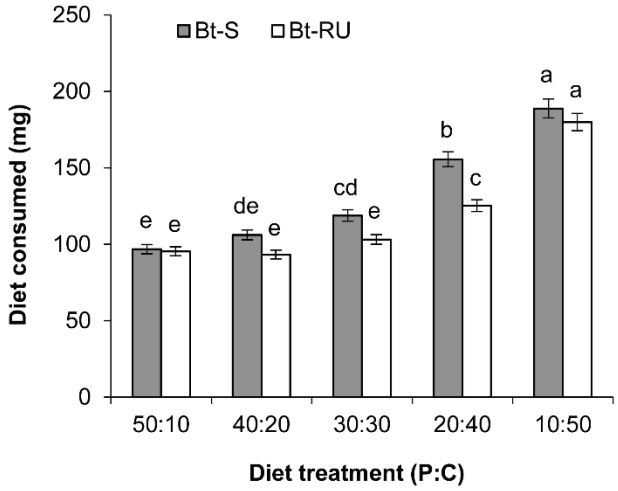

Figure 3.2. Fitness costs in *T. ni* are associated with reduced consumption.
Diet consumption (protein and carbohydrate combined) by final instar Bt-S and Bt-RU larvae across the five P:C ratio diets over the full larval stadium. Consumption is presented as least squares means (±SE) adjusted for initial larval weight and sex. Different letters indicate significant differences (Tukey HSD comparison).

Insects will select specific amounts of required nutrients to achieve an optimal blend of nutrients when they are given a choice of foods differing in nutritional content. This has been termed the intake target (see review by Behmer 2009). Here, *T. ni* larvae were prevented from reaching their intake target (obtained from Shikano & Cory 2014) because they were restricted to one of five P:C ratios. When I examined the amount of protein and carbohydrate that they consumed on each P:C ratio and compared it to the optimal amount that they required (intake target), I found that for both *T. ni* strains, carbohydrate consumption deviated more from the intake target than protein consumption (Figure 3.3). This suggests that maintaining protein intake close to their intake target is more important than maintaining carbohydrate intake.

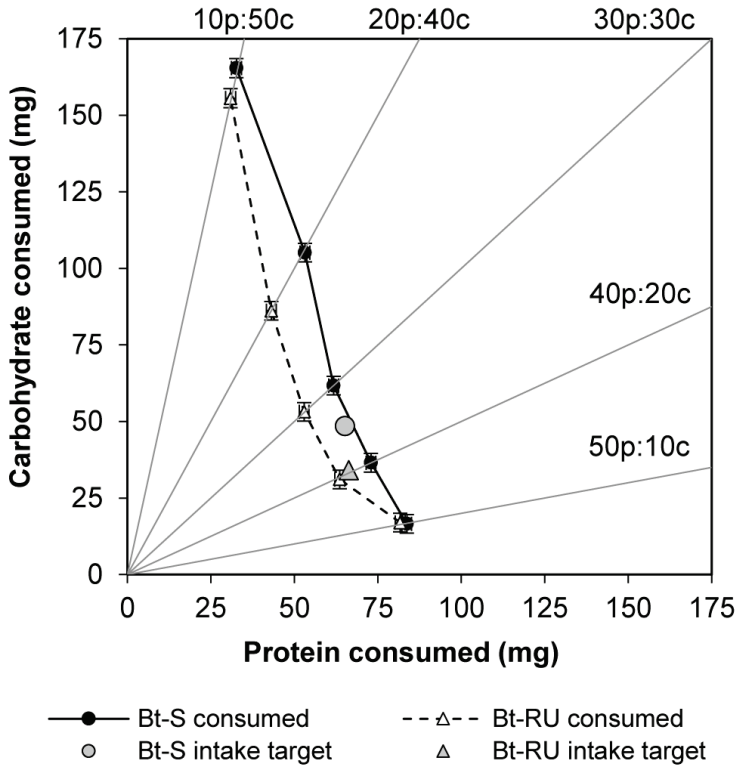

Figure 3.3. Compensatory protein-carbohydrate consumption.
Bivariate least squares means (±SE) for protein and carbohydrate intake for final instar Bt-S and Bt-RU larvae adjusted for initial larval weight and sex. Points along each trajectory correspond to the cumulative intake of protein and carbohydrate over the entire final larval stadium. Solid gray lines represent nutrient ratios for the five food treatments (P:C = 50:10, 40:20, 30:30, 20:40, 10:50). Intake points for each day are connected by solid black lines and dashed black lines for Bt-S and Bt-RU respectively. Optimal intake targets (Bt-S, grey circle; Bt-RU, grey triangle) were obtained from Shikano and Cory (2014).

Lipid and nitrogen content of the resulting pupae were plotted against the quantity of carbohydrate and protein ingested to assess the effect of macronutrient intake on body composition (Figure 3.4). Pupal lipid content increased and gradually came to a plateau as the P:C ratio of the diet moved from protein-rich to carbohydrate-rich (P:C ratio, $F_{4,182}$=141.34, p<0.0001). Although overall lipid content did not differ between Bt-RU and Bt-S pupae (Colony, $F_{1,182}$=0.38 p=0.54), susceptible insects accumulated more lipid than resistant ones as the amount of carbohydrate in the diet increased; however, this trend was reversed on the most carbohydrate-rich diet (P:C ratio by Colony, $F_{4,182}$=4.75, p=0.001). Both initial mass and sex affected total lipid content (Covariates: initial mass, $F_{1,182}$=22.20, p<0.0001; sex, $F_{1,182}$=14.40, p=0.0002).

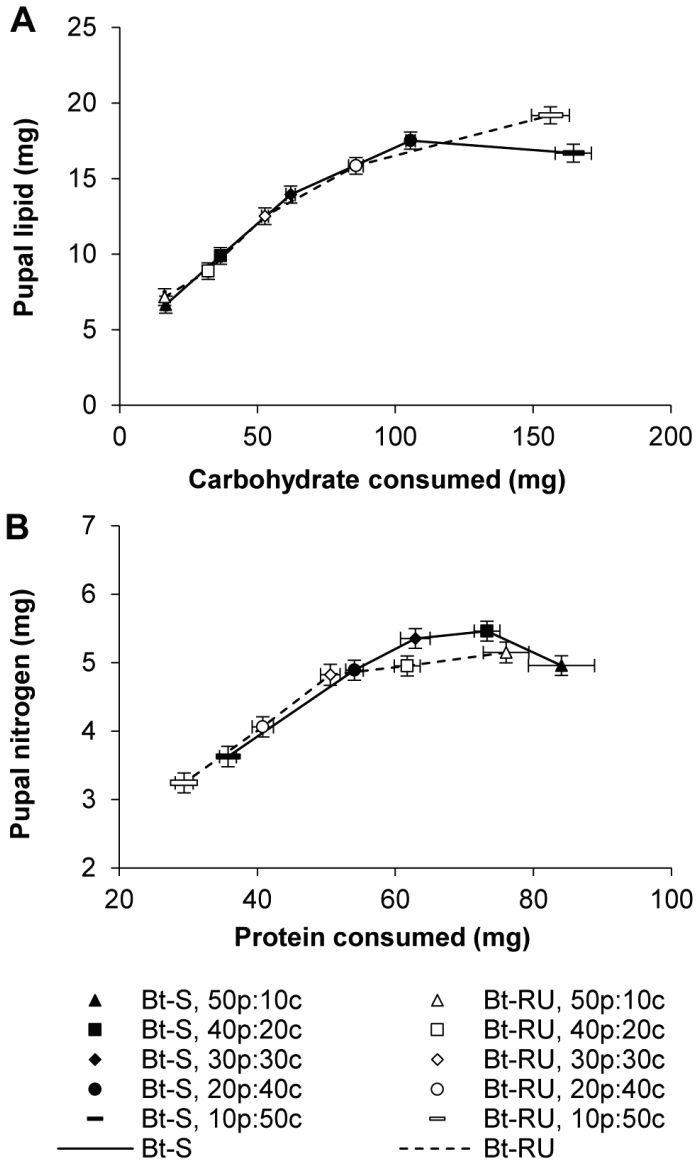

Figure 3.4. Bodily lipid and nitrogen accumulated across diet treatments.
(A) Pupal lipid content plotted against carbohydrate intake and (B) pupal nitrogen content plotted against protein intake across the five P:C ratio diets for final instar Bt-S and Bt-RU larvae. Data are presented as least squares means (±SE) adjusted for initial larval weight and sex.

To compare the efficiency of conversion of carbohydrate to lipid between colonies, I added the amount of carbohydrate consumed by each individual larva as a covariate. This adjusts the carbohydrate consumed, such that I can compare the amount of lipid that was produced if all larvae consumed the same amount of carbohydrate. The efficiency of conversion of carbohydrate to lipid decreased as the

P:C ratio of the diet became carbohydrate-rich (Figure 3.5A, Table 3.2). Furthermore, Bt-RU converted carbohydrate more efficiently into lipid than Bt-S on the two carbohydrate-rich diets.

Table 3.2. ANCOVA results comparing the efficiency of conversion of ingested carbohydrate into body lipid and ingested protein into body nitrogen between *Bt*-resistant and susceptible *T. ni* larvae

	Lipid (n=194)			Nitrogen (n=100)		
Source	DF	F	p	DF	F	p
Initial larval weight	1,177	104.96	**<0.001**	1,83	33.71	**<0.001**
Sex	1,177	0.97	0.33	1,83	0.18	0.67
Colony	1,177	87.31	**<0.001**	1,83	10.12	**<0.01**
P:C ratio	4,177	69.34	**<0.001**	4,83	27.16	**<0.0001**
Colony x P:C ratio	4,177	10.85	**<0.001**	4,83	3.01	**0.02**
Covariate	1,177	213.54	**<0.001**	1,83	196.76	**<0.001**
Colony x Covariate	1,176	1.61	0.21	1,82	0.079	0.78
P:C ratio x Covariate	4,177	21.55	**<0.001**	4,83	3.85	**<0.01**
Colony x P:C ratio x Covariate	4,172	1.06	0.38	4,78	0.73	0.58

Covariate = Amount of carbohydrate or protein ingested.
Colony = Bt-S vs Bt-RU
Values in boldface are significant at $p < 0.05$.

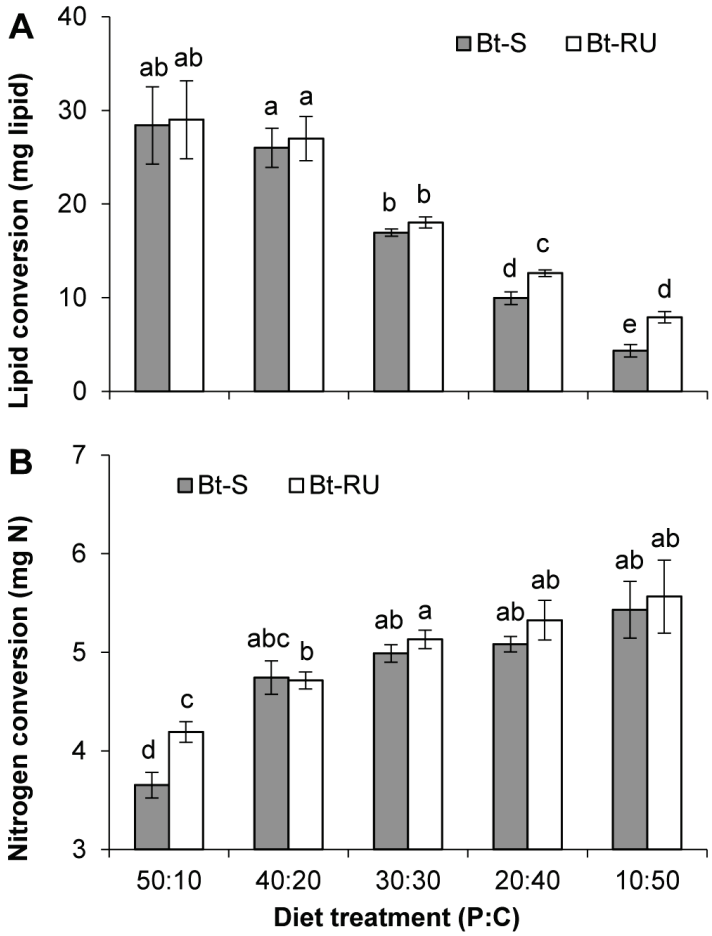

Figure 3.5. Comparisons of nutrient conversion efficiency between *Bt*-resistant and susceptible *T. ni.*

(A) Efficiency of conversion of ingested carbohydrate to pupal lipid content and (B) ingested protein to pupal nitrogen content of pupated Bt-S (solid bars) and Bt-RU (open bars) across the five P:C diet treatments. Efficiency of conversion of ingested macronutrients to body content are presented as least squares means of body content adjusted for the amount of ingested macronutrient (carbohydrate or protein), initial larval weight, and sex. Different letters indicate significant differences (Tukey HSD comparison).

Pupal nitrogen content was consistent across P:C ratios except on the most protein-poor diet (P:C ratio of diet, $F_{4,88}$=51.66, p<0.0001; Figure 3.4B). Overall, nitrogen content was significantly lower in Bt-RU than Bt-S pupae (Colony, $F_{1,88}$=19.00 p<0.0001). The interaction between P:C ratio and *Bt* resistance (P:C ratio by Colony, $F_{4,88}$=3.33, p=0.01) indicates greater differences in nitrogen content between Bt-RU and Bt-S on the three more balanced diets with a significant difference on the 20p:40c diet. The efficiency of conversion of protein to nitrogen decreased as the P:C ratio of the diet

became protein-rich (Figure 3.5B, Table 3.2). Furthermore, Bt-RU converted protein more efficiently than Bt-S on the most protein-rich diet.

3.4.3. *Bt*-challenge

The composition of the diet consumed prior to *Bt* challenge altered the resulting mortality levels. Survival of Bt-S larvae increased significantly with increasing dietary P:C ratio (Table 3.3; Figure 3.6; X_4^2=165.77, p<0.001). Mortality rose more rapidly with *Bt* concentration as the proportion of carbohydrate in the diet increased, but declined on the most carbohydrate-rich diet (10p:50c) (*Bt* concentration by P:C ratio, X_4^2=10.80, p=0.03; Figure 3.6; Table 3.3). As expected, mortality increased with increasing *Bt* concentration (X_1^2=522.15, p<0.001). For Bt-RU, survival also increased with increasing P:C ratio (X_4^2=62.34, p<0.001); however, in contrast to Bt-S, it declined sharply on the most protein-biased diet (by 44% based on LC_{50}; Table 3.3) from its peak on the 40p:20c diet (pairwise contrast between 40p:20c and 50p:10c, X_1^2=12.11, p<0.001). Again, mortality increased with increasing *Bt* concentration (X_1^2=337.15, p<0.001). There was no interaction between *Bt* concentration and diet (X_4^2=2.04, p=0.73), indicating that while levels of mortality differed across P:C ratio diets, the rate at which mortality increased with *Bt* concentration was the same on each diet (i.e. equal slopes). There was no control mortality for both Bt-S and Bt-RU.

Table 3.3. Median lethal concentration of *Bt* (KIU ml^{-1} diet) for final instar Bt-S and Bt-RU larvae that were pre-fed one of five diets differing in P:C ratios.

	Bt-S			Bt-RU			
P:C ratio	LC_{50}	(95% CI)	Slope	LC_{50}	(95% CI)	Slope	*Resistance ratio
50p:10c	2.05	(1.64 – 2.72)	3.24±0.50	21.80	(17.14 – 27.47)	3.06±0.20	10.61
40p:20c	0.91	(0.76 – 1.09)	4.33±0.48	38.62	(30.78 – 48.44)	3.06±0.20	42.39
30p:30c	0.67	(0.56 – 0.78)	5.24±0.56	31.83	(25.30 – 39.91)	3.06±0.20	47.77
20p:40c	0.58	(0.49 – 0.68)	5.53±0.60	24.78	(19.57 – 31.15)	3.06±0.20	42.48
10p:50c	0.33	(0.25 – 0.40)	4.16±0.56	10.93	(8.22 – 14.23)	3.06±0.20	33.34

*Resistance ratio = (LC_{50} of Bt-RU) / (LC_{50} of Bt-S) (Gassmann *et al.* 2009)

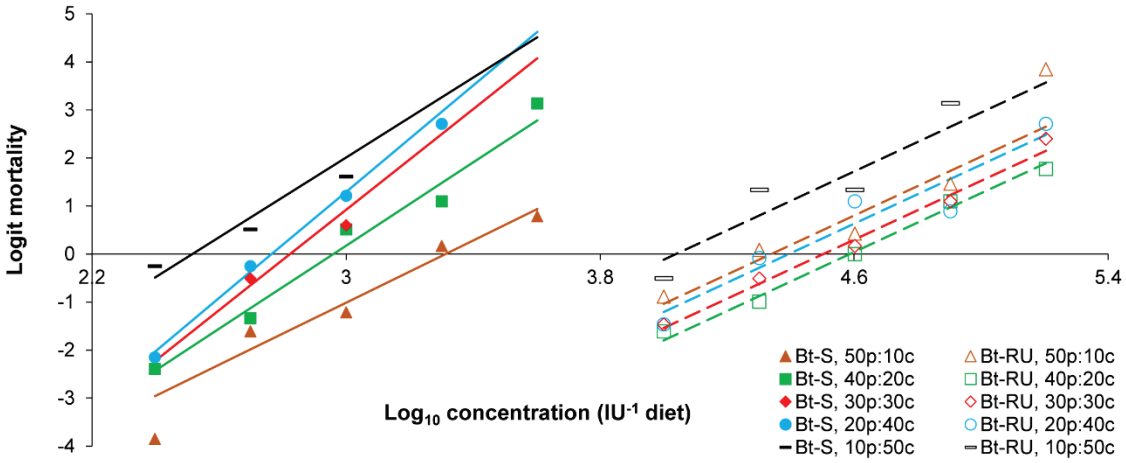

Figure 3.6. Diet composition before *Bt*-challenge affects survival.
Variation in mortality of Bt-RU and Bt-S to *Bt* after pre-feeding on one of five P:C ratio diets. Symbols show the actual data points (solid symbols, Bt-S; open symbols, Bt-RU) and lines (solid line, Bt-S; dashed line, Bt-RU) are the fitted models. The statistical analyses were performed separately for Bt-S and Bt-RU. Values of 0% or 100% are not represented in logits.

3.5. Discussion

Bt-resistance in several species is associated with significant costs such as reduced pupal weight and slower growth rate. My findings show that, in my strain of *T. ni,* these costs are incurred primarily by reduced food consumption and not impaired conversion of ingested nutrients into body mass. In fact, under certain dietary conditions, *Bt*-resistant larvae converted nutrients more efficiently than susceptible larvae. However, greater efficiency of converting protein into bodily nitrogen on the most protein-rich diet was associated with a significant increase in mortality compared to more balanced diets when challenged with *Bt*. This demonstrates a detrimental effect of consuming excess dietary protein in *Bt*-resistant insects.

Insects, like other animals, have evolved behavioural and physiological mechanisms to obtain an optimal mixture and blend of nutrients (Behmer 2009). Here, I restricted *T. ni* larvae to one of five P:C ratios so that they only had three options: (1) consume the diet until it meets its requirement for protein even though it takes in too much or too little of carbohydrate, (2) consume until it meets the requirement for carbohydrate while suffering an excess or deficit of protein, or (3) feed to an intermediate point where the excesses and deficits of both nutrients are less extreme (Behmer 2009).

As none of the P:C ratio diets I provided exactly matched the intake target ratio of Bt-S and Bt-RU (although 40p:20c was very close for Bt-RU), both strains of *T. ni* consumed excess amounts of the plentiful nutrient to obtain sufficient amounts of the deficient nutrient. This behaviour is typical of generalist herbivores (Lee, Behmer & Simpson 2006a). Excess carbohydrate consumption was greater than excess protein consumption, resulting in higher variability in pupal lipid content, suggesting the greater importance of nitrogen regulation in its diet. This is consistent with nutrient regulation in other lepidopteran species (Lee *et al.* 2002, 2003; Lee, Simpson & Raubenheimer 2004; Lee *et al.* 2006a). Generalists such as *T. ni* are likely to encounter dietary heterogeneity, and thus have flexible metabolic strategies to deal with nutrient imbalances (Lee *et al.* 2006a). In *T. ni,* nitrogen accumulation is likely to be regulated by a post-ingestive mechanism, as excess protein consumption did not result in continued increases in bodily nitrogen content (Zanotto, Simpson & Raubenheimer 1993; Thompson & Redak 2000). Contrary to my hypothesis, Bt-RU showed higher efficiency of conversion of dietary protein into bodily nitrogen on the extremely protein-rich diet. This was associated with no change in pupal mass on the protein-rich diet, whereas the pupal mass of susceptible *T. ni* was negatively affected. The higher conversion efficiency of protein to nitrogen by Bt-RU, coupled with the negative effects of excess protein on pupal mass of susceptible *T. ni,* could explain the higher protein to carbohydrate ratio selected by Bt-RU in my previous study (Shikano & Cory 2014).

Body lipid content increased consistently in both strains as the amount of carbohydrate ingested increased. However, the efficiency with which carbohydrate was converted to lipid decreased as dietary carbohydrate content increased. This indicates that *T. ni* larvae regulate body lipid content, possibly through a post-ingestive regulatory mechanism that releases overeaten carbohydrate from their body (Lee *et al.* 2002). Interestingly, Bt-RU had a better conversion efficiency of carbohydrate to lipid on the carbohydrate-rich diets. Accumulating more body lipid can be advantageous as it can prolong survival during starvation (Stockhoff 1991). *Bt* resistance in Bt-RU is not an all-or-nothing response. High concentrations of *Bt* still inhibit feeding and slow growth due to the damaging effects of *Bt* toxins on the insect gut. Therefore, surviving starvation could be an important component of *Bt* resistance in this strain.

However, this notion is contradicted by higher survival of both strains after *Bt*-challenge when pre-fed higher protein diets (except 50p:10c for Bt-RU). The *Bt* formulation used in this study contains *Bt* spores in addition to toxins. Once the toxins breach the midgut, *Bt* spores act synergistically with the toxins by germinating in the insect haemolymph causing septicaemia (Johnston & Crickmore 2009; Raymond *et al.* 2010). Studies on lepidopteran larvae have found altered immune activity following oral inoculations with formulations of *Bt* spores and toxins, such as increased phagocytic activity and encapsulation rate (Dubovskiy, Krukova & Glupov 2008), and changes in haemocyte density and phenoloxidase activity (Ericsson *et al.* 2009). Furthermore, inducing an immune response (rate of melanization reaction) with a low concentration of a *Bt* formulation was associated with a subsequent increase in survival to challenge with the same *Bt* formulation (Rahman *et al.* 2004). Since higher haemocyte densities (Povey *et al.* 2014), as well as higher antimicrobial, encapsulation and phenoloxidase activities (Lee *et al.* 2006b) were found in the larval haemolymph of two *Spodoptera* species after consuming higher P:C ratio diets, it is possible that *T. ni* that have fed on higher protein diets had higher baseline immune activity at the time of *Bt*-challenge.

I observed a 44% decrease in LC_{50} in Bt-RU on the highest protein diet, compared to a peak on the 40p:20c diet, whereas the LC_{50} of Bt-S larvae continued to increase with protein levels, resulting in a dramatic decline in the resistance ratio (Table 3.3). Bt-S, however, had significantly lower pupal weight and growth rate on the most protein biased diet. Thus, excess dietary protein is deleterious to each *T. ni* line, but in different ways. This could be due to the higher costs of catabolizing excess ingested protein (Raubenheimer & Simpson 2003; Lee *et al.* 2003). Negative effects of excess dietary protein on performance have been observed in other lepidopteran species (Scriber 1984; Schroeder 1986), but this is the first study to show an increase in the susceptibility of an insect resistant to a microbial insecticide.

Lastly, the fitness costs associated with *Bt*-resistance in my *T. ni* strain were due to reduced food consumption rather than less efficient nutrient processing. Reduced consumption could result from a change in the feedback mechanism that involves peripheral contact chemoreception. It provides information about the nutrient content of food and chemical composition of the haemolymph, which reflects the quality and

quantity of nutrient uptake and metabolic activities within the insect (Thompson & Redak 2000). *Bt*-resistant *T. ni* have lower densities of haemocytes, and lower concentrations of protein, and phenoloxidase in the haemolymph than susceptible *T. ni* (Ericsson *et al.* 2009), although this may have been a function of larval size. Lower requirements for these haemolymph components might influence the feedback mechanism, thereby reducing nutrient intake. However, it is important to keep in mind that nutrient intake is a dynamic process that will be influenced by environmental stressors. For example, *Bt*-resistant *T. ni* were shown to increase food consumption and weight gain when fed artificial diet treated with low doses of *Bt* compared to a control (Janmaat, Bergmann & Ericsson 2014).

An important limitation of the present study is the use of casein as the only protein source (from an animal) and sucrose as the only carbohydrate source as these are not representative of the variety of nutrients available to *T. ni* feeding on plants. Therefore, the critical question that remains concerns the extent to which macronutrient ratios affect the performance of *Bt*-resistant and susceptible *T. ni* on natural host plants, since most herbivorous caterpillars have access to a variety of plants and plant parts that vary in nutritional content (Bernays & Chapman 1994). More studies of fitness costs incorporating nutritional ecology using other *Bt* resistant strains are needed to determine the generality of my findings.

3.6. Acknowledgements

I gratefully acknowledge the technical assistance of Rafel Sulaiman and Jutta Buchhop. I also thank Yves Carrière for useful discussion of the data, Kwang P. Lee for statistical advice, Judy Myers for providing the original *T. ni* colonies, Carl Lowenberger and Jerry Ericsson for providing the insects to initiate my colonies, Zamir Punja for the use of his Mini-BeadBeater, and Michelle Franklin and Michelle Tseng for their comments on an earlier version of the manuscript. Financial support was provided by the Natural Sciences and Engineering Research Council of Canada (NSERC) Discovery Grant to J.S.C. and an NSERC Alexander Graham Bell Canada Graduate Scholarship-Doctoral, the Pacific Century Graduate Entrance Scholarship from the government of BC and a Targeted Special Graduate Entrance Scholarship from SFU to I.S.

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3.8. Connecting statement

In Chapters 2 and 3, I demonstrated the effects of nutrient intake on fitness costs associated with evolved resistance to *Bt.* I also showed that nutrient intake prior to *Bt* challenge has a significant impact on survival for both *Bt*-resistant and susceptible *T. ni.* In the following two chapters, I no longer focus on evolved resistance; *Bt*-resistant *T. ni* are no longer used. In Chapter 4, I focus on the interactive effects of two environmental factors on the interaction between *T. ni* and two baculoviruses. I manipulate temperature and host nutrition simultaneously after baculovirus challenge, to determine their interactive effects on within generation resistance and costs associated with resistance. I also examine the effects of temperature and host nutrition on virus productivity.

Chapter 4.

Host-pathogen interactions in a complex world: temperature and nutrition interact to alter performances of the cabbage looper and two baculoviruses

4.1. Abstract

Interactions with pathogens are unavoidable in nature. Pathogens are an important selective force on their hosts and often play key roles in their population dynamics. The interactions between host and pathogen involve biochemical, physiological and behavioural processes that can be influenced differently by environmental factors. The impact of single factors on host-pathogen interactions are well studied, but in nature, numerous environmental factors can vary simultaneously. I simultaneously manipulated two key environmental factors, temperature (16, 24 and 32°C) and nutrient quality (protein to carbohydrate ratio; P:C), to assess their impact on the interaction between the cabbage looper, *Trichoplusia ni*, and a nucleopolyhedrovirus (NPV) after pathogen challenge. To determine the influence of pathogen identity on the host's response to environmental variability, I compared two species of NPV that had different host ranges; one that specializes on *T. ni* (*T. ni* single NPV; TnSNPV) and another that has a broad host range (*Autographa californica* multiple NPV; AcMNPV). Optimal host performance occurred at higher P:C ratios when challenged by virus as resistance to both viruses increased with dietary protein content. The degree with which optimal performance shifted to a higher P:C ratio was strongly affected by temperature when challenged by AcMNPV but not TnSNPV. Virus performance was also affected by environmental variability. TnSNPV showed a distinct peak in performance (host mortality and virus yield) at a narrow P:C ratio range at 24°C, whereas AcMNPV showed more comparable host use across two temperatures and a wider range of P:C ratios.

My study highlights the complex effects of interacting environmental variables and pathogen specificity on the outcomes of host-pathogen interactions.

4.2. Introduction

Pathogens are a ubiquitous threat to all organisms and can be a major influence on the ecology and evolution of their hosts (Wilson 2005). The interaction between host and pathogen are influenced by numerous environmental factors. Host nutrition is an important component of pathogen-dependent population dynamics, because the depletion of nutritional resources and increase in the probability of disease transmission often occur in sync as population densities increase (Gulland 1992; Knell *et al.* 1996; Ryder *et al.* 2005; Boots & Roberts 2012). Recent research on warming temperatures associated with climate change has revealed complex effects of temperature on host-pathogen interactions (Thomas & Blanford 2003; Paull, LaFonte & Johnson 2012). While host nutrition and temperature are individually known to alter pathogen virulence and host immune defense, how interactions between the two environmental factors will influence disease outcome and host and pathogen fitness is difficult to predict.

Host resistance to pathogens is condition-dependent such that resources available for immune defense must be traded-off with costs to other life-history traits (Sheldon & Verhulst 1996; Lochmiller & Deerenberg 2000; Moret & Schmid-Hempel 2000; Schmid-Hempel 2005a). Costs associated with surviving pathogen challenge in insects can include prolonged development, and reduced adult size and fecundity (Milks, Burnstyn & Myers 1998; Fellowes *et al.* 1999; Agnew *et al.* 1999; Myers, Malakar & Cory 2000). Dietary nutrient availability of the infected host is critical to its survival, because it can alter the availability of key resources necessary for components of the host's resistance mechanisms to mount a response and may reduce the costs of allocating resources away from other traits (Coop & Kyriazakis 1999, 2001; Siva-Jothy & Thompson 2002; Myers *et al.* 2011; Hood & Skaar 2012; Povey *et al.* 2014). Therefore, reduced nutrient availability or quality prior to infection can often result in reduced investment in immune defence (Siva-Jothy & Thompson 2002; Shikano *et al.* 2010; Lord 2010; Myers *et al.* 2011) and physical barriers to pathogens such as the structure of the peritrophic matrix (Plymale *et al.* 2008) and melanisation of the cuticle (Lee *et al.* 2008b),

consequently lowering resistance to pathogens. Changes to nutrient availability after pathogen challenge can also alter the likelihood of survival (Lee *et al.* 2006b; Povey *et al.* 2009, 2014). This could be due to changes in the availability of resources needed to produce effector molecules for resistance (Lee *et al.* 2006b), to replenish the costs of resistance such as the sloughing of midgut cells in response to baculovirus infection, or to repair damaged host tissues inflicted by invading pathogens.

Host nutrition is also particularly important from an ecological and evolutionary perspective for the parasite because it can affect the condition of the host, which is the ecological system or environment that it is invading. The dietary nutrients of the infected host may influence both the supply of nutrients available to the parasite for growth and metabolism, as well as affect the availability of nutrients necessary for components of the host's immune system to mount a response (Smith *et al.* 2005). For example, spore production by the microsporidian parasite *Vavraia culicis* increased with the food quantity of its host *Aedes aegypti*, indicating that the parasite benefited from the resources available to its host. However, as the quantity of the host's food continued to increase, host survival increased (increased ability to fight off the parasite), thereby reducing the fitness of the parasite (Bedhomme *et al.* 2004). Therefore, there is a fine balance between whether host nutritional components benefit parasite growth or whether host nutrition improves host survival. Interestingly, in low-food treatments, the parasite showed adaptation to host condition such that it varied its replication rate. This would be beneficial to the parasite because faster replication would consume more host resources, increasing the risk of host death before parasite spores are produced (Bedhomme *et al.* 2004). There are several insect-parasite systems where the host's food quantity or quality has been shown to affect the growth, production, and ultimately fitness of the parasite (bumblebees-trypanosomes (Logan, Ruiz-González & Brown 2005), caterpillars-viruses (Raymond *et al.* 2002), mosquitoes-microsporidians/protozoans (Bedhomme *et al.* 2004; Tseng 2006), reduviid bug-trypanosomes (Kollien and Schaub 1998; 2002; 2003)).

For insects, which are ectotherms, environmental temperature affects the maintenance of almost all bodily functions, including the ability of the host to mount an immune response (Blanford & Thomas 1999; Thomas & Blanford 2003; Linder *et al.*

2008), and consequently affects both host resistance and pathogen growth after infection (Inglis *et al.* 1996; Arthurs & Thomas 2001; Olsen & Hoy 2002; Subramanian *et al.* 2006; Cevallos & Sarnow 2010). Additionally, any differences in the thermal sensitivity of host and pathogen performance can significantly alter the outcome of their interaction (Thomas & Blanford 2003). The most well studied examples of temperature affecting insect-pathogen interactions come from orthopteran species and their fungal pathogens where the pathogen can appear highly virulent under certain temperatures but become completely ineffective at high temperatures. Most interesting is the orthopteran's ability to enhance survival by elevating its body temperature beyond the critical temperature of the pathogen (Boorstein & Ewald 1987; Inglis *et al.* 1996; Blanford, Thomas & Langewald 1998; Blanford *et al.* 2003; Blanford & Thomas 1999, 2001; Elliot, Blanford & Thomas 2002; Bundey *et al.* 2003; Ouedraogo, Goettel & Brodeur 2004). However, such effects of temperature on virulence may not apply to all pathogen groups. For insect baculoviruses, which are obligate intracellular pathogens, temperature has strong effects on speed of kill and replication, but the effects on host mortality are mixed (Ribeiro & Pavan 1994; van Beek, Hughes & Wood 2000; Frid & Myers 2002; Subramanian *et al.* 2006) and may depend on the developmental stage of the insect at the time of viral-challenge and whether the temperature was manipulated throughout, prior to or after viral-challenge.

Here I examine the combined effects of temperature and nutrition, in the form of macronutrient ratios, on the interaction between an insect and two of its pathogens. The host is the cabbage looper *Trichoplusia ni* (Hübner; Lepidoptera: Noctuidae) and the pathogens are two species of baculovirus: *Autographa californica* multiple nucleopolyhedrovirus (AcMNPV) and *Trichoplusia ni* single nucleopolyhedrovirus (TnSNPV). Both viruses occur naturally in *T. ni* populations (Jaques 1970; Theilmann *et al.* 2005) and belong to the same genera (Alphabaculovirus) within the family Baculoviridae (Harrison *et al.* 2012). A major difference between the two viruses is their host range. AcMNPV has a broad host range and can infect species within at least 15 Lepidopteran families (Cory & Myers 2003), while TnSNPV is not known to infect any other hosts as it was only able to infect *T. ni* larvae from among five species tested spanning five families (Del Rincón-Castro & Ibarra 1997).

My main objective was to assess how two environmental factors, nutrition and temperature, interact to influence the interaction between *T. ni* and two NPVs that vary in host specificity. To determine the effects of environmental conditions on hosts challenged with virus, I manipulated temperature and dietary macronutrient ratios of *T. ni* after virus challenge, and measured host survival and the costs to life-history traits associated with resistance. To assess the impact of host environmental conditions on the viruses, I measured host mortality, speed of kill and the productivity of viruses within hosts. *T. ni* is a generalist pest that is widespread in tropical and subtropical regions around the world. It is native to subtropical areas of North America and migrate annually to establish summer breeding populations as far north as Canada (Franklin, Ritland & Myers 2011). I selected a wide temperature range of 16, 24 and 32°C to capture the geographic and seasonal variation that would be experienced by different *T. ni* populations.

4.3. Materials and Methods

4.3.1. Insects and viruses

Eggs of *Trichoplusia ni* were obtained from a laboratory colony maintained at Simon Fraser University at 25°C and L16:D8 photoperiod on a standard wheat-germ based artificial diet. Larvae are routinely reared in 160 ml Styrofoam cups (Dixie) at a density of 14 larvae per cup. Inbreeding was minimized by mating approximately 200 moths each generation. Eggs and pupae were surface sterilized with 0.2% and 1% bleach respectively every generation. To minimize density effects on larval development and virus resistance (Goulson & Cory 1995), larvae used for the experiment were reared individually from egg-hatch until the late fourth instar in 32-well plastic insect rearing trays (BioServ).

The AcMNPV strain E2 and the TnSNPV strain FV#34 were both obtained from Dr. Martin Erlandson (Agriculture and Agri-Food Canada, Saskatoon Research Centre). The TnSNPV strain was collected from greenhouse populations of *T. ni* in the Fraser Valley, British Columbia in 2000 (Janmaat & Myers 2003; Erlandson *et al.* 2007).

4.3.2. Experimental diets

Five chemically defined artificial diets were made following the protocol in Chapter 3. All five diets were isocaloric, such that the sum total of protein (casein) and digestible carbohydrate (sucrose) were kept constant at 60% of the dry weight of diet. The ratios of protein and digestible carbohydrate were manipulated as follows: 50% protein with 10% digestible carbohydrate (50:10), 40:20, 30:30, 20:40, 10:50. The wide range of protein to carbohydrate ratios was selected to capture realistic variability in *T. ni* host plants (described in Chapter 3).

4.3.3. Experimental protocol

Late fourth instar larvae that had initiated their moult were weighed to the nearest 0.1 mg and placed in individual wells of 24-well tissue culture plates without food. Upon completing their moult, 900 freshly moulted final (fifth) instar larvae were assigned to a control group or one of two NPV treatments (AcMNPV or TnSNPV). To challenge larvae with NPV, an equivalent number of infectious units (360 occlusion bodies suspended in 2 µl of distilled water) of AcMNPV or TnSNPV were applied to a 3 x 2 mm plug of wheat-germ based artificial diet. Virus was replaced with 2 µl of distilled water for the untreated control insects. Each larva was given 24 hrs to consume the entire diet plug.

Virus-challenged and control larvae were then placed in individual 30 ml plastic cups and provided with a block of one of five treatment diets (approx. 0.7 g). These larvae were reared at one of three constant temperature regimes (16, 24 or 32°C), yielding a combination of 45 treatments. Diet blocks were replaced every 24 hrs until pupation to minimize desiccation. The stadium duration of each larva was recorded every 12 hrs. Immediately after a hard pupal case was formed, pupae were collected and dried in a desiccating oven at 50°C for 48 hours, and then weighed to the nearest 0.1 mg. Insect performance was estimated by multiplying growth rate (dry pupal weight divided by development time) and proportionate survival (Lee *et al.* 2006b; Lee & Roh 2010).

Larvae that were challenged with NPV were monitored every 12 hrs for death. Larvae that died or were near death and had stopped feeding were weighed to the

nearest 0.1 mg and placed in individual 2 ml centrifuge tubes. If the larva was still alive, a small piece of kimwipe was placed under the lid such that the lid would not close fully, allowing for ventilation. Upon death, all cadavers were stored at -20°C. To count NPV yield, cadavers were macerated for 2 min each in their tubes using a plastic pestle. Distilled water was added to the macerated cadaver to produce a 1 ml suspension. From this suspension, 1:100 or 1:1000 dilutions were made and virus yield was counted using an improved Neubauer brightline haemocytometer (0.1 mm deep; Hausser Scientific) at 400x magnification. The diluted suspension was allowed to settle for 2 min in the haemocytometer before counting OBs. If no OBs were visible at the 1:100 dilution, the virus yield was considered to be zero and death of the larva was considered not a result of virus infection. A 1:10 dilution contained too much debris to search for OBs. Virus performance was estimated by multiplying proportion of hosts killed and OB yield.

4.3.4. Statistical analyses

Measures of *T. ni* pupal weight, development time and performance were analyzed by analyses of covariance (ANCOVA). Larval survival was analyzed using a generalized linear model (GLM) with a binomial error structure and a logit link function. Temperature and virus treatment were included as factors, diet (P:C ratio) as both linear and quadratic terms was included as an independent variable, and initial larval mass and sex were included as covariates. Two larvae did not feed and were excluded from analyses. For speed of kill by AcMNPV and TnSNPV, ANCOVA was performed with virus species and temperature as factors, initial larval mass as a covariate, and diet as only a linear term since the patterns were clearly linear. Due to the dramatically higher OB yield per cadaver for TnSNPV compared to AcMNPV, measures that were calculated using OB yield (OB yield, efficiency, rate of production and performance) were analyzed separately for the two NPVs. Therefore, ANCOVAs were performed with temperature as a factor, initial mass as a covariate, and diet as both linear and quadratic terms. Data were transformed where necessary to meet the assumptions for ANCOVA and differences between means were compared by Tukey HSD test. For all ANCOVA and GLM analyses, non-significant interaction terms were removed sequentially to produce the final minimal model using JMP 10 (SAS Institute, 2010, Cary, NC, USA). Non-

parametric thin plate splines were used to fit a three-dimensional response surface of the bivariate effects of temperature and dietary P:C ratio on insect performance using the fields package in R (version 3.0.1). I used this non-parametric technique for illustrative purposes as it does not constrain the shape of the surface (Lee *et al.* 2008a; Lee & Roh 2010).

4.4. Results

4.4.1. Insect performance

Survival. Virus challenge with 360 OBs of TnSNPV or AcMNPV significantly lowered the survival of larvae to pupation (*virus treatment*: $X^2_{2,885}$=79.17, P<0.0001; Figure 4.1A). There was no difference in host mortality after challenge by the two viruses (pairwise contrast: X^2_1=2.78, P=0.10). Average mortality, across temperature and host dietary P:C ratios, caused by AcMNPV was 42.0 ± 2.4% and 37.3 ± 3.4% for TnSNPV. Temperature did not affect survival (*temperature*: $X^2_{2,885}$=1.67, P=0.43), and did not influence resistance to virus (*temperature*virus treatment*: $X^2_{4,877}$=3.60, P=0.46). Larval resistance to virus-challenge increased nonlinearly as the dietary P:C ratio changed from carbohydrate-rich to protein-rich (*diet*: $X^2_{1,885}$=15.23, P<0.0001; *diet*^{2}: $X^2_{1,885}$=10.35, P=0.001). Resistance to AcMNPV rose steadily as the P:C ratio increased and peaked at 50p:10c, while resistance to TnSNPV rose more rapidly with increased protein and reached a plateau at 40p:20c. Survival of control larvae decreased as the P:C ratio became increasingly unbalanced (*virus treatment*diet*: $X^2_{2,885}$=1.08, P=0.58; *virus treatment*diet*^{2}: $X^2_{2,885}$=9.37, P=0.009). Diet did not interact with temperature (*temperature*diet*: $X^2_{2,883}$=4.72, P=0.09; *temperature*diet*^{2}: $X^2_{2,881}$=3.57, P=0.17) and there were no 3-way interactions (*temperature*virus treatment*diet*: $X^2_{4,873}$=1.66, P=0.80; *temperature*virus treatment*diet*^{2}: $X^2_{4,869}$=3.84, P=0.43). Heavier larvae at the start of the experiment were more likely to survive (*initial larval mass*: $X^2_{1,885}$=5.27, P=0.02), and sex did not influence the outcome (*sex*: $X^2_{1,885}$=0.37, P=0.54).

Development time. Surviving larvae reached pupation faster as temperature increased (*temperature*: $F_{2,627}$=3096.70, P<0.0001; Figure 4.1B), and this was not affected by virus challenge (*virus treatment*: $F_{2,627}$=1.73, P=0.18; *temperature*virus*

treatment: $F_{4,615}$=1.30, P=0.27). P:C ratio affected development time nonlinearly such that it was fastest between the 40p:20c and 30p:30c diets, and took longer as the P:C ratio became increasingly unbalanced; more so as the diet became carbohydrate-rich (*diet*: $F_{1,627}$=101.45, P<0.0001; *diet*^{2}: $F_{1,627}$=219.50, P<0.0001). The effect of diet on development time was not influenced by virus challenge (*virus treatment*diet*: $F_{2,625}$=1.27, P=0.28; *virus treatment*diet*^{2}: $F_{2,623}$=2.68, P=0.07) or temperature (*temperature*diet*: $F_{2,621}$=2.41, P=0.09; *temperature*diet*^{2}: $F_{2,619}$=1.17, P=0.31), and there were no 3-way interactions (*temperature*virus treatment*diet*: $F_{4,611}$=0.78, P=0.54; *temperature*virus treatment*diet*^{2}: $F_{4,607}$=1.48, P=0.43). Females pupated earlier than males (*sex*: $F_{1,627}$=69.22; P<0.0001), and larvae that started heavier pupated earlier (*initial mass*: $F_{1,627}$=51.91; P<0.0001).

Pupal weight. A cost of surviving TnSNPV-challenge was evident as pupal weight was significantly lower than control pupae (*pairwise contrast:* $F_{1,619}$=15.55, P<0.0001; *virus treatment*: $F_{2,619}$=7.81, P<0.001; Figure 4.1C). However, pupae of AcMNPV-challenged *T. ni* were not significantly different from controls (*pairwise contrast:* $F_{1,619}$=1.70, P=0.19). Pupal weight increased significantly as temperature cooled (*temperature*: $F_{2,619}$=55.11, P<0.0001; Figure 4.1D), while P:C ratio of the diet influenced pupal weight nonlinearly (*diet*: $F_{1,619}$=49.90, P<0.0001; *diet*^{2}: $F_{1,619}$=48.92, P<0.0001). At 16 and 24°C, pupal weight peaked on the balanced diet and declined as the P:C ratio became increasingly unbalanced. However, at 32°C, pupal weight increased from protein-rich to carbohydrate-rich and peaked at 10p:50c (*temperature*diet*: $F_{2,619}$=3.87, P=0.02; *temperature*diet*^{2}: $F_{2,619}$=15.05, P<0.0001; Figure 4.1D). Virus challenge also influenced the nonlinear effect of diet. Pupae from all three treatments had similar weights on the most protein-rich diet. However, the weights of control and AcMNPV-challenged pupae increased sharply until reaching a peak at 30p:30c, while TnSNPV-challenged pupae increased more gradually until reaching a plateau and similar pupal weights as control and AcMNPV-challenged pupae on the two carbohydrate-rich diets. Therefore, the cost of lower pupal weight of TnSNPV occurred primarily on the 40p:20c and 30p:30c diets (*treatment*diet*: $F_{2,619}$=0.36, P=0.70; *treatment*diet*^{2}: $F_{2,619}$=3.14, P=0.04; Figure 4.1C). There was no interaction between temperature and virus challenge (*temperature*virus treatment*: $F_{4,615}$=1.82, P=0.12), and no 3-way interactions (*temperature*virus treatment*diet*: $F_{4,611}$=0.29, P=0.88;

*temperature*virus treatment*diet*^{2}: $F_{4,607}$=1.55, P=0.19). Male pupae were heavier than female (*sex*: $F_{1,619}$=21.93; P<0.0001), and heavier larvae at the start of the experiment achieved heavier pupal weight (*initial mass*: $F_{1,619}$=63.02; P<0.0001).

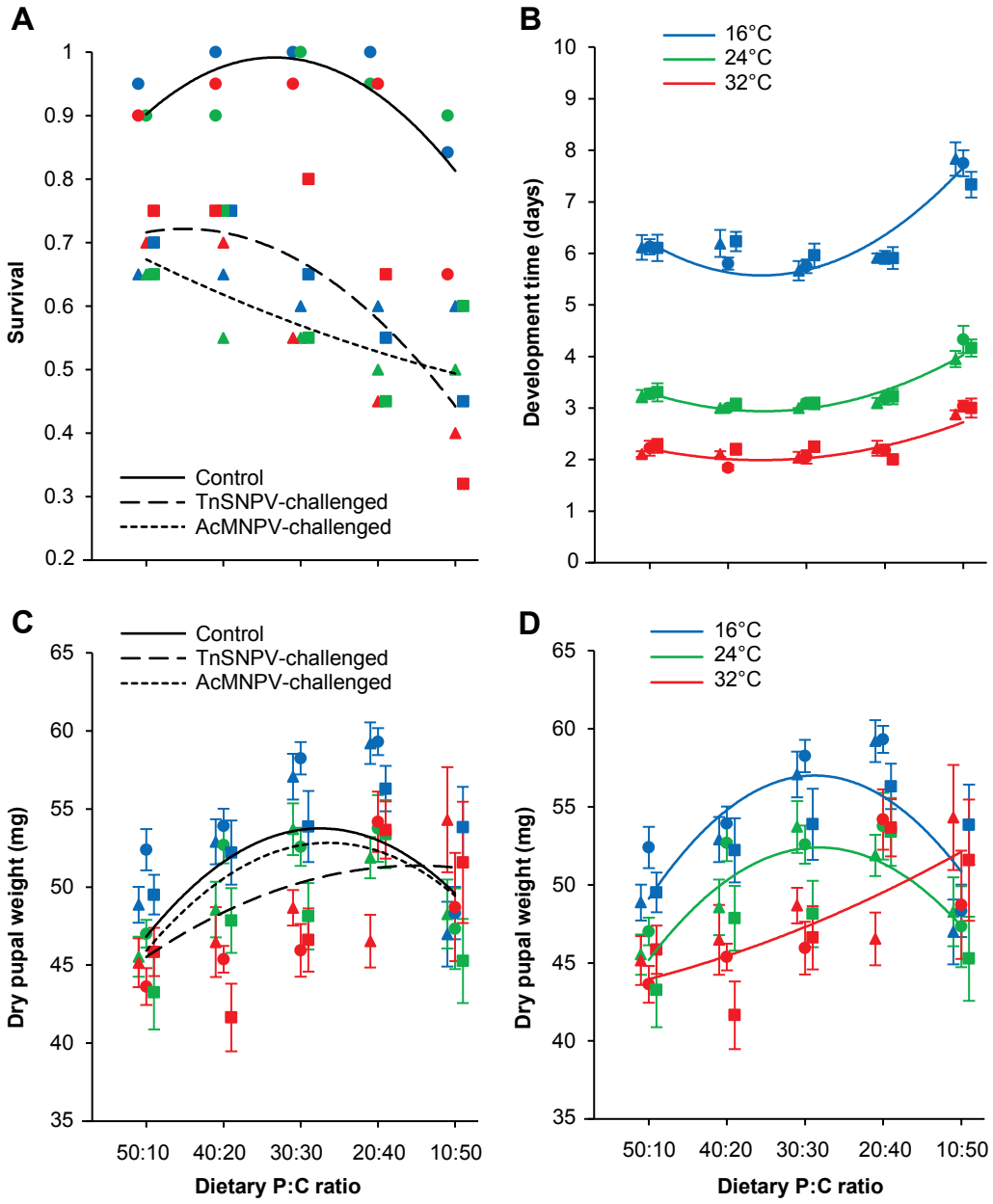

Figure 4.1. Effects of NPV challenge, temperature and nutrition on host development.

The effects of NPV challenge and simultaneous manipulations of temperature and dietary P:C ratio on *Trichoplusia ni* (A) survival, (B) days to pupation (±SE) and (C, D) dry pupal weight (±SE). Data points for control (circle), AcMNPV-challenged (triangle) and TnSNPV-challenged (square) are jittered to prevent overlap. Lines represent the fitted minimal models and have not been jittered.

Performance. Larval performance decreased with virus challenge relative to the control (*treatment*: $F_{2,607}$=213.86, P<0.0001; Figure 4.2 and 4.3), and increased with temperature (*temperature*: $F_{2,607}$=339.31, P<0.0001). Larvae challenged by TnSNPV or AcMNPV achieved equal performance at 16 and 24°C. However, at 32°C, AcMNPV-challenged larvae had significantly lower performance than TnSNPV-challenged larvae (*pairwise contrast:* $F_{1,607}$=25.26, P<0.0001; *temperature*treatment*: $F_{4,607}$=6.88, P<0.0001). Performance was affected nonlinearly by dietary P:C ratio (*diet*: $F_{1,607}$=98.87, P<0.0001; *diet*^{2}: $F_{1,607}$=231.35, P<0.0001), and this effect was strongly influenced by both virus challenge (*treatment*diet*: $F_{2,607}$=6.49, P=0.002; *treatment*diet*^{2}: $F_{2,607}$=15.02, P<0.0001) and temperature (*temperature*diet*: $F_{2,607}$=11.95, P<0.0001; *temperature*diet*^{2}: $F_{2,607}$=6.78, P=0.001) such that there was a 3-way interaction between diet^2, treatment and temperature (*temperature*treatment*diet*: $F_{4,607}$=2.32, P=0.06; *temperature*treatment*diet*^{2}: $F_{4,607}$=2.56, P=0.04). The differences in optimal performance are clearly presented in the three-dimensional plot of dietary P:C ratio against temperature, separately constructed for each treatment (Figure 4.2). Peak performance at each temperature for control larvae were consistently on the balanced 30p:30c. Challenge with TnSNPV pushed the P:C ratio of peak performance slightly more protein-biased at all temperatures relative to control larvae. In contrast, temperature strongly affected AcMNPV-challenged larvae such that at 32°C peak performance was achieved at the extremely protein-biased ratio and steadily shifted to a more balanced P:C ratio as temperature declined to 16°C. Heavier larvae at the start of the experiment performed better (*initial larval mass*: $F_{1,607}$=81.96, P<0.0001), and females outperformed males (*sex*: $F_{1,607}$=6.57, P=0.01).

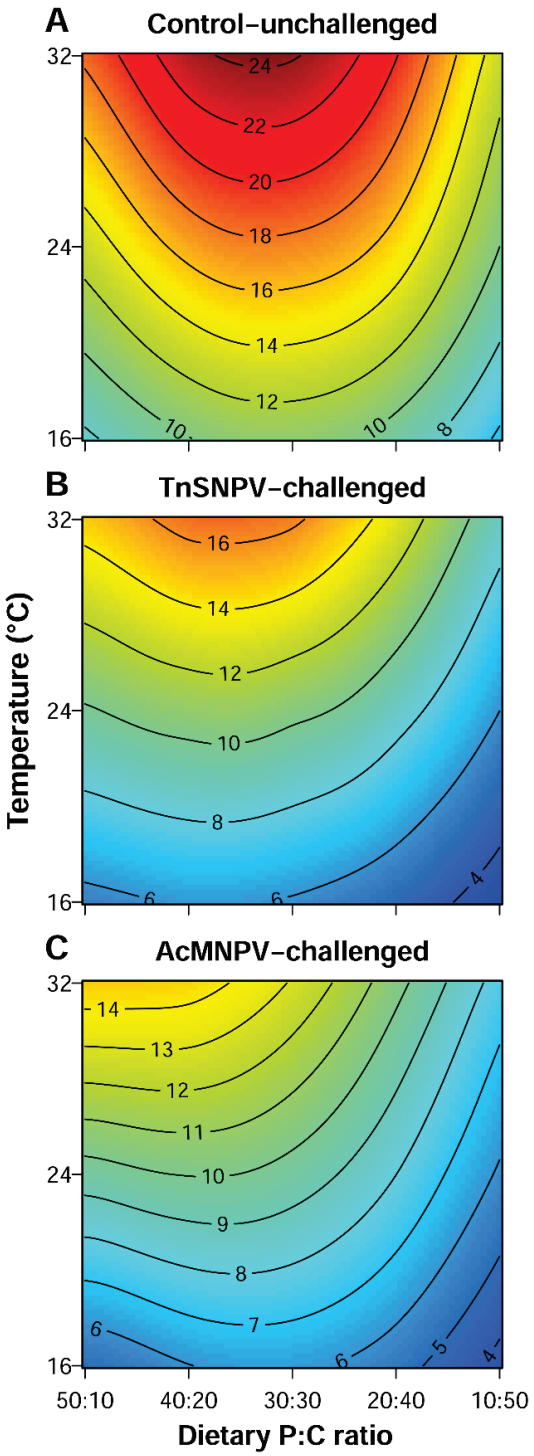

Figure 4.2. Response surface of the effects of NPV challenge, temperature and nutrition on host performance.

Response surface fitted using non-parametric thin-plate splines illustrating the bivariate effects of temperature and P:C ratio on *Trichoplusia ni* performance after (A) no virus challenge, (B) TnSNPV challenge and (C) AcMNPV challenge. Performance was calculated by multiplying growth rate (dry pupal weight divided by days to pupation) and survival.

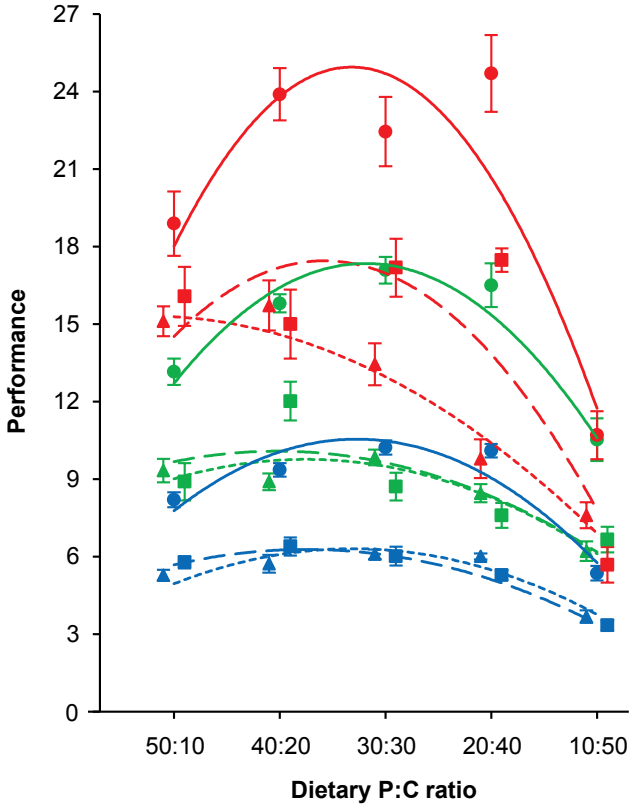

Figure 4.3. Effects of NPV challenge, temperature and nutrition on host performance.

Effects of temperature and P:C ratio on *Trichoplusia ni* performance after no virus challenge (circle), TnSNPV challenge (square) and AcMNPV challenge (triangle). Performance was calculated by multiplying growth rate (dry pupal weight divided by days to pupation) and survival. Data points are jittered to prevent overlap. Lines represent the fitted minimal models for control (solid), TnSNPV-challenged (long dashed) and AcMNPV-challenged (short dashed) and have not been jittered. Colours represent temperatures: blue, 16°C; green, 24°C; red, 32°C.

4.4.2. Virus performance

Speed of kill. AcMNPV and TnSNPV took the same amount of time to kill their hosts (*virus*: $F_{1,214}$=0.15, P=0.70; Figure 4.4), and killed significantly faster as the temperature increased (*temperature*: $F_{1,214}$=826.15, P<0.0001). P:C ratio of the host's diet also had an effect (*diet*: $F_{1,214}$=23.91, P<0.0001), such that at 16°C both viruses took longer to kill as the P:C ratio decreased, while no differences across P:C ratios were observed at 24 and 32°C (*temperature*diet*: $F_{2,214}$=6.71, P=0.002). There was no interaction between virus and temperature (*virus*temperature*: $F_{2,212}$=1.14, P=0.32), virus and diet (*virus*diet*: $F_{1,211}$=0.004, P=0.95), or three-way interaction

(*temperature*virus*diet*: $F_{2,209}$=1.06, *P*=0.35), and the mass of larvae at the time of virus challenge did not affect speed of kill (*initial mass*: $F_{1,214}$=0.32, *P*=0.57).

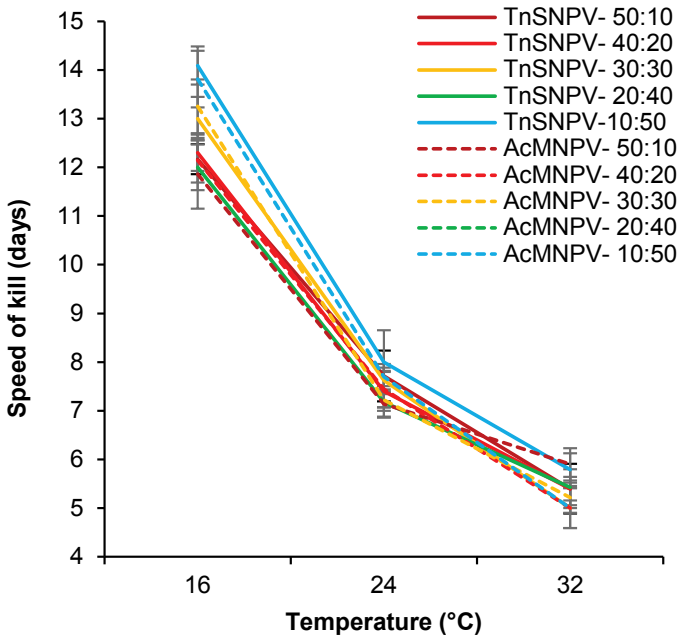

Figure 4.4. Effects of temperature and host nutrition on speed of kill by NPVs.
Effects of rearing temperature and dietary P:C ratio on the speed of kill (days ± SE) of orally inoculated *Trichoplusia ni* larvae by TnSNPV and AcMNPV.

OB yield per cadaver. Analyses were performed separately for each virus as TnSNPV produced approximately 5-fold more OBs in its hosts than AcMNPV. OB yield of TnSNPV was strongly affected by temperature, producing significantly more at 24°C than at 16 and 32°C (*temperature*: $F_{2,104}$=8.85, *P*<0.001; Figure 4.5A). Yield tended to peak on the most balanced dietary P:C ratio of the host and was skewed such that more OBs were produced at higher P:C ratios than lower ratios (*diet*: $F_{1,104}$=63.18, *P*<0.0001; *diet*^{2}: $F_{1,104}$=24.58, *P*<0.0001). This pattern was consistent across temperatures (*temperature*diet*: $F_{2,102}$=2.34, *P*=0.10; *temperature*diet*^{2}: $F_{2,100}$=1.78, *P*=0.17). In contrast, AcMNPV yield was equally high at 16 and 24°C, and significantly lower at 32°C (*temperature*: $F_{2,106}$=6.80, *P*=0.002; Figure 4.5D). AcMNPV OB yield changed nonlinearly with P:C ratio as more OBs were produced at protein-rich and balanced P:C ratios compared to carbohydrate-rich (*diet*: $F_{1,106}$=16.75, *P*<0.0001; *diet*^{2}: $F_{1,106}$=10.02, *P*=0.002). Unlike TnSNPV, OB yield did not peak at any one P:C ratio, and this pattern was not altered by temperature (*temperature*diet*: $F_{2,104}$=0.06, *P*=0.94;

*temperature*diet*^{2}: $F_{2,102}$=1.03, P=0.36). OB yields were not affected by the larval mass at the time of challenge (*initial mass*: TnSNPV, $F_{1,104}$=0.39, P=0.53; AcMNPV, $F_{1,106}$=1.08, P=0.30).

Virus efficiency. The production of OBs per unit host tissue by TnSNPV was significantly greater at 24°C than at 16 or 32°C (*temperature*: $F_{2,102}$=10.07, P=0.0001; Figure 4.5B). Efficiency increased nonlinearly with P:C ratio of the host diet, and tended to plateau as the diet became balanced and protein-rich (*diet*: $F_{1,102}$=6.65, P=0.01; *diet*^{2}: $F_{1,102}$=9.12, P=0.003). The magnitude with which efficiency improved with P:C ratio was greater at 24 and 32°C compared to 16°C (*temperature*diet*: $F_{2,102}$=0.52, P=0.01; *temperature*diet*^{2}: $F_{2,100}$=2.07, P=0.13). Conversely, AcMNPV had equally high efficiency at 16 and 24°C, and lower at 32°C (*temperature*: $F_{2,106}$=7.59, P<0.001; Figure 4.5E). Like TnSNPV, efficiency improved with increasing P:C ratio, reaching a plateau as the host's diet became balanced and protein-rich (*diet*: $F_{1,106}$=15.88, P<0.001; *diet*^{2}: $F_{1,106}$=7.20, P<0.01). However, unlike TnSNPV, the magnitude with which efficiency improved with P:C ratio was consistent across temperatures (*temperature*diet*: $F_{2,104}$=0.03, P=0.97; *temperature*diet*^{2}: $F_{2,102}$=0.62, P=0.54). There was no effect of larval initial mass (*initial mass*: TnSNPV, $F_{1,102}$=0.44, P=0.51; AcMNPV, $F_{1,106}$=0.0005, P=0.98).

Average virus production per day. TnSNPV had equally high OB production per day at 32 and 24°C, and significantly lower at 16°C (*temperature*: $F_{2,102}$=15.72, P<0.0001; Figure 4.5C). OB production was fastest on the most balanced host diet, and slowed as the diet became unbalanced; more so as P:C ratio declined (*diet*: $F_{1,102}$=10.09, P=0.002; *diet*^{2}: $F_{1,102}$=25.23, P<0.0001). OB production per day on the most carbohydrate-rich diet was similarly low across temperatures, but at 24 and 32°C increased more with increasing P:C ratio (*temperature*diet*: $F_{2,102}$=4.96, P=0.009; *temperature*diet*^{2}: $F_{2,100}$=2.78, P=0.07). AcMNPV also had equally high production per day at 32 and 24°C, and significantly lower at 16°C (*temperature*: $F_{2,106}$=15.30, P<0.0001; Figure 4.5F). Unlike TnSNPV, the production per day was consistent across four higher dietary P:C ratios and dropped on the most carbohydrate-rich diet (*diet*: $F_{1,106}$=17.24, P<0.0001; *diet*^{2}: $F_{1,106}$=9.49, P=0.003). Furthermore, the change in production per day with P:C ratio was consistent across temperatures (*temperature*diet*:

$F_{2,104}$=0.15, P=0.87; *temperature*diet*^{2}: $F_{2,102}$=1.18, P=0.31). OB production per day was not affected by larval mass at the time of challenge (*initial mass*: TnSNPV, $F_{1,102}$=0.25, P=0.62; AcMNPV, $F_{1,106}$=0.30, P=0.58).

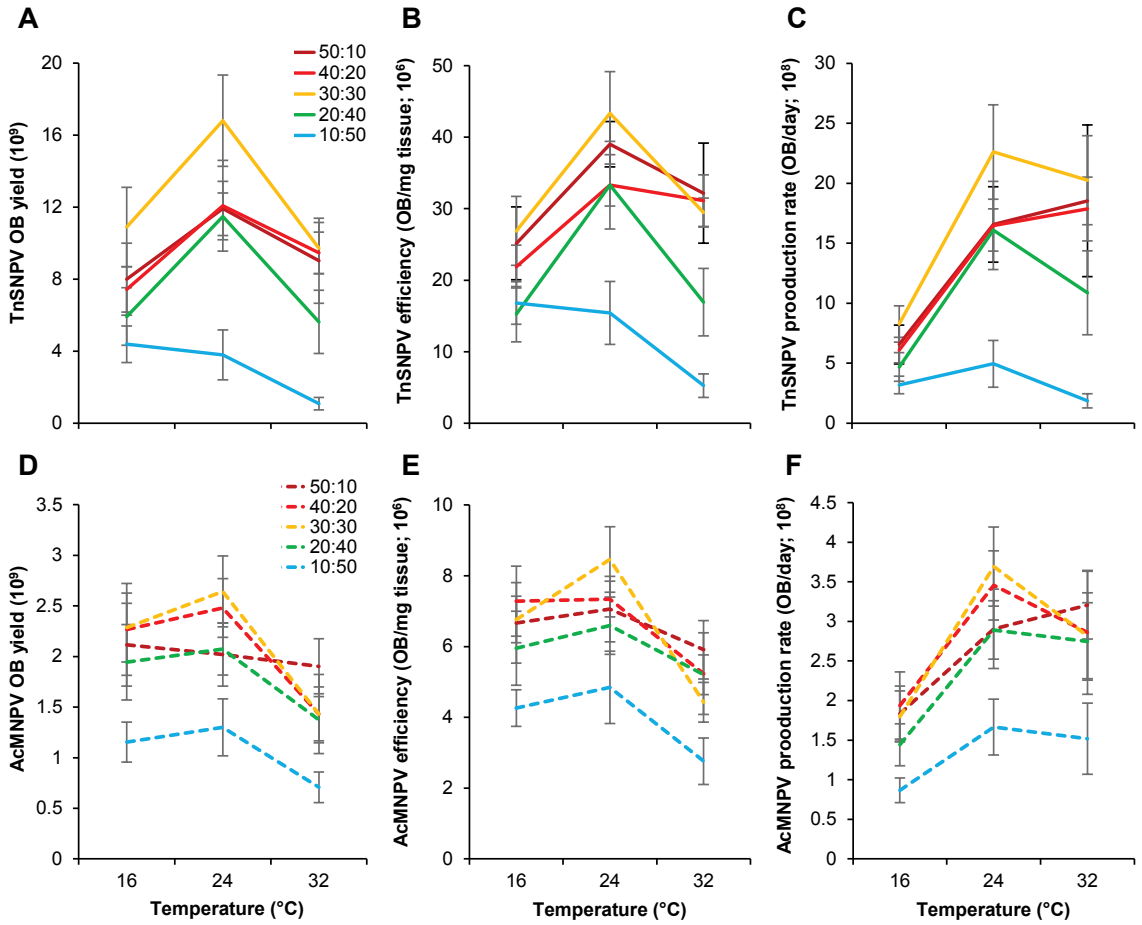

Figure 4.5. Effects of temperature and host nutrition on NPV productivity.
Effects of host rearing temperature and dietary P:C ratio on the mean (±SE) (A, D) occlusion body (OB) yield, (B, E) efficiency of OB production and (C, F) OB production rate of TnSNPV and AcMNPV respectively.

Virus performance. TnSNPV performance was significantly higher at 24°C than at 16 and 32°C, by more than 2.3 and 3.1 fold respectively (*temperature*: $F_{2,100}$=13.27, P<0.0001; Figure 4.6A). P:C ratio had no main effect on performance (*diet*: $F_{1,100}$=0.41, P=0.52; *diet*^{2}: $F_{1,100}$=0.91, P=0.34) but interacted with temperature (*temperature*diet*: $F_{2,100}$=3.23, P=0.04; *temperature*diet*^{2}: $F_{2,100}$=3.37, P=0.04). Performance was similar across P:C ratios at 16°C peaking only slightly at a balanced ratio, whereas at 32°C performance was the same at the four higher P:C ratios and declined on the most

carbohydrate-rich diet. At 24°C, where performance was highest, there was a clear optimum as performance on the 30p:30c and 20p:40c diets peaked dramatically above any other temperature and diet combinations. On the other hand, performance of AcMNPV was significantly higher at 24°C than at 16°C, and significantly higher at 16°C at 32°C (*temperature*: $F_{2,106}$=19.51, P<0.0001; Figure 4.6B). In contrast to TnSNPV, the differences in AcMNPV performance at 24°C compared to 16 and 32°C were considerably smaller (1.2 and 1.9 fold respectively). While TnSNPV performance showed a clear optimum, AcMNPV performance was relatively more consistent across diets, such that performance was equally high across the four higher P:C ratios while declining on the 10p:50c diet (*diet*: $F_{1,106}$=5.31, P=0.02; *diet*^{2}: $F_{1,106}$=19.50, P<0.0001). This pattern was consistent at each temperature (*temperature*diet*: $F_{2,104}$=0.15, P=0.86; *temperature*diet*^{2}: $F_{2,102}$=1.23, P=0.30). Performance was not affected by larval weight at the time of challenge (*initial mass*: TnSNPV, $F_{1,100}$=0.26, P=0.61; AcMNPV, $F_{1,106}$=1.41, P=0.24).

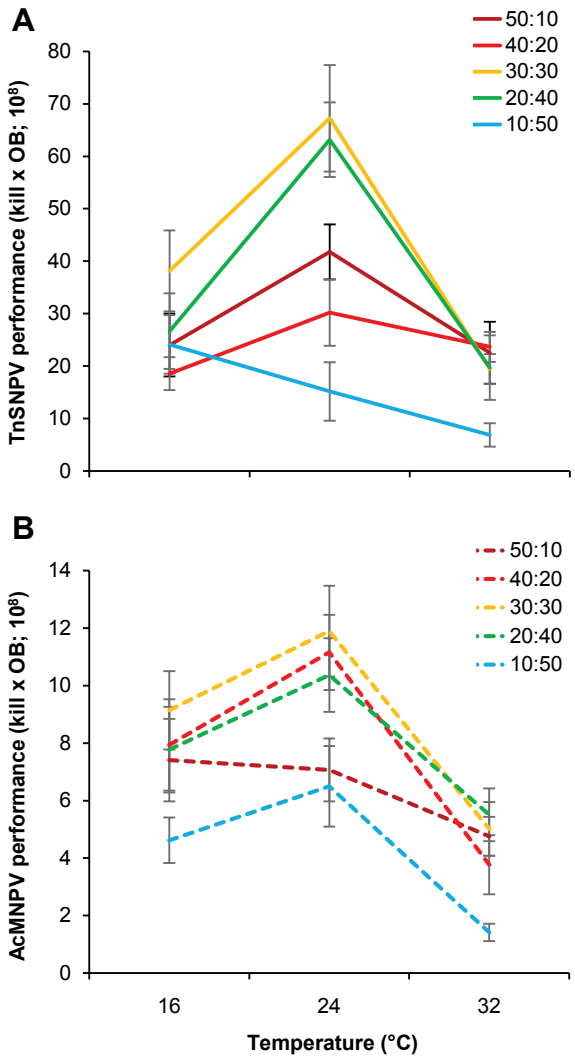

Figure 4.6. Effects of temperature and host nutrition on NPV performance. Effects of host rearing temperature and dietary P:C ratio on the performance (±SE) of (A) TnSNPV and (B) AcMNPV. Performance was calculated by multiplying the proportion of hosts killed and OB yield.

4.5. Discussion

4.5.1. Host performance

My results show that host performance varied with P:C ratio, temperature and virus-challenge. Peak performance by unchallenged *T. ni* was achieved at relatively balanced P:C ratios, and performance increased as a result of faster development with

temperature. As expected, virus challenge significantly reduced host survival and hence performance. Challenge by the 'narrow host range virus' (TnSNPV) caused peak host performance to shift slightly more protein-biased (between 40p:20c and 30p:30c) at each temperature. In contrast, challenge by the 'broad host range virus' (AcMNPV) shifted peak host performance to extremely protein biased P:C ratios at 32°C, but only slightly at 24°C and not at all at 16°C. The shift of peak performance to a more protein-biased P:C ratios after viral-challenge was due to greater survival, indicating that host resistance against virus challenge depends more on protein acquisition than energy. This is consistent with findings in other lepidopteran species that have shown higher disease resistance in larvae feeding on higher P:C ratio diets post-pathogen challenge (Lee *et al.* 2006b; Povey *et al.* 2009, 2014). My study demonstrates that optimal P:C intake is influenced by both temperature and virus identity. It also highlights the importance of examining a range of values, as this may indicate different host responses over a host's geographical range or different seasons. I did not investigate the mechanisms behind these effects, but they could be attributable to differences in infection strategies and temperature tolerance of the viruses.

There was a significant cost associated with surviving TnSNPV challenge, as measured by reduced pupal weight, but not AcMNPV challenge. This difference might result from different infection strategies of the multiple nucleocapsid (MNPV) and single nucleocapsid (SNPV) phenotypes. For instance, comparing occlusion-derived virus (ODV) fractions enriched for the MNPV or SNPV phenotypes of AcMNPV revealed that while SNPV established more primary midgut cell infections than MNPV, MNPV infected tracheal cells more quickly and efficiently to initiate a systemic infection (Washburn *et al.* 1995). Since one of the key defenses against NPVs is the sloughing of infected midgut cells (Engelhard & Volkman 1995), the higher establishment of midgut infection foci and slower infection of tracheal cells by SNPV may result in higher rates and longer periods of midgut sloughing. Therefore, the reduced pupal weight associated with TnSNPV challenge could be a cost of replacing sloughed midgut cells or result from impaired nutrient absorption due to midgut sloughing. Another possibility is that *T. ni* may have evolved a stronger immune response to TnSNPV challenge. As the vast majority of *T. ni* cadavers collected from multiple greenhouses and field sites contained TnSNPV compared to only a few that contained AcMNPV (Jaques 1970; Erlandson *et al.* 2007),

T. ni and TnSNPV likely share a closer evolutionary relationship. A recent study showed that *S. exigua* cells infected by its specialist virus *Spodoptera exigua* MNPV (SeMNPV) had 38 immune-related transcripts differentially up-regulated 36 hours after infection, but none were up-regulated after AcMNPV infection (Jakubowska, Vogel & Herrero 2013). Since the activation of the immune system can be costly (Siva-Jothy & Thompson 2002; Schmid-Hempel 2005a; Sadd & Siva-Jothy 2006; McKean *et al.* 2008), the potential difference in the level of immune activation to the two viruses might explain the higher cost of surviving TnSNPV-challenge.

T. ni reached pupation more quickly with increasing temperature at a cost of significantly lower pupal weight. My results are in line with commonly observed phenotypic plasticity responses to temperature termed the temperature-size rule, where species achieve a smaller size at higher developmental temperatures, potentially through thermal sensitivity of growth rates and smaller cell size (Atkinson & Sibly 1997; Kingsolver & Huey 2008). Lower pupal weights at higher temperature could also be due to reduced lipid storage for energy since more energy would be used to fuel the increased basal metabolic rate associated with rapid growth (Lee & Roh 2010). This change in energy allocation might explain the increase in pupal weights at 32°C with increasing carbohydrate in the diet, since there would be more energy from ingested carbohydrate to allocate to both metabolism and lipid storage (Chapter 3).

4.5.2. Virus performance

Disease progression is strongly affected by temperature (Ribeiro & Pavan 1994; Frid & Myers 2002), primarily because pathogen replication is expected to increase as a function of host growth rate (van Beek *et al.* 2000). I found that increasing temperature after virus challenge did not affect host mortality but shortened speed of kill by both viruses, consistent with the thermal ecology of the western tent caterpillar and its NPV (Frid & Myers 2002). Infectivity of the budded form of AcMNPV, which spreads infection between cells, is known to be unaffected by exposure to high temperatures up to 45°C (Michalsky *et al.* 2008). The temperature tolerance of TnSNPV budded virions is not known, but is also likely to be high based on my findings. As expected, the more specialized TnSNPV was far more productive than AcMNPV in *T. ni* larvae, producing

approximately 5-fold more OBs. This likely occurred in part because TnSNPV OBs are much smaller than AcMNPV OBs (observation), typical of the differences between the SNPV and MNPV phenotypes that package a single or multiple nucleocapsids within a single ODV envelope, respectively (Washburn *et al.* 1999, 2003). TnSNPV showed a more restricted temperature range as both OB yield and efficiency of OB production peaked at only 24°C, whereas AcMNPV had a broader peak (16 and 24°C). For NPVs, negative genetic correlations between the speed of kill and yield of OB production often produce trade-offs. The longer the NPV takes to kill the host the more OBs are produced, with yield often reaching a plateau (Hernández-Crespo *et al.* 2001; Hodgson *et al.* 2001). Longer time to kill by AcMNPV with declining temperature resulted in a plateau for OB yield at 16 and 24°C, consistent with a trade-off. This was not the case for TnSNPV.

The rate of growth of *T. ni* and *in vivo* rate of increase of AcMNPV OBs were previously shown to increase linearly with temperature, such that temperature had no effect on the balance between the growth of *T. ni* larvae and growth of AcMNPV (van Beek *et al.* 2000). However, my study suggests a more complex thermal relationship between *T. ni* and NPV growth. While host growth rate (pupal weight divided by development time) increased with temperature, the average daily production of OBs for both AcMNPV and TnSNPV reached a maximum rate at 24°C and reached a plateau (same rate at 32°C). As both NPVs killed their hosts more quickly at 32°C, they consequently produced fewer OBs per killed host.

Optimal performance of TnSNPV was strongly influenced by both temperature and host diet. There was a distinct peak in performance at 30p:30c and 20p:40c at 24°C, supportive of the specialized nature of TnSNPV. The optimal P:C range for TnSNPV was slightly more carbohydrate-biased than for unchallenged hosts, while the optimal P:C ratio for TnSNPV-challenged hosts was slightly more protein-biased than unchallenged hosts. Thus, the optimal P:C range for TnSNPV and TnSNPV-challenged hosts shifted in opposite directions from unchallenged hosts. This carbohydrate-biased optimal P:C range for TnSNPV might reflect an ideal balance between a compromised host immune system, resulting from lower protein availability, and the quality of the host as a nutritive resource for the virus (Bize *et al.* 2008). Performance of AcMNPV was

less variable across temperatures and P:C ratios than TnSNPV. The ability of AcMNPV to perform more consistently with variable host environments is likely attributable to their ability to infect numerous insect species that vary in geographic distribution and diet breadth. For example, lepidopteran larvae which are known hosts of AcMNPV self-compose optimal dietary P:C ratios as high as 4p:1c and as low as 0.8p:1c (Behmer 2009; Shikano & Cory 2014).

The generalism of AcMNPV and specificity of TnSNPV in host range are accompanied by trade-offs. The advantage of specificity is that the NPV should be adapted to use its host more efficiently, whereas generalism will increase the availability of hosts. Specificity, however, is accompanied by a greater risk of extinction in unpredictable or patchy environments, while generalists will make poorer use of their hosts (Straub, Ives & Gratton 2011). I found similar trade-offs with my two NPVs infecting the same host species under different environmental conditions. While TnSNPV showed greater productivity in its *T. ni* host, AcMNPV was less affected by environmental variability. However, it is important to keep in mind that there are likely more differences between TnSNPV and AcMNPV than just host range and nucleocapsid packaging, as the TnSNPV genome is 30kb larger than AcMNPV (Fielding *et al.* 2002). For instance, evolved resistance to TnSNPV in *T. ni* resulted in little cross-resistance to AcMNPV, suggesting significant genetic variation and different routes of infection (Milks & Myers 2003).

4.5.3. Conclusions

Temperature and dietary nutrients had dramatic effects on host development and consequently affected its interactions with viruses. The two virus species showed differential productivity when invading hosts in variable environmental conditions. Important limitations of the present study are the use of artificial diets and constant temperatures. Daily temperatures can fluctuate considerably in nature, and it has been shown that the temperature extremes during fluctuation diminish the performance of the butterfly, *Pieris napi*, but not the means (Bauerfeind & Fischer 2013). However, I feel that my results highlight the importance of studying interactions of numerous environmental factors on host-pathogen relationships. In nature, organisms are likely to

encounter multiple pathogens and variable environments. Therefore, predicting outcomes of host-pathogen interactions based on single pathogens and individual environmental variables are likely not applicable. Interactions between environmental factors are likely to have significant consequences for the efficacy of viruses as biological control agents, and may also influence viral epizootics and population dynamics in nature.

4.6. Acknowledgements

I thank Martin Erlandson for providing the AcMNPV and TnSNPV. Financial support was provided by a Natural Sciences and Engineering Research Council of Canada (NSERC) Discovery Grant to J.S.C. and a NSERC Alexander Graham Bell Canada Graduate Scholarship-Doctoral, Graduate Fellowship and President's PhD Scholarship from Simon Fraser University to I.S.

4.7. References

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4.8. Connecting statement

In Chapter 4, I showed that nutrition interacted with temperature to influence within generation resistance of *T. ni* to two species of baculoviruses. Also, host nutrition and temperature had differential impacts on the productivity of the two baculoviruses. In Chapter 5, I focus on the impact of nutritional stress on trans-generational immune priming. Since both nutritional stress and resisting pathogen challenge can be costly, I looked for trade-offs between the parental transfer of nutritional stress tolerance to their offspring and trans-generational immune priming.

Chapter 5.

Trade-offs between trans-generational transfer of nutritional stress tolerance and immune priming

A modified version of this chapter has been submitted to the Journal of Animal Ecology: Shikano, I., Halpert-Scanderbeg, O., Oak, M.C. & Cory, J.S. Trade-offs between trans-generational transfer of nutritional stress tolerance and immune priming (JAE-2014-00223).

5.1. Abstract

Trans-generational effects are often assumed to have adaptive value as a driver of variation in offspring and parental fitness. The adaptive value of trans-generational immune priming (TGIP) depends on the match between the pathogen experienced by the parents and offspring. Similarly, nutritional stress can lead to the transfer of nutritional stress tolerance to offspring. Studies of trans-generational effects often focus on individual environmental variables. However, in nature, it is unlikely for one environmental factor to vary independently from others. Since reacting to a pathogen challenge and developing under nutritional stress are both costly, if encountered together, a trade-off between TGIP and the transfer of nutritional stress tolerance is expected. I altered the nutritional environment of both parents and offspring to examine how nutritional stress influences TGIP in the cabbage looper, *Trichoplusia ni*. I challenged parents with the bacterial pathogen *Bacillus thuringiensis* and measured TGIP by determining offspring resistance to the same pathogen and testing immune

activity. I assessed the costs associated with TGIP by measuring life-history traits for both parents and offspring, and the susceptibility of immune primed offspring to a different pathogen (baculovirus, TnSNPV). Lastly, I examined whether the induced phenotypic changes in the offspring were associated with altered egg size. TGIP was adaptive when offspring encountered the same pathogen experienced by their parents. However, parents suffered significant costs for TGIP, such as longer development time and reduced pupal weight, thereby decreasing parental fitness if the same pathogen is absent in the offspring environment. Interestingly, nutritional stress in the parents enhanced both nutritional stress tolerance of offspring and heightened resistance to both *B. thuringiensis* and TnSNPV. Elevated pathogen resistance was linked to increased egg sizes. There was a significant trade-off between TGIP and the transfer of nutritional stress tolerance such that nutritionally stressed parents transferred immunity but not nutritional stress tolerance. These results highlight trade-offs that can mediate trans-generational effects and illustrate the importance of assessing interactions between multiple environmental variables. At high population densities, disease risk increases and resources become depleted. Thus my findings could have significant implications for population dynamics.

5.2. Introduction

Trans-generational effects occur when the environment experienced by the parents determines offspring phenotype. These effects have an intrinsic time delay from parent to offspring that is believed to influence population dynamics because of the variability of environments over time (Beckerman *et al.* 2002; Plaistow *et al.* 2006; Tidbury, Best & Boots 2012). There is growing evidence for the prevalence of trans-generational effects in nature, and its implications for animal and human health. Such effects have recently garnered much attention from both evolutionary biologists and ecologists as it is becoming clear that they can involve indirect genetic effects (Wolf *et al.* 1998; Gómez-Díaz *et al.* 2012), and serve as an important mechanism for a multi-generational response to changing environments (Mousseau & Fox 1998; Beckerman *et al.* 2002). However, many evolutionary life-history studies often assume that trans-generational effects have adaptive value as a driver of variation in offspring and

maternal fitness without rigorously testing life-history or considering environmental context (Sheriff & Love 2013).

One type of trans-generational effect can occur when parental exposure to a pathogen acts as a cue to increase offspring immune defences; a phenomenon known as trans-generational immune priming (TGIP; Little *et al.* 2003; Sadd *et al.* 2005; Moret 2006). In vertebrates, offspring immunocompetence is often determined by the level of immune stimulation of their parents (Brinkhof *et al.* 1999), and is mediated by maternal antibody transfer to the offspring that provides early protection before the maturation of its own immune system (Carlier & Truyens 1995). Invertebrates have been assumed to have no capacity for memory in their response to pathogens because they lack the immune cells that are needed for the acquired immunity found in vertebrates (Arala-Chaves & Sequeira 2000; Janeway & Medzhitov 2002). However, heightened immune activity in the offspring of immune-challenged parents has been found in insect orders of Coleoptera (Moret 2006; Roth *et al.* 2010; Zanchi *et al.* 2011, 2012; Moreau *et al.* 2012), Hymenoptera (Sadd *et al.* 2005; Sadd & Schmid-Hempel 2007, 2009; Cisarovsky, Koch & Schmid-Hempel 2012) and Lepidoptera (Freitak, Heckel & Vogel 2009a; Freitak *et al.* 2009b; Tidbury *et al.* 2011; Trauer & Hilker 2013), and offspring are clearly more resistant to the same pathogen experienced by their parents (Rahman *et al.* 2004; Roth *et al.* 2009, 2010; Tidbury *et al.* 2011).

An individual's evolutionary fitness depends heavily on the effectiveness of its immune system. However, the level of investment into immune related traits can depend on the environment and parasite pressure experienced by the host (Sadd & Schmid-Hempel 2009) as life-history costs associated with immune defense create trade-offs between immunity and other traits (Moret & Schmid-Hempel 2000; Siva-Jothy & Thompson 2002; Sadd & Siva-Jothy 2006; Zanchi *et al.* 2012). Therefore, the heightened immunity in offspring acquired through TGIP should be maladaptive if infection risks do not persist beyond the parental generation (Sadd & Schmid-Hempel 2009). Fitness costs associated with TGIP in insects, such as increased susceptibility to an unrelated pathogen or reduced reproductive output, have been found in primed offspring (Sadd & Schmid-Hempel 2009; Roth *et al.* 2010; Zanchi *et al.* 2011; Trauer & Hilker 2013).

Immune investment can be costly and is likely to be influenced by other environmental factors such as nutritional stress, because fewer resources are available to invest in the immune system (Siva-Jothy & Thompson 2002; Myers *et al.* 2011; Triggs & Knell 2012). Nutritional stress, either by starvation or nutrient dilution, is also known to have trans-generational effects in invertebrates that improve offspring tolerance to nutritional stress (Plaistow *et al.* 2006; Vijendravarma *et al.* 2010; Hafer *et al.* 2011; Triggs & Knell 2012; Saastamoinen *et al.* 2013). It can be associated with significant costs such as reduced fecundity to the parents (eg. Fox & Mousseau 1996; Azevedo, Partridge, & French 1997; Mousseau & Fox 1998; Vijendravarma *et al.* 2010).

It is therefore clear that individually, parental pathogen challenge and nutritional stress induce trans-generational effects that can be both adaptive and costly depending on the environmental match between parent and offspring. However, in nature, it is unlikely for one environmental factor to vary independently of others. To my knowledge no studies have investigated trade-offs between these trans-generational effects. Understanding such a trade-off is crucial for studies of population dynamics, because reduced availability of nutritional resources is often a consequence of high population densities where there is also a higher probability of disease transmission (Knell *et al.* 1996; Ryder *et al.* 2005; Boots & Roberts 2012).

Here, I examined trade-offs between two costly parental stresses, nutritional stress and pathogen challenge by the bacteria *Bacillus thuringiensis*, on parentally induced phenotypic change in the offspring of the cabbage looper, *Trichoplusia ni.* Offspring were also reared under poor and ideal nutrient conditions to examine how this would influence the expression of parentally-induced traits. I predicted that parents would be able to transfer either immune priming or nutritional stress tolerance to their offspring if each stress was encountered on its own. However, if parents were exposed to both stressors at the same time, there would be a trade-off between TGIP and transfer of nutritional stress tolerance such that only one would be transferred.

I measured the occurrence of TGIP in the offspring by determining their resistance to *B. thuringiensis*, and by conducting a rigorous test of their immune activity. I also estimated the costs of TGIP by measuring life-history characteristics for both

parents and offspring, and the susceptibility of offspring to a different pathogen. Lastly, I examined if egg size, a proxy for investment in individual offspring, was associated with the induced phenotypic changes in the offspring.

5.3. Materials and methods

5.3.1. Study animal

The cabbage looper, *Trichoplusia ni*, is a common pest of cruciferous vegetables, but is a generalist feeder on many other plant species. My *T. ni* colony was originally collected from a commercial tomato greenhouse in British Columbia, Canada in 2001 (Janmaat & Myers 2003) and has subsequently been maintained on a wheat-germ based diet (Table 5.1) at 25°C and 16:8 (L:D) photoperiod. 200 moths were mated each generation to minimize inbreeding. Eggs and pupae were surface sterilized with 0.2% and 1% bleach respectively every generation to prevent disease in the colony.

5.3.2. Parental treatments

Freshly hatched neonate larvae were placed in individual 30 ml plastic cups (P1000100, Solo Cup Company, Lake Forest, IL, USA) with one of two diet treatments and reared at 25°C and 16:8 (L:D) photoperiod. Resource quality was manipulated by diluting the standard wheat-germ based artificial diet 1:1 with the non-nutritive bulking agent, cellulose (Table 5.1). Immediately after moulting to the final (fifth) larval instar, 140 larvae from "good" diet (standard diet) and 140 larvae from "poor" diet (diluted diet) were weighed, then allocated equally to the control group or an immune priming group. Immune priming consisted of feeding larvae on a sublethal concentration (determined from preliminary assays) of the gram-positive entomopathogenic bacteria, *Bacillus thuringiensis* var. *kurstaki* (*Bt*; 125 International Units ml^{-1} diet; DiPel 2x DF, Valent Biosciences, Libertyville, IL, USA) for 48 hrs on their respective diets. As high concentrations of *Bt* will shut-down larval feeding, I ensured that the concentration was low enough that larvae continued to feed over the 48 hr period, albeit at a reduced rate, and did not cause any mortality. The control group fed on freshly made untreated diets for 48 hrs. Larvae were then transferred back to pathogen-free diets and left to pupate.

Development time from the start of the final instar to pupation was recorded, and pupae were sexed and weighed four days after initiation of pupation. Only one larva failed to pupate (poor diet-control treatment).

Table 5.1. Ingredients used to produce standard wheat-germ artificial diet for *T. ni.* Poor (diluted) diet consists of 1:1 dilution of standard diet with cellulose.

Ingredients	Grams
Wheat germ	105
Alfalfa meal	20
Cellulose	63
Casein	73.5
Sucrose	38.5
Cholesterol	6.5
Sodium alginate	10.5
Wesson salt mix	21
Ascorbic acid	7
Sorbic acid	2.5
Vanderzant vitamins	34.5
Wheat germ oil	5
Total	387

Ingredients combined in 1.35% agar solution in a 5:1 agar solution:dry diet ratio.

5.3.3. Mass mating and egg collection

All female pupae from each treatment were placed in 23 x 30 (D x H) cm cages constructed with wire mesh. Male pupae were placed in groups of 10-12 in 2.5 L plastic containers until emergence, and 10% sucrose solution was placed in cages and containers to feed emerging adult moths. When all moths in a treatment had emerged, the male moths were placed into the cage containing the females, with 69-70 individuals split approximately equally between the sexes in each treatment cage. Moths can pass their ovipositor through the wire mesh to lay eggs on paper towel wrapped around the outside. Eggs were only collected on the third day after mating as this is the peak egg-laying date for *T. ni* (Shikano, Hua & Cory, unpublished). Eggs were surface sterilized by submersion in 1% bleach solution for 3 min, then rinsing in distilled water for 5 min

and air drying at 25°C for 5 hrs. Eggs were stored in 4 L plastic containers for 3 days until they hatched.

To determine the relative number and sizes of eggs laid on the third day, I placed two 2 x 30 cm strips of black construction paper on opposite sides of the cage between the wire mesh and the paper towel for 24 hrs. Any eggs laid on the black paper were scanned with an output resolution of 1000 dpi using a Canon CanoScan LiDE210 Color Image Scanner. The number of eggs on the black paper strips was counted and the spherical volume of each egg was estimated by measuring the diameter of the eggs using ImageJ 1.46. The number of eggs on the black paper strips was used to estimate the number of eggs laid in each treatment cage, which was then divided by the number of female moths to estimate average female fecundity on day three. Eggs that were touching other eggs or had blemishes/markings could not be measured for size.

5.3.4. Offspring rearing

Newly hatched larvae from each parental treatment were reared on good or poor diet in individual 30 ml plastic cups. Sixty larvae per treatment were assigned to immune assays, and 100 larvae were assigned to measure offspring development. These were weighed 7 days after egg-hatch and 4 days after the initiation of pupation, and development time and survival were recorded. The remaining larvae were reared in groups of 25 in 180 ml Styrofoam cups containing good or poor diet until the start of the bioassays to estimate disease resistance. All offspring were reared at 25°C and 16:8 (L:D) photoperiod.

5.3.5. Offspring disease resistance

Offspring Bt resistance

Freshly moulted second instar larvae from each offspring treatment were placed in individual wells of a 128-well insect rearing tray (BioServ) containing one of six concentrations of *Bt* (125, 250, 500, 1000, 2000, 4000 IU ml^{-1} diet; 64 larvae per concentration) or a control mixed into their respective diets as described in Janmaat &

Myers (2003). Trays were placed at 24ºC with 16L:8D photoperiod, and mortality was recorded after 72 hrs.

Offspring virus resistance

Five doses of occlusion bodies (OB) of a naturally occurring DNA virus, *Trichoplusia ni* single nucleopolyhedrovirus (TnSNPV), were applied to 2 x 4 mm (D x L) diet plugs made of the respective offspring diets. As larvae reared on poor diet are more susceptible to TnSNPV, it was necessary to adjust the virus doses for each diet (good diet, 0, 32, 80, 200, 500, 1000 OB larva^{-1}; poor diet, 0, 13, 32, 80, 200, 500 OB larva^{-1}; 48 larvae dose^{-1}). Larvae were allowed to feed on the diet plugs for 24 h in individual wells of a 48-well tissue culture plate. After 24 hrs, all larvae that consumed the entire diet plug were transferred to individual 30 ml plastic cups with their respective diets and allowed to feed *ad libitum* until death or pupation.

5.3.6. Offspring immune activity

Thirty final instar larvae from each of eight offspring treatments were weighed, and their haemolymph was collected individually by piercing the middle proleg with a fine needle and pooling haemolymph on Parafilm (Parafilm "M" laboratory film, Bemis Company Inc.). Enough haemolymph was collected from most individual larvae to measure all three immune factors (antibacterial activity, phenoloxidase (PO) activity, and haemocyte density) and a measure of insect condition (protein concentration).

Antibacterial activity

Lytic zone of inhibition was measured according to the methodology described in Wilson *et al.* (2002) and modified for *T. ni* (Olson, Shikano & Cory, Unpublished). Plates of the gram-positive bacteria *Micrococcus lysodeikticus* were made by suspending freeze-dried *M. lysodeikticus* (5 mg ml^{-1}; ATCC No. 4698, Sigma-Aldrich) in a 50°C, 10 ml solution of 1% agar in potassium phosphate buffer (67 mM, pH 6.4) and streptomycin sulphate (0.1 mg ml^{-1}). The *M. lysodeikticus* suspension was evenly dispersed in a 100 mm diameter petri dish and allowed to cool and solidify. A 6 µl mixture of 2.4 µl haemolymph and 3.6 µl anticoagulant buffer [17 mM KCl, 54 mM NaCl, 2 mM $NaHCO_3$, 100 mM D(+)-glucose, 30 mM tripotassium citrate, 26 mM citric acid, 20 mM Na_2-EDTA,

pH 4.6] saturated with phenylthiourea (PTU) was pipetted into 2 mm diameter wells in the *M. lysodeikticus* plates. Plates were incubated for 24 hrs at 33°C and zones of inhibition were measured with Vernier calipers. Dilutions of lysozyme from chicken egg white (1, 0.750, 0.500, 0.250, 0.125, 0.062 mg ml^{-1}; Sigma-Aldrich) were used to produce standard curves and the concentration of chicken egg white lysozyme equivalents was then calculated.

PO activity and protein concentration

I followed the protocol of Shikano *et al.* (2010) with some minor modifications. A 5 µl haemolymph sample was added to 100 µl of ice-cold PBS (pH 7.4) and initially frozen at -12°C for 24 hrs to rupture the haemocytes, and subsequently stored at -80°C for two weeks until analyses of PO activity and protein concentration. Prior to the PO and protein assays, the samples were thawed on ice, centrifuged, then vortexed. For the PO assay, 50 µl of each sample, along with 150 µl of dopamine hydrochloride solution (11.3mM) were loaded in individual wells of a 96-well microplate. Absorbance measurements were taken at a wavelength of 492nm every 45 s for 40 min on a 25°C SpectraMax M2 microplate reader (SoftMax Pro 5 Software, MDS Analytical Technologies, Sunnyvale, CA). The enzymatic rate of PO for each sample, represented by the change in optical density per min (slope) during the linear phase of the reaction, was recorded.

The Bradford assay was used to measure total protein concentration. Five µl of each haemolymph–PBS sample were placed in individual wells of a 96-well microplate, and 200 µl of Bradford reagent dye (BioRad protein kit, BioRad Inc.) was added to each sample. The plate was then shaken in the microplate reader to mix the sample and dye, and incubated at 25°C for 15 min. Two sets of dilution series of bovine serum albumin (BSA) ranging from 0 to 2 mg ml^{-1} was included on each microplate to establish a standard curve of known protein concentrations to calculate the total protein concentration of each sample. After incubation, an end point reading at 595 nm was taken for each sample.

Haemocyte densities

Two µl of haemolymph was mixed thoroughly with 11 µl of ice-cold PBS, loaded in an improved Neubauer brightline haemocytometer (0.1 mm deep; Hausser Scientific), and counted immediately at 100X magnification.

5.3.7. Statistical analyses

Measures of development and immunity were analyzed by analysis of covariance (ANCOVA) using a full factorial design. The fixed effects for parental development were diet quality and *Bt*-challenge, and initial larval weight and sex were included as covariates. Offspring diet quality was added as a third fixed effect for the offspring generation. For offspring development time and pupal weight, sex was included as a covariate, but not for weight at day 7 because some larvae that were weighed did not survive until sex was identified. For all immunity measures, weight at bleeding was included as a covariate. PO activity was square root transformed, and antibacterial activity and protein concentration were $\log_{10}$ transformed. Tukey HSD comparisons were performed when significant interactions were detected. Egg sizes were $\log_{10}$ transformed and analyzed by generalized linear model (GLM) using a normal distribution and identity link function because the numbers of eggs measured for each parental treatment were unbalanced. Mortality data for the *Bt* and virus bioassays were analysed using generalized linear models, using a binomial error structure and a logit link function and checked for overdispersion. As overdispersion was detected in the virus bioassay, a quasi-likelihood approach was used. This approach uses the same binomial error distribution but adjusts the standard errors and test statistics such that hypothesis testing is conducted with an F-test instead of Chi-squared (Littell, Stroup & Feund 2002). The highest concentration in the *Bt* assay was excluded from analysis as mortality was at or near 100% in all treatments. For all ANCOVA and GLM analyses, all fixed factors and their interactions were fitted initially in the model and non-significant interaction terms were removed sequentially to produce the final minimal model using JMP (version 10, SAS Institute, Cary, NC, USA). Figures showing mortality data were simplified by presenting LC_{50} (median lethal concentration of *Bt*) and LD_{50} (median lethal dose of

virus) and their 95% confidence intervals obtained from the final, minimal GLM using the inverse prediction option in JMP.

5.4. Results

5.4.1. Parental generation

Exposure to *Bt* for 48 hours significantly delayed pupation (Figure 5.1a; $F_{1,273}$=1242.64, p<0.001), as did poor diet quality ($F_{1,273}$=187.63, p<0.001). However, the difference between the diets decreased when the larvae were exposed to *Bt* ($F_{1,273}$=12.58, p<0.001). Pupal weight, which is correlated with fecundity in female *T. ni*, decreased with *Bt* exposure ($F_{1,274}$=145.18, p<0.001), but not diet quality ($F_{1,274}$=0.01, p=0.92; Figure 5.1a) and there was no interaction ($F_{1,273}$=3.38, p=0.07). Females pupated earlier and were smaller than males (development time, $F_{1,273}$=21.33, p<0.001; pupal weight, $F_{1,274}$=140.57, p<0.001); initial larval weight only influenced development time (development time, $F_{1,273}$=184.13, p<0.001; pupal weight, $F_{1,274}$=1.00, p=0.32).

Moths laid larger eggs after exposure to *Bt* (parental *Bt*: $X^2_{1,1024}$=49.94, p<0.001) and when they had been reared on the poor diet (parental diet: $X^2_{1,1024}$=79.79, p<0.001, Figure 5.1b), although there was no interaction between these factors (parental diet by parental *Bt*: $X^2_{1,1023}$=3.24, p=0.07). The estimated number of eggs laid by individual females on day three, suggests that females reared on poor diet and females challenged with *Bt* laid fewer eggs and is supportive of a trade-off between egg number and egg size.

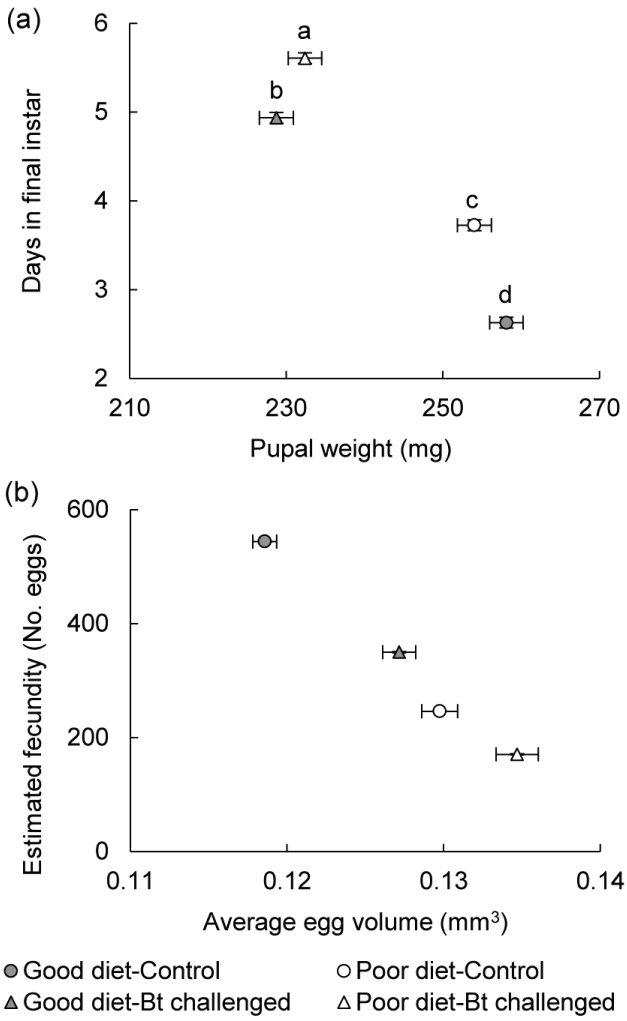

Figure 5.1. Diet quality and *Bt*-challenge affects parental growth and fecundity. Effect of diet quality and *Bt*-challenge on *Trichoplusia ni*: (a) least squares means (±SE) of parental development time (days) and pupal weight (mg), and (b) means (±SE) of egg size (mm^3) and the estimated number of eggs laid per female (no SE). Different letters indicate significant differences for the interaction between diet quality and *Bt*-challenge on development time ($p<0.05$). Number of eggs measured for size: good diet-control, 406; good diet-*Bt*, 280; poor diet-control, 199; poor diet-*Bt*, 142.

5.4.2. Offspring generation

Offspring development

Although offspring diet quality dominated all growth and weight measurements, there were other more subtle effects. Weight of offspring 7 days after egg-hatch was associated with egg size such that larger eggs laid by nutritionally stressed parents and

Bt-challenged parents resulted in heavier offspring. The smallest eggs laid by parents reared on good quality control diet resulted in the lightest offspring (parental diet by parental *Bt*-challenge; Figure 5.2a; Table 5.2). However, the positive effect of parental *Bt*-challenge on early-life growth occurred only if the offspring were fed a good diet, indicating the absence of transfer of nutritional stress tolerance by nutritionally stressed, *Bt*-challenged parents (parental *Bt*-challenge by offspring diet).

The initial growth increase on good diet did not result in faster pupation time, as all offspring on good diet took the same time to pupation regardless of parental treatment (Figure 5.2b; Table 5.2). Parental *Bt*-challenge and diet only affected offspring development time if the offspring were fed a poor diet (parental *Bt*-challenge by offspring diet, parental diet by offspring diet), such that offspring from *Bt*-challenged parents pupated later and offspring from parents fed a poor diet pupated earlier.

Offspring fed good diet produced heavier pupae than offspring fed poor diet, and offspring of parents fed a good diet were heavier than those from poor-diet fed parents (Figure 5.2c; Table 5.2). Interestingly, parental *Bt*-challenge reduced offspring pupal weight on good offspring diet but increased pupal weight on poor diet, resulting in a smaller difference between offspring diets compared to offspring from control parents.

Table 5.2. **Summary of results of the analyses of covariance (*F*-statistics and their significance) on measures of offspring development and immune activity.**

	Day 7 weight	[a]Development time	[a]Pupal weight	[b]Antibacterial activity	[b]PO activity	[b]Protein	[b]Haemocytes
	N=398	N=392	N=392	N=240	N=227	N=240	N=240
Covariate	-	$\boldsymbol{F_{1,385}= 9.72^{**}}$	$\boldsymbol{F_{1,386}=150.28^{***}}$	$\boldsymbol{F_{1,231}= 6.17^{*}}$	$\boldsymbol{F_{1,221}=12.45^{***}}$	$\boldsymbol{F_{1,231}=391.66^{***}}$	$F_{1,234}= 0.01$
Parent diet	$\boldsymbol{F_{1,392}= 9.98^{**}}$	$\boldsymbol{F_{1,385}= 6.92^{**}}$	$\boldsymbol{F_{1,386}= 25.67^{***}}$	$F_{1,231}= 1.17$	$\boldsymbol{F_{1,221}= 4.22^{*}}$	$F_{1,231}= 0.13$	$\boldsymbol{F_{1,234}= 8.28^{**}}$
Parent *Bt*	$F_{1,392}= 0.12$	$\boldsymbol{F_{1,385}= 5.90^{*}}$	$F_{1,386}= 0.09$	$\boldsymbol{F_{1,231}=114.75^{***}}$	$F_{1,221}= 0.35$	$F_{1,231}= 2.34$	$F_{1,234}= 1.69$
Offspring diet	$\boldsymbol{F_{1,392}=1803.21^{***}}$	$\boldsymbol{F_{1,385}=1104.11^{***}}$	$\boldsymbol{F_{1,386}=309.48^{***}}$	$\boldsymbol{F_{1,231}=110.25^{***}}$	$F_{1,221}= 0.61$	$\boldsymbol{F_{1,231}= 4.21^{*}}$	$\boldsymbol{F_{1,234}=71.89^{***}}$
Parent diet x Parent *Bt*	$\boldsymbol{F_{1,392}= 7.84^{**}}$	$F_{1,384}= 1.32$	$F_{1,385}= 1.06$	$F_{1,231}= 0.13$	$F_{1,220}= 2.44$	$\boldsymbol{F_{1,231}= 8.12^{**}}$	$F_{1,232}= 0.01$
Parent diet x Offspring diet	$F_{1,391}= 0.38$	$\boldsymbol{F_{1,385}= 6.04^{*}}$	$F_{1,384}= 0.49$	$\boldsymbol{F_{1,231}= 14.28^{***}}$	$F_{1,219}= 0.30$	$F_{1,231}= 0.20$	$\boldsymbol{F_{1,234}= 5.79^{*}}$
Parent *Bt* x Offspring diet	$\boldsymbol{F_{1,392}= 14.00^{***}}$	$\boldsymbol{F_{1,385}= 9.42^{**}}$	$\boldsymbol{F_{1,386}= 10.40^{**}}$	$\boldsymbol{F_{1,231}= 13.63^{***}}$	$\boldsymbol{F_{1,221}= 8.48^{**}}$	$\boldsymbol{F_{1,231}= 5.32^{*}}$	$F_{1,233}= 0.95$
Parent diet x Parent *Bt* x Offspring diet	$F_{1,390}= 0.21$	$F_{1,383}= 0.49$	$F_{1,383}= 0.20$	$\boldsymbol{F_{1,231}= 9.04^{**}}$	$F_{1,218}= 0.00$	$\boldsymbol{F_{1,231}= 6.45^{*}}$	$F_{1,231}= 3.49$

$^{*}p<0.05$, $^{**}p<0.01$, $^{***}p<0.001$
Covariate: [a]sex, [b]weight at bleeding

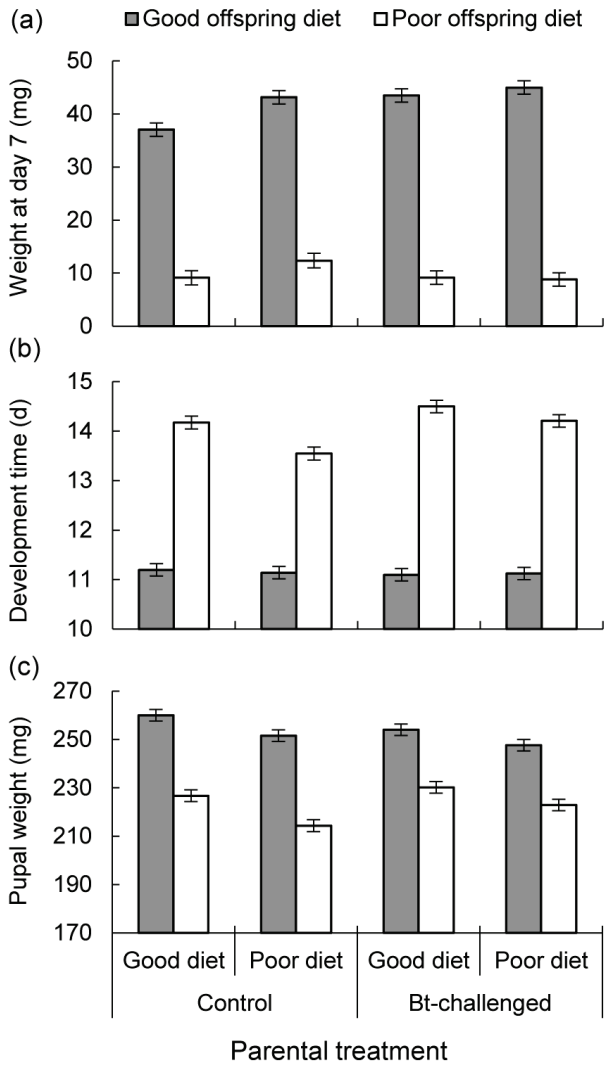

Figure 5.2. Parental diet quality and *Bt*-challenge affects offspring development. Effects of parental diet quality, parental *Bt*-challenge, and offspring diet quality on offspring (a) early-life development measured as weight at day 7 (mg), (b) development time (days) to pupation from egg-hatch, and (c) pupal weight (mg) (Least squares means (±SE)).

Offspring disease resistance

There was clear evidence of trans-generational immune priming as the offspring of *Bt*-challenged parents were more resistant to *Bt* infection (Figure 5.3a; Table 5.3). However, the complex interplay between nutritional stress and trans-generational immune priming is evident in the three-way interaction between parental diet, parental *Bt*-challenge and offspring diet. Pair-wise contrasts reveal that when parental diet quality was good and were not challenged with *Bt,* offspring were more susceptible to *Bt*

when fed poor diet. However, if unchallenged parents were fed poor diet, their offspring were more resistant to *Bt* and showed no differences between offspring diets. While offspring of *Bt*-challenged parents showed significantly higher resistance, parental and offspring diet qualities impacted the level of offspring resistance. Therefore, if parental diet quality was good, nutritionally stressed offspring showed higher resistance to *Bt* than offspring on good diet. On the other hand, if parental diet was poor, offspring were highly resistant to *Bt* regardless of offspring diet. Higher resistance on poor offspring diet was attributable to a slower rise in mortality with dose (offspring diet by dose).

Interestingly, as with *Bt* resistance, nutritionally stressed parents produced offspring that were significantly more resistant to the virus (Figure 5.3b; Table 5.3). Offspring reared on poor diet were significantly more susceptible to TnSNPV than those reared on good diet. Mortality rose more rapidly with virus dose if parents were fed good diet. *Bt*-challenge affected the impact of parental diet such that if parents were reared on poor diet, *Bt*-challenge had no effect on offspring resistance to virus. However, if the parents were reared on good diet, offspring from *Bt*-challenged parents were significantly more susceptible to virus than offspring from unchallenged parents (pairwise contrast, X_1^2=4.75, p=0.03). There was no control mortality in both *Bt* and virus assays.

Table 5.3. Summary of results of the generalized linear model on offspring mortality in the *Bt* and virus bioassays.

	Bt bioassay (N = 2556)		Virus bioassay (N = 1897)	
Dose	**$X^2_{1, 27}$ = 1397.54**	**p < 0.0001**	**$F_{1, 32}$ = 493.34**	**p = 0.03**
Parent diet	**$X^2_{1, 27}$ = 11.70**	**p < 0.001**	**$F_{1, 32}$ = 27.37**	**p < 0.0001**
Parent *Bt*	**$X^2_{1, 27}$ = 57.80**	**p < 0.0001**	$F_{1, 32}$ = 1.58	p = 0.21
Offspring diet	$X^2_{1, 27}$ = 0.03	p = 0.87	**$F_{1, 32}$ = 112.11**	**p < 0.0001**
Parent diet x parent *Bt*	$X^2_{1, 27}$ = 1.47	p = 0.22	**$F_{1, 32}$ = 3.87**	**p = 0.05**
Parent diet x offspring diet	$X^2_{1, 27}$ = 0.18	p = 0.67	**$F_{1, 32}$ = 5.66**	**p = 0.02**
Parent *Bt* x offspring diet	**$X^2_{1, 27}$ = 13.27**	**p < 0.001**	$F_{1, 29}$ = 0.25	p = 0.62
Parent diet x dose	$X^2_{1, 27}$ = 0.06	p = 0.81	**$F_{1, 32}$ = 4.80**	**p = 0.03**
Parent *Bt* x dose	$X^2_{1, 27}$ = 0.03	p = 0.86	$F_{1, 31}$ = 3.21	p = 0.07
Offspring diet x dose	**$X^2_{1, 27}$ = 7.35**	**p < 0.01**	$F_{1, 30}$ = 0.86	p = 0.35
Parent diet x parent *Bt* x offspring diet	**$X^2_{1, 27}$ = 5.70**	**p = 0.02**	$F_{1, 27}$ = 1.12	p = 0.29
Parent diet x parent *Bt* x dose	**$X^2_{1, 27}$ = 4.44**	**p = 0.04**	$F_{1, 25}$ = 0.00	p = 0.96
Parent *Bt* x offspring diet x dose	$X^2_{1, 25}$ = 0.04	p = 0.85	$F_{1, 26}$ = 0.07	p = 0.79
Parent diet x offspring diet x dose	$X^2_{1, 26}$ = 1.54	p = 0.21	$F_{1, 28}$ = 2.10	p = 0.15
Parent diet x parent *Bt* x offspring diet x dose	$X^2_{1, 24}$ = 0.11	p = 0.75	$F_{1, 24}$ = 0.03	p = 0.86

Dose = $\log_{10}$ *Bt* (conc) or $\log_{10}$ virus (dose)
Pearson goodness of fit test: *Bt* bioassay, X^2 = 35.49, p = 0.13; Virus bioassay, X^2 = 50.23, p = 0.02
Overdispersion of virus bioassay = 1.57
Significant results (p < 0.05) are in bold font.

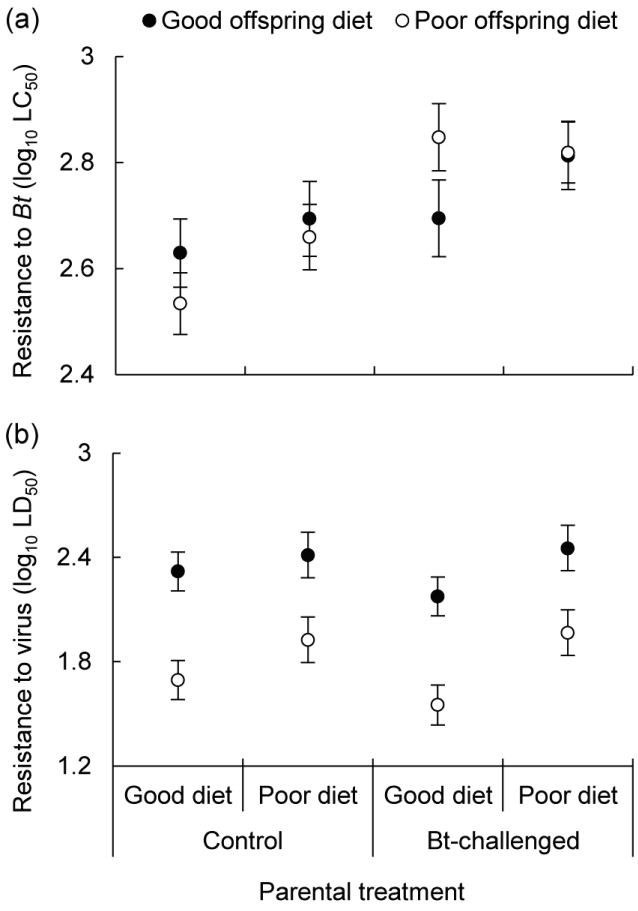

Figure 5.3. Parental diet quality and *Bt*-challenge affects offspring disease resistance.

Effects of parental diet quality, parental *Bt*-challenge, and offspring diet quality on offspring resistance to: (a) the parental pathogen *Bt*, measured as the lethal concentration of *Bt* incorporated into artificial diet that kills 50% of the offspring [$\log_{10}(LC_{50})$; IU ml^{-1} diet], and (b) a different pathogen TnSNPV (naturally occurring DNA virus), measured as the lethal dose of virus that kills 50% of the offspring [$\log_{10}(LD_{50})$; OB $larva^{-1}$]. Error bars represent 95% confidence intervals.

Offspring immune activity

As expected, trans-generational immune priming by parental exposure to bacteria (*Bt*) resulted in significantly higher antibacterial activity in their offspring's haemolymph; even more so if the offspring were fed a poor diet (Figure 5.4a; Table 5.2). However, parental *Bt*-challenge had no effect on offspring haemocyte densities (Figure 5.4b; Table 5.2). Instead, diet quality had a strong effect. Offspring on good diet had roughly 40% higher haemocyte counts than offspring on poor diet. Offspring diet

interacted with parental diet such that whereas offspring on poor diet had similarly low haemocyte counts, offspring on good diet had about 20% more haemocytes if their parents were also fed good diet. Poor parental diet resulted in increased offspring PO activity, but this effect appears to be driven by parents not challenged with *Bt* (Figure 5.4c; Table 5.2). If the parents were *Bt*-challenged, the offspring had higher PO activity on good diet than on poor diet, but offspring diet had no effect if parents were not challenged (parental *Bt*-challenge by offspring diet). Haemolymph protein concentration was used to assess offspring condition, and revealed a three-way interaction showing that offspring on poor diet from nutritionally stressed, *Bt*-challenged parents had significantly less protein than the other treatments (Figure 5.4d; Table 5.2).

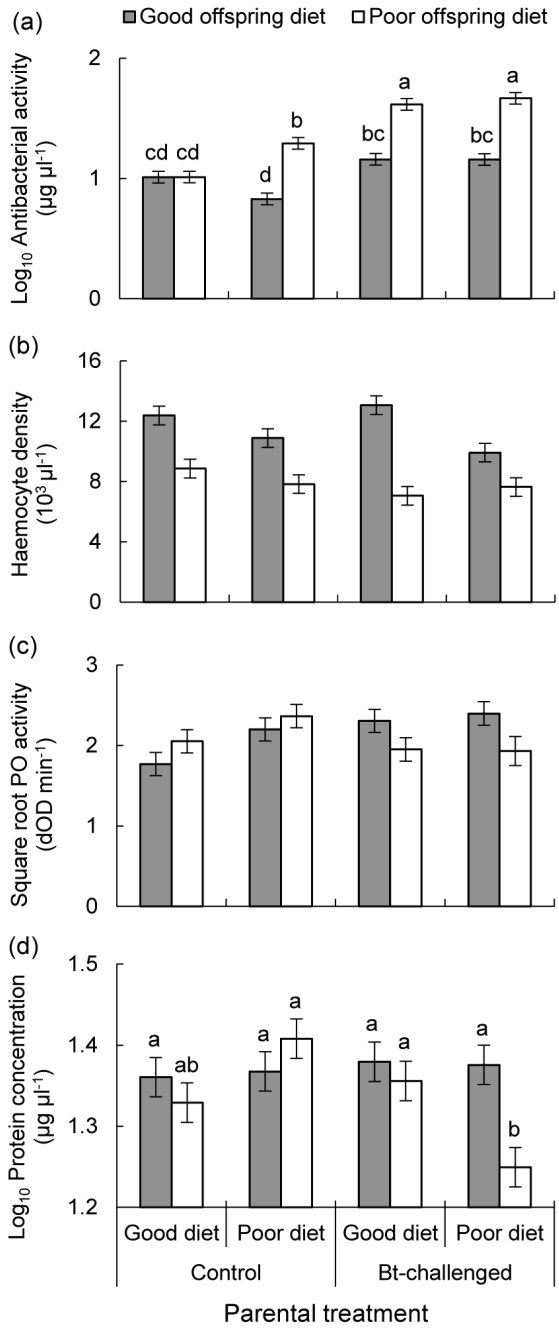

Figure 5.4. Parental diet quality and *Bt*-challenge affects immune activity of offspring haemolymph.

Effects of parental diet quality, parental *Bt*-challenge, and offspring diet quality on the immune activity of offspring haemolymph, and on offspring condition: (a) Antibacterial activity to gram-positive bacteria measured as the diameter of the lytic zone on agar plates with lyophilized *M. lysodiekticus* and transformed into lysozyme equivalents (µg μl^{-1} haemolymph), (b) number of haemocytes (10^3 μl^{-1} haemolymph), (c) phenoloxidase activity measured as change in optical density per min (dOD min^{-1}), and (d) offspring condition measured as protein concentration of haemolymph (µg μl^{-1}). Different letters indicate significant differences for the 3-way interaction ($p<0.05$). Least squares means (±SE) are presented.

5.5. Discussion

By manipulating both parental and offspring nutritional environments, I was able to detect trade-offs between the parental transfer of nutritional stress tolerance and immune priming. Nutritionally stressed, *Bt*-challenged parents produced offspring with greater resistance to *Bt* and higher antibacterial activity in their haemolymph, but these offspring weighed less early in development and took longer to pupate under nutritional stress than the offspring of nutritionally stressed, unchallenged parents. Therefore, when confronted with both nutritional stress and *Bt*-challenge, parents were able to transfer immune priming to offspring but not nutritional stress tolerance. Interestingly, parental nutritional stress enhanced the resistance of offspring to both *Bt* and virus challenge. There was evidence of a cost associated with TGIP such that parental *Bt* exposure had a negative effect on offspring virus resistance, but only if the parents had been reared on high quality diet. Increased egg provisioning (i.e. larger egg size) was associated with the positive trans-generational effects on pathogen resistance. As expected, there were significant costs to the parents associated with both nutritional stress and *Bt*-challenge, such as longer development time, reduced pupal weight and potentially reduced fecundity (estimated from pupal weight and my one-time egg count), thereby decreasing parental fitness if the pathogen is absent in the offspring environment. These results highlight the complex interactions between environmental cues that mediate trans-generational effects.

The associated costs and the relationship between parental and offspring environment determine the fitness consequences of TGIP (Sadd & Schmid-Hempel 2009). Therefore, if there are strong costs associated with TGIP, the trait is only likely to evolve where offspring experience a high likelihood of encountering the same parental pathogen environment (Sadd & Schmid-Hempel 2009). Such a temporal match between parental and offspring environments vary depending on the developmental mode of the organism. For an organism like *T. ni* that has a short life-span and little time between generations, there is a high probability of the same pathogen being encountered by parent and offspring. This may select for mechanisms whereby the parent primes the immune system of its offspring to reduce both the impact of pathogen exposure and the costs of inducing an immune response from a naive level (Little & Kraaijeveld 2004;

Rowley & Powell 2007). I found that TGIP in *T. ni* with a gram-positive bacteria (*Bt*) heightened the offspring's resistance to the pathogen and elevated the baseline gram-positive antibacterial activity of the haemolymph.

Trade-offs between the parental transfer of nutritional stress tolerance and pathogen immunity were detected. Parents reared on poor diet without *Bt*-challenge transferred nutritional stress tolerance to their offspring such that offspring developed faster under the same nutritional conditions as their parents. If nutritionally stressed parents were challenged with *Bt*, they transferred immunity to *Bt* but not nutritional stress tolerance, indicating that in *T. ni* providing protection against the immediate threat of disease is favoured over enhanced competition under nutritional stress. Development under poor nutrient conditions is usually slow, such that faster development under these conditions can be especially advantageous. Competition for food in a poor nutrient environment will favour the individual that can reach maturity before the few available resources are used up, as well as escape pathogen infection and parasitism during the most vulnerable life-stages. Interestingly, the parental transfer of nutritional stress tolerance through faster offspring development was traded-off with reduced offspring pupal mass. This corroborates a finding in *Drosphila melanogaster* that the effects of parental nutrient stress on offspring performance involve both adaptive plasticity and maladaptive effects of parental stress (Vijendravarma *et al.* 2010).

Although the heightened resistance to *Bt* and elevated baseline antibacterial activity would be adaptive in the presence of *Bt*, I found that it is costly and maladaptive in the absence of the pathogen. High antibacterial activity was found in nutritionally-stressed offspring of *Bt*-challenged parents, and this coincided with slow development time and low offspring condition (ie. low haemolymph protein concentration). Furthermore, the transfer of immunity by parents fed a good diet appears to be specific (at least to bacteria) such that resistance of offspring to a different pathogen (baculovirus) did not increase, indicating the specific nature of immune priming (Little *et al.* 2003; Sadd & Schmid-Hempel 2006; Roth *et al.* 2009; Roth & Kurtz 2009). In fact, the parental *Bt*-challenge on good diet resulted in offspring that were more susceptible to virus, confirming costs incurred by increased offspring susceptibility to a parasite distinctly unrelated to the maternal challenge (Sadd & Schmid-Hempel 2009). This

could also indicate trade-offs between resistances to different pathogen groups, due to trade-offs in the activation of different immune pathways, though little is known about insect immunity to baculoviruses (Saejeng, Siva-Jothy & Boots 2011). The ecological relevance of such specificity is uncertain in nature where there are complex interactions between multiple hosts and multiple pathogens (Rigaud, Perrot-Minnot & Brown 2010) and high environmental variability (Cisarovsky *et al.* 2012). Parental exposure to pathogens can have other trans-generational effects such as altering the variance in susceptibility of offspring which could affect the stability of host-parasite interactions (Ben-Ami, Ebert & Regoes 2010).

Nutritional stress itself can also act as a cue for higher disease risk in the next generation (Boots & Roberts 2012), because a reduced availability of nutritional resources is often a consequence of high population densities where there is also a higher probability of disease transmission (Knell *et al.* 1996; Ryder *et al.* 2005). In *T. ni*, parental nutrient stress resulted in a general heightened resistance to both pathogens. This effect was not traded-off against TGIP to *Bt* such that offspring of nutritionally stressed, *Bt*-challenged parents had additional protection against *Bt* and still maintained resistance to the virus. Recent evidence for the trans-generational effects of parental nutrient stress on offspring disease resistance has been unclear. Heightened resistance in the offspring of nutritionally stressed parents to a baculovirus was found in the Indian meal moth, *Plodia interpunctella* (Boots & Roberts 2012), while reduced immune activity in the offspring (ie. haemocyte density and phenoloxidase activity) was also found in the same species (Triggs & Knell 2012). Interestingly, my findings with *T. ni* corroborate both studies such that parental nutritional stress resulted in increased offspring resistance to pathogens even though haemocyte density declined. This shows that parental nutrient stress has a general increasing effect on pathogen resistance in their offspring, but that the effects on offspring immune activity are complex, likely due to trade-offs between immune system components (Cotter, Kruuk & Wilson 2004). Although there is evidence of haemocoelic immune defenses against baculoviruses (McNeil *et al.* 2010; Saejeng *et al.* 2011; Hori *et al.* 2013), mechanisms that are better known to defend against baculovirus infection takes place in the midgut through changes in gut pH, antiviral digestive enzymes (Nakazawa *et al.* 2004), the peritrophic matrix (Plymale *et al.* 2008), and by the sloughing of infected midgut cells (Engelhard &

Volkman 1995). Therefore, offspring of nutritionally stressed parents may incur alterations at the gut level, which might explain the enhanced resistance to both baculovirus and *Bt,* as both pathogens are orally inoculated and infect through the gut membrane.

Parental transfer of disease resistance to offspring was linked to increased egg size. Egg size is an important character in life history evolution because it characterizes both mother and offspring (Sinervo 1990; Bernardo 1996). It can be quite variable and have large effects on the fitness of offspring in both invertebrates (eg. Tauber, Tauber, & Tauber 1991; Sota & Mogi 1992; Fox 1994; Fox & Mousseau 1996; Vijendravarma *et al.* 2010) and vertebrates (eg. Williams 1994; Krist 2011). Increasing egg size while reducing egg number would reduce competition between offspring in poorer environments, while the increased provisioning of eggs may improve the fitness of individual offspring. Larger eggs from my study may have contained more immune molecules (Sadd & Schmid-Hempel 2007; Zanchi *et al.* 2012; Moreau *et al.* 2012) that provided offspring with heightened pathogen resistance, rather than just increased lipid and protein reserves.

The adaptive value of trans-generational effects depends on the match between parental and offspring environments. I have highlighted the importance of costs associated with environmental mismatches that can have significant consequences in terms of the offspring's fitness. The intrinsic time delay accompanying trans-generational effects could potentially impact population dynamics in nature (Beckerman *et al.* 2002; Plaistow *et al.* 2006; Tidbury *et al.* 2012), due to the variability of environments and the complex interaction between multiple hosts and pathogens (Rigaud *et al.* 2010). It is clear that parental experience plays a critical role in host-parasite interactions.

5.6. Acknowledgements

Financial support was provided by an NSERC Discovery Grant to J.S.C. and an NSERC-CGS PhD fellowship, Mutual Fire Insurance Company of BC Graduate

Scholarship in Biological Sciences, and the Sulzer (Bingham) Pumps Inc. Graduate Scholarship to I.S.

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Chapter 6.

Discussion

In this thesis, I investigated the effects of dietary nutrients on the interaction between an herbivorous caterpillar, *Trichoplusia ni*, and its pathogens. I started by examining the effects of nutrient availability on the degree of a fitness cost associated with evolved resistance to *Bt* and the impact of nutrition prior to *Bt* challenge on host survival. I then assessed the interactive effects of host nutrition and temperature after baculovirus challenge on host and virus performance. Lastly, I examined the effects of nutritional stress on trans-generational immune priming. It is clear from the results of this thesis that nutrition plays a critical role in both within and trans-generational pathogen resistance in *T. ni.* In this final chapter, I will summarize the main conclusions from the thesis and discuss some questions that may encourage future studies.

6.1. Nutrition affects evolved resistance and associated fitness costs

Resistance to the bacterium *Bt* has evolved in some target herbivores due to strong selection pressure from its repeated use as a microbial insecticide (Heckel *et al.* 2007; Bravo & Soberón 2008; Carrière *et al.* 2010). Fitness costs associated with evolved resistance has limited its evolution to fixation in the absence of *Bt* (Gassmann *et al.* 2009). These costs often include reduced pupal weight and growth rate, and slower development time. I showed in Chapter 2 that when given a choice, *Bt*-resistant *T. ni* will compose a higher ratio of protein to carbohydrate (P:C ratio) than *Bt*-susceptible *T. ni*. The higher P:C ratio was not a result of greater protein intake, but was due to reduced carbohydrate intake. This higher P:C ratio, even though it reduced overall food intake, eliminated a fitness cost (reduced pupal weight) in resistant males, but not in females as

females reduced carbohydrate intake to a greater extent. Using a no choice feeding experiment in Chapter 3, reduced overall food intake was confirmed to be the mechanism behind fitness costs (reduced pupal weight and growth rate) associated with *Bt*-resistance in *T. ni*.

The first question that comes to mind is, how were *Bt*-resistant males able to achieve equal pupal weights as susceptible *T. ni* while consuming significantly less carbohydrate (ie. less food)? One possibility is that *Bt*-resistant *T. ni*, converted ingested nutrients into body mass more efficiently than susceptible *T. ni.* In Chapter 3, I showed that *Bt*-resistant *T. ni* converted dietary protein into bodily nitrogen more efficiently than susceptible *T. ni* on the most protein-rich diet (50p:10c), and were more efficient at converting dietary carbohydrate to bodily lipid on the two carbohydrate-rich diets (10p:50c and 20p:40c). Considering that the 50p:10c diet was used as the main source of protein in all three choice experiments and the two carbohydrate-rich diets as the primary carbohydrate source in two of the choice experiments, it is possible that *Bt*-resistant males required less food overall to produce an equivalent mass of bodily nitrogen and lipid. Thus, I may have inadvertently used P:C ratios in Chapter 2 that were easier for *Bt*-resistant *T. ni* to utilize than susceptible *T. ni.* It would be interesting to see if consistent results could be obtained using diet choices that were less extreme where the efficiency of converting nutrients into body mass would be the same between resistant and susceptible *T. ni.*

Also in Chapter 3, I found that the probability of surviving *Bt*-challenge increases in both *Bt*-resistant and susceptible *T. ni* if pre-fed higher P:C ratios, although it declines for *Bt*-resistant *T. ni* on the most protein-rich diet. The P:C ratio of approximately 2p:1c self-composed by *Bt*-resistant *T. ni* was the same ratio that maximized survival when consumed prior to *Bt* challenge. Consumption of higher P:C ratios is associated with higher constitutive immune activity in two *Spodoptera* species (Lee *et al.* 2006b; Povey *et al.* 2009, 2014). Therefore, it is possible that *Bt*-resistant *T. ni* compose a higher protein diet as a means of increasing constitutive immune activity and maximizing *Bt*-resistance. This would be particularly important since *Bt*-resistant *T. ni* is more susceptible to a nucleopolyhedrovirus (Sarfraz, Cervantes & Myers 2010) and exhibited significantly lower haemocyte density and phenoloxidase activity in their haemolymph,

compared to susceptible *T. ni* (Ericsson *et al.* 2009) when restricted to a single host plant or diet. A follow-up study should examine whether the higher P:C ratio composed by *Bt*-resistant *T. ni* is a form of compensatory feeding that also eliminates such immune costs.

While *Bt*-resistant *T. ni* showed potential for eliminating a fitness cost (males only) and improved nutrient utilization at some dietary P:C ratios, whether they are capable of this on plants, and hence in nature, is unknown. Fitness costs associated with *Bt*-resistance are often magnified by defensive phytochemicals that can reduce feeding performance through direct toxic effects (Gassmann *et al.* 2009). Since reproductive and younger parts of plants that tend to have higher protein content are also higher in defensive phytochemicals, the pursuit of a higher P:C ratio diet may do more harm than good. An alternative source of protein could be obtained through cannibalism (Mayntz & Toft 2006; Simpson *et al.* 2006), or by intraguild predation on other herbivorous insects or mites (van Maanen *et al.* 2011). A follow-up study should investigate the extent to which *Bt*-resistant *T. ni* compose their diet between plants, between different parts within a plant, or by engaging in intraguild predation.

6.2. Nutrition interacts with temperature to affect host susceptibility and virus production

Host nutrition and temperature are individually known to alter pathogen virulence and host immune defense. However, it is not known how interactions between these two environmental factors will influence disease outcome and host and pathogen fitness. In Chapter 4, I simultaneously varied temperature and dietary P:C ratio to determine their interactive effects on the performance of *T. ni* and two species of nucleopolyhedroviruses. Host performance was strongly influenced by dietary P:C ratio, temperature and virus-challenge. Virus challenge significantly reduced host survival and performance. Optimal host performance was consistently achieved at slightly more protein-biased P:C ratios (between 40p:20c and 30p:30c) at each temperature when challenged by the 'narrow host range virus' (TnSNPV) compared to controls. Optimal performance of hosts challenged by the 'broad host range virus' (AcMNPV) were strongly influenced by temperature such that peak host performance shifted to extremely

protein biased P:C ratios at 32°C, but only slightly at 24°C and not at all at 16°C. There was a significant cost of reduced pupal weight associated with surviving TnSNPV challenge but not AcMNPV challenge. Virus performance was also affected by changes in temperature and host nutrition. TnSNPV had a distinct peak in performance (host mortality and virus yield) at a narrow P:C ratio range at 24°C, whereas AcMNPV showed more similar host use across two temperatures and a wider range of P:C ratios.

The different patterns of optimal host performance after challenge by the two NPVs and the differences in the two NPV species' peak performances, are likely due to the effects of nutrition and temperature variation on host immune response as well as the differences in effectiveness of the immune response against the two NPVs. A number of studies using *Spodoptera frugiperda* cell lines have shown that host genes are differentially regulated after AcMNPV infection (as much as 70% out 42,000 probes in a microarray) with nearly all genes down-regulated. These down-regulated genes included those involved in pattern recognition and immune responses (Ooi & Miller 1988; Nobiron, O'Reilly & Olszewski 2003; Salem *et al.* 2011). A recent study showed that *S. exigua* cells infected by its specialist virus SeMNPV had almost 3-fold more transcripts differentially regulated 36 hours after infection compared to cells infected with the generalist AcMNPV. Of these, a number of immune-related genes were up-regulated after SeMNPV infection but none were up-regulated after AcMNPV infection (Jakubowska *et al.* 2013). *T. ni* might also exhibit a greater immune response through effector molecules to its specialist virus TnSNPV than to AcMNPV. Haemocyte density and phenoloxidase activity, which are typically used to assess immunity to baculoviruses, are both known to decline in *T. ni* haemolymph after TnSNPV infection (Scholefield, Shikano, Fung & Cory, unpublished). Therefore, to determine if the presence of a cost associated with surviving TnSNPV (ie. reduced pupal weight) is due to a greater immune response to TnSNPV, it is necessary to conduct a thorough comparison of the regulation of immune-related genes after TnSNPV and AcMNPV challenge. Examining how the regulation of these immune-related genes respond to viral challenge under different temperature and host nutritional conditions may reveal some mechanisms behind differences in optimal host and virus performances observed in this chapter.

Another factor that needs further investigation is the degree to which host nutrition and temperature alters the host as a nutritional resource for the viruses. I expected host nutrition to have a strong effect since larval feeding on different P:C ratio diets in Chapter 3 resulted in significant differences in pupal nitrogen and lipid content. Thus, the accumulation of different proportions of nutrients in the host may alter the blend of nutrients available for the growth of cells, which are the environments the viruses are invading. Development of larval *Drosophila* on low nutrient diet resulted in reduced cell size in their wings as adults (Vijendravarma, Narasimha & Kawecki 2011). Cell size is likely important for the production of baculovirus OBs, as the cell size of a *S. frugiperda* cell line prior to infection with recombinant baculoviruses was shown to positively correlate with the quantities of recombinant protein produced (Chai *et al.* 1996). Cell size is determined by the cell metabolic state, which can be strongly influenced by nutrient availability. Glutamine, yeast extract and fetal bovine serum added to cell culture media provide key nutrients such as vitamins, trace elements, lipids, and hormones that determine cellular metabolism and promote cell growth (reviewed in Palomares, Pedroza & Ramírez 2001). Carbon sources such as glucose are also important for cell growth (Drews, Paalme & Vilu 1995). Continued delivery of an adequate supply of nutrients after viral infection is necessary to maintain culture viability and substrates for recombinant protein production (Palomares *et al.* 2001). Temperature can also affect cell size. I found that increasing the developmental temperature of *T. ni* resulted in lighter pupal weights. In *Drosophila*, higher developmental temperature was associated with smaller body parts and smaller cell sizes in the adults (French, Feast & Partridge 1998). Therefore reduced virus yields obtained at high temperature (32°C) and unbalanced P:C ratios, may have been in part due to smaller host cell sizes.

6.3. Nutrition affects trans-generational immune priming

Trans-generational effects occur when the parental environment determines offspring phenotype. Studies of trans-generational effects tend to ignore environmental context and often only focus on individual environmental variables. However, environmental factors such as nutrient availability and parasite exposure are often

linked. One type of trans-generational effect, called trans-generational immune priming (TGIP), can occur when the parasite environment experienced by the parents acts as a cue to increase offspring immune defences (Little *et al.* 2003; Sadd *et al.* 2005; Moret 2006). Similarly, the nutritional environment experienced by parents can have a trans-generational effect, such that nutritional stress can lead to the transfer of nutritional stress tolerance to offspring. Therefore in Chapter 4, I investigated the effects of parental nutritional stress on TGIP. Reacting to a pathogen challenge and developing under nutritional stress are both costly, thus I hypothesized that if encountered together there would be a trade-off between TGIP and the transfer of nutritional stress tolerance to offspring.

I found clear evidence of TGIP and the trans-generational transfer of nutritional stress tolerance. However, both pathogen challenge and nutritional stress inflicted significant costs to parents, such as longer development time and reduced pupal weight. When both stressors were experienced simultaneously, a trade-off between TGIP and the transfer of nutritional stress tolerance was detected such that nutritionally stressed parents transferred immunity but not nutritional stress tolerance. Under these circumstances, why would it be more beneficial to transfer immunity to the pathogen than nutritional stress tolerance? One possibility is that *T. ni* moths may be able to avoid nutritional stress for their offspring by preferentially ovipositing on optimal host plants, whereas they may not be able to avoid ovipositing on parasite contaminated plants. *T. ni* moths are known to preferentially oviposit on host plant species that are optimal for offspring development (Shikano, Akhtar & Isman 2010). Another lepidopteran species (*Spodoptera exigua*) has shown the ability to detect and oviposit on higher nitrogen containing plants within the same species (Chen, Ruberson & Olson 2008). While larvae of the gypsy moth (*Lymantria dispar*) are known to avoid feeding on virus contaminated foliage (Parker, Elderd & Dwyer 2010), to my knowledge there is no evidence to suggest that adult lepidopterans are able to detect pathogens on plant surfaces and avoid ovipositing on them. However, monarch butterflies (*Danaus plexippus*) infected with a protozoan parasite has been shown to preferentially lay eggs on medicinal plants that mitigate parasite infection in their offspring, termed trans-generational medication (Lefèvre *et al.* 2010, 2012).

Heightened resistance to both *Bt* and TnSNPV in offspring of nutritionally stressed and *Bt*-challenged parents was linked to increased egg sizes. I hypothesized that this increased allocation of resources into individual eggs may have resulted in the transfer of more immune molecules (Sadd & Schmid-Hempel 2007; Zanchi *et al.* 2012; Moreau *et al.* 2012) that provided offspring with heightened pathogen resistance, rather than just increased lipid and protein reserves. This could be the case as parents that were simultaneously nutritionally stressed and *Bt*-challenged, produced larger eggs than parents that experienced only one of the stressors. Another possible mechanism that may have contributed to higher pathogen resistance and antibacterial activity of the haemolymph is imprinting. Imprinting might occur through epigenetic marks that are passed to the offspring and alter gene expression of immune molecules. Epigenetic marks can occur through methylation of DNA and the post-translational modification of histone proteins (Hunt *et al.* 2013), and are known to be influenced by environmental factors such as diet in mammals, and consequently influence post-embryonic development (Jaenisch & Bird 2003). Genomic imprinting through DNA methylation has been lost in some lineages of insects (reviewed in Hunt *et al.* 2013), but it has been found in a lepidopteran species, *Bombyx mori* (Xiang *et al.* 2010; Zemach *et al.* 2010).

Interestingly, nutritional stress in the parents enhanced both nutritional stress tolerance of offspring and heightened resistance to both *B. thuringiensis* and TnSNPV. If trans-generational effects are adaptive when the environment experienced by the parents matches that of the offspring, why would parents that experienced nutritional stress but not pathogen challenge produce offspring that are more resistant to pathogens? It would seem more adaptive for unchallenged nutritionally stressed parents to produce offspring that have reduced investment in pathogen resistance, so that the offspring could redirect those unused resources to faster development on nutritionally poor food. Furthermore, nutritionally stressed parents might suffer less fecundity costs by only transferring nutritional stress tolerance and not pathogen resistance. One possibility is that nutritional stress tolerance is achieved by alterations to the gut that improve the utilization of nutrients, and these changes at the gut level may consequently affect the pathogenesis of gut entering pathogens such as *Bt* and TnSNPV. Another possibility is that because nutritional stress-tolerant offspring are developing at a faster rate, they may simply be able to escape lethal infection through faster growth. The rate

of midgut sloughing of virus challenged *T. ni* increases in an age-dependent manner, and larvae may be able to escape lethal infection if they can reach ecdysis more quickly because all infection foci on the midgut epithelium is cleared during ecdysis (Engelhard & Volkman 1995).

6.4. Concluding remark

My thesis demonstrates that host nutrition is a key environmental factor that is likely to have significant consequences for the outcomes of host-pathogen interactions. My findings clearly indicate that the resource quality of hosts can influence the efficacy of microbial biological control agents, and may also influence viral epizootics and population dynamics in nature.

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