Effect of host nutrition on the outcome of mixed infections in an insect-pathogen association

by

Pauline Deschodt

M.Sc, Montpellier SupAgro, 2014

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 2022

Declaration of Committee

Name:	**Pauline Deschodt**
Degree:	**Doctor of Philosophy**
Title:	**Effect of host nutrition on the outcome of mixed infections in an insect-pathogen association**
Committee:	**Chair: Jonathan Moore** Professor, Biological Sciences
	Jenny Cory Supervisor Professor, Biological Sciences
	David Green Committee Member Professor, Biological Sciences
	Steve Perlman Committee Member Professor, Biology University of Victoria
	Gerhard Gries Examiner Professor, Biological Sciences
	Angela Smilanich External Examiner Associate Professor, Biology University of Nevada, Reno

Abstract

Hosts are commonly exposed to multiple pathogens. Co-infecting pathogens may compete for resources, interact through the host immune system or interfere directly with each other. Moreover, resource heterogeneity can alter host investment in defence mechanisms and thus is likely to be a major force shaping interactions between pathogens and altering pathogen epidemiology. I investigated the impact of nutrition on host resistance to co-infections in an insect system, using the cabbage looper*, Trichoplusia ni*, and two of its pathogens, the *T. ni*-specific nucleopolyhedrovirus (TniSNPV), and the generalist entomopathogenic fungus, *Beauveria bassiana*. I first evaluated the role of resource quantity and quality on the outcome of mixed infection*,* by introducing *T. ni* larvae to artificial diets post-pathogen challenge. While neither diet quality (Protein-to-Carbohydrate ratio) nor quantity affected overall larval mortality, fungus-induced mortality did increase with increasing protein in the diet. *Beauveria bassiana* was the dominant pathogen in our system, negatively affecting virus-induced mortality in all mixed infections. While fungus yield increased when co-infected with TniSNPV at a low dose, virus yield did not differ in mixed infections but decreased with increasing protein. Production of virus transmission stages and virus virulence after mixed infection were influenced by complex interactions between nutritional resources, competition, and infection dose. Next, I investigated the effect of host plant on the outcome of mixed infections after pathogen challenge. Mortality was generally higher on the poorer host plant. Moreover, the order of introduction altered the outcome of co-infections, revealing synergistic, antagonistic, and neutral relationships depending on plant identity. Lastly, both pathogens can be used as microbial control agents. Hence, I explored potential benefits of combining TniSNPV and *B. bassiana* to manage *T. ni* populations in the field and asked whether crop plant identity could alter the outcome of mixed pathogen applications. Overall, larval mortality was comparable between mixed applications and TniSNPV alone. Virus-induced mortality decreased by 50% when applied with the fungus while *B. bassiana* was shown to be more sensitive to host plant. These results demonstrate that pathogen competition is highly dependent on the environment and could have major implications for pathogen epidemiology and disease outbreaks in wild populations.

Keywords: Baculovirus, *Beauveria bassiana*, fitness costs, mixed infection, pathogen replication, tritrophic interactions

Dedication

To my beloved grandparents. Mamie, Papi, Mémé and Pépé, I will never forget you.

Acknowledgements

Thank you to everyone who helped me through my PhD journey.

First, I would like to thank my senior supervisor Dr. Jenny Cory for believing that a young student from France could be a good addition to her lab. Thank you, Jenny, for your guidance, for sharing with me your passion for pathogen ecology and evolution and for allowing me to challenge myself in many ways. You have always pushed me to look at the bigger picture and I do think I grew so much as a researcher and person working with you.

I'm extremely grateful to my committee members, Dr's. David Green and Steve Perlman. Your patience and feedback throughout my degree were extremely valuable. You helped me stay focused and make sure I was heading in the right direction. Thank you.

When I arrived in Vancouver, I knew I was going to be ok when I saw my first labmate, Paul MacDonald. Paul thank you for picking me up at the airport that day, you are such a generous person, you showed me around, introduced me to everyone in the lab and you were always there if I needed to talk. Joyce Leung, Kari Zurowski, Kevin Colmenares-Di Maria, Dennis Quach Danielle Ogilvie and Paul, I am proud to have been part of the Cory lab family, you are all so nice and my time here would not have been the same without you. I'm looking forward to another karaoke night, it has been long overdue. Danielle, I'm happy you came and joined us. I'm impressed by the work you are putting out and I'm glad you were around in the last bits of my degree to help keep me sane. I know the pandemic has made it hard to get a sense of community, but I enjoyed our time together either in the field looking for egg masses and caterpillars, making diet for the 100th time or sharing plant cuttings. To Sean McCann, I have to say, my presentations have not been the same since you were kind enough to take pictures of my cabbage loopers. You were always ready to try something new to get the best shot of those green caterpillars and I cannot say how much I appreciate it. Shout out to all the undergraduate students who worked with me on those projects, it is not always easy to spend hours transferring larvae from one cup to another or try to find them on a plant, but you all worked so hard. Special thank you to Alana Breitkreutz, Olivia Walker,

Jessi Li, Soobin Yim, Jess Hercus and Brandon Dang, I hope you enjoyed building and running your projects as much as I did.

I would not have gone this far without finding my family away from home. Heather Coatsworth, Ahdia Hassan and Kyle Bobiwash, I cannot thank you enough for your support and for making me feel at home. You have been there for the hardest and the best moments of my time in Vancouver. I loved living with you all, binge-watching shows, cooking for each other and yelling over a game of Yahtzee. Thank you, Joyce, Heather and Lydia Stepanovic, for the great craft nights and hangouts. You are so supportive and loving. I will never forget our sleepover and game nights. Jaime Chalissery, Don Wiggins, Stacey Hrushowy, Nicholas Salcedo, Sydney, Heather, Joyce and Kevin, I have so many great memories of crazy discussion topics, drinks after work, camping trips and hikes with you all. You were so much fun to be around and I know I could count on you if I was ever in doubt or if I needed advice with my project. A special thank you to Liz Steves for pushing me to get out and discover the amazing outdoors of Vancouver, I have never owned that much gear and I am ready for our next adventure. Throughout my years at SFU, I have met so many amazing graduate students and although many are already gone now, our lunchtime outside on the picnic table that we build together, our frisbee games and cake Monday made a huge impact on my graduate student experience in the Biology department. Cheers and good luck to you all.

As a graduate student, I did spend a few semesters working as a teaching assistant and although it was something very new to me, I would like to give a special thank you Mala Fernando and Miranda Meents for everything. I loved teaching plant biology and working with you both. The labs were so much fun, and I like to believe I am a better instructor now. An extra thank you to Leslie Dodd, our paths crossed when I was teaching plant biology but your guidance and advice on the best way to take care of my plants were a lifesaver, my caterpillars would have had nothing to eat without your help. I hope you know how much I appreciate the time you took to show me around the greenhouse.

I have left a lot of friends back home and I know I haven’t been very present in the last few years but Marion, Helene, Louise and Zeneibe you have always understood me. You were patient and so supportive no matter what. I’m a better person thanks to you. I am sorry I bothered you so much talking about insects and pathogens but you

were kind enough to still hang out with me anytime we had a chance. To my current roommates, Caroline and Quincy, thank you for being there when I needed distractions, I really enjoyed our nights binge-watching true crime documentaries.

I cannot write these acknowledgements without thanking those who without knowing were the cutest support and cuddlers. Acorn, Miss No, Pepe, Zulu, Elliot, Debu and Lupita I don't think you understand how much your cute mischievous little faces have helped me.

Lastly, thank you to my amazing family for their support. Mom, Dad, I know you always knew that one day I was going to move far away to start something crazy. You never hold me back or questioned my decision. You have set such a great example and I hope I have made you proud (I swear I am almost done!). Merci pour tout, vous êtes les meilleurs. Agathe, Maïté, you have always pushed me to do my best and were ready to make the best time out of my short visits. You are two amazing sisters. To my little nephews Mathis and Timothé, I know you have no clue what I have been doing here but your kindest hugs and cheers meant the world to me. I will show you around Vancouver one day I'm sure you will love it.

Table of Contents

List of Tables

List of Figures

List of Acronyms

Bt	*Bacillus thurigiensis*
NPV	Nucleopolyhedrovirus
OB	Occlusion body
ODV	Occlusion-derived virion
P:C	Protein-to-carbohydrate ratio

Chapter 1.

Introduction

1.1. Background

Invertebrate pathology has traditionally focused on single host-pathogen interactions on a limited number of pathogen groups and species with high virulence and insect pest control potential (Hajek et al., 2007; St. Leger et al., 2011; Lacey et al., 2015). However, from an ecological perspective, it is clear that both insects and their pathogens, including viruses, bacteria and fungi, are rarely found in isolation in natural situations (e.g. Malakar et al., 1999; Cox, 2001; Hajek and Tobin, 2011; Bartolomé et al., 2020; Trzebny et al., 2020). Pathogens are often isolated from individual species, and the use of sensitive molecular tools and next-generation sequencing has made it clear that communities of microorganisms with varying impacts on their hosts can be detected in single organisms. In addition, host-pathogen interactions can also be modulated by trophic interactions, and in particular, the host plant that the insect feeds on can alter the outcome (Cory and Hoover, 2006; Shikano, 2017). Thus, biotic interactions are likely to play a major role in how invertebrate pathogens (entomopathogens) impact their host populations. Despite this role, there is still a lack of studies on more complex interactions within pathogen communities at all spatial scales. This information is important to the development and use of microbial insecticides and the prevention of disease among managed invertebrates, in addition to expanding our knowledge about the roles played by pathogens in natural communities. Entomopathogens are increasingly being used in pest management programs, and yet there is no clear way to accurately predict whether the use of pathogen combinations will be likely to enhance or impede biological control and assist in successful pest management. Understanding the mechanisms underlying pathogen-pathogen interactions would allow us to develop a framework for mixed microbial applications.

1.2. Pathogen-Pathogen Interactions

Invertebrates are attacked by a wide range of natural enemies, including numerous species of predators, parasitoids, and many groups of pathogens. These can interact with a host at the individual level (within-host dynamics), the population level through intraguild

interactions (Rosenheim, 1995), and potentially via other members of the community in which these species are embedded. Different natural enemies can compete with one another either directly (interference) or indirectly via competition for resources or by co-opting the immune system (apparent competition) (Mideo, 2009) (Fig. 1.1). Alternatively, they can act independently or even enhance the susceptibility of one natural enemy to another via trait-mediated effects, whereby a host's behaviour, physiology, or morphology is changed, making it more vulnerable to other natural enemies (Sih et al., 1998). Entomopathogens encompass a wide range of very diverse species (Blissard and Rohrmann, 1990; Shanchez-Contreras and Vlisidou, 2008; Junglen and Drosten, 2013; Araújo and Hughes, 2016) differing in their life cycle, infection pathways, resources needed, as well as their virulence to the host, potentially creating diverse and variable interactions. Increased interest in understanding the factors influencing the evolution of virulence has resulted in significant attention being devoted to within-host pathogen interactions, particularly from a theoretical perspective (e.g. Alizon, 2008; Choisy and de Roode, 2010; Ben-Ami et al., 2011; Ben-Ami and Routtu, 2013; Clay and Rudolf, 2019).

Studies on mixed entomopathogen infections extend back many decades, particularly for baculoviruses (Family: Baculoviridae) (Harper et al., 1986). The main focus of earlier mixed-infection studies was on the overall impact on insect host mortality and mortality rate when pests were challenged with two pathogens to determine whether synergism, interference, or additive effects occurred. All of these outcomes have been demonstrated (Cory and Bishop, 1997; Mantzoukas et al., 2013); however, co-exposing a host to two pathogens does not necessarily mean that they will co-infect (both infect and potentially develop and reproduce within the host, at least to some measurable extent), although the second pathogen could still have an impact on the first, even if one dominates at the end of the infection process. In terms of pathogen fitness, it is the production of transmissible stages which is ultimately important, but this has rarely been measured. Laboratory studies have shown that the timing and order of infection are important for the outcome of mixed infections (Marchetto and Power, 2018; Zilio and Koella, 2020), as are the relative concentrations of different pathogens (Ebert et al., 2000; Fellous and Koella, 2009; Georgievska et al., 2010). For example, in first and second instar of the corn earworm, *Helicoverpa zea* (Lepidoptera: Noctuidae) larvae, co-infection of a "fast kill" *H. zea* single nucleopolyhedrovirus (HzSNPV) (average speed of kill 5-6 days) with a "slow kill" strain of *H. armigera* granulovirus (HearGV) (average speed of kill 16 days) showed

that the two viruses competed and that HearGV appeared to inhibit HzSNPV replication, even when the challenge with HearGV came 36 hours after HzSNPV infection (Hackett et al., 2000). Migratory locusts (*Locusta migratoria*) infected with a microsporidian (*Paranosema locustae*) followed by the fungus *Metarhizium acridum* died more rapidly compared to what was expected based on the mortality and speed of kill in single pathogen infections, but only when the second pathogen was added 9 days after the initial challenge, which corresponded with microsporidian spore maturation (Tokarev et al., 2011).

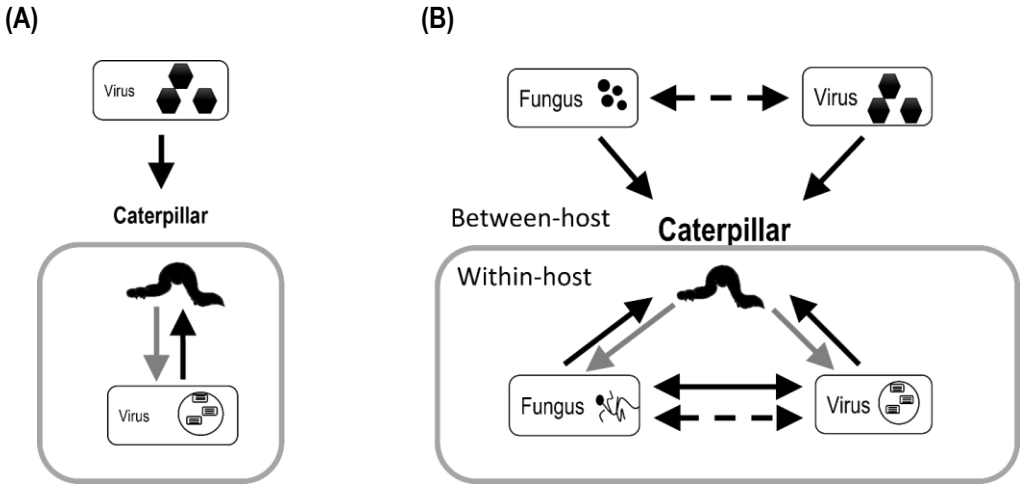

Figure 1.1 **Entomopathogens have a negative interaction with their host as they infect individuals and consume host resources (solid black arrows). In (A) single pathogen infections, the infecting pathogen only interacts with the host resources (black arrow) or the host immune response (grey arrow). (B) When multiple pathogens are infecting the same host, interactions can take place at two levels: within the host and at the population level. Within the host, pathogens will have an indirect impact on each other by competing for host resources (exploitation competition). Alternatively, they could interact directly, such as by the production of toxins (interference competition) (dashed line). In addition, the host immune system will have a direct negative effect on a pathogen (grey arrows), and this response could also indirectly influence co-infecting organisms, either positively (by making the host more vulnerable) or negatively (by fighting off the second infection with an enhanced immune response; immune-mediated competition). At the host population level, a generalist pathogen can directly remove potential susceptible hosts from the population. All natural enemies compete for uninfected hosts in the population (dashed line), and population persistence may depend on the nature of pathogen transmission (whether or not it is density-dependent) and the critical population threshold (modified from Cory and Deschodt 2017)**

Similarly, interactions among entomopathogenic fungi and the bacterium, *Bacillus thuringiensis* (Bt), may be additive (the mortality observed in the mixed infection treatment is similar than the mortality predicted using single pathogen treatments), synergistic, or antagonistic (the mortality in the mixed treatment is higher or lower than expected, respectively) depending on the pathogen dose and species involved (Mantzoukas et al., 2013). Although the majority of mixed-infection studies have focused on host mortality or speed of kill, some (but not many) have measured impacts on pathogen fitness, in particular the production of transmission stages. For example, laboratory investigation of co-infection of the smaller tea tortrix, *Adoxophyes honmai* Yasuda (Lepidoptera: Tortricidae), with two virus species commonly found co-infecting the host in the field (Nakai et al., 1998), *Adoxophyes honmai* nucleopolyhedrovirus (AdhoNPV) and *A. honmai* entomopoxvirus (AdhoEPV) demonstrated that simultaneous co-infection reduced AdhoEPV yield whereas AdhoNPV yield was not affected, and prior infection of *A. honmai* larvae with AdhoEPV reduced AdhoNPV yield but did not alter AdhoEPV final yield (Ishii et al., 2002). Co-infection of two entomophthoralean fungi, *Pandora blunckii* and *Zoophthora radicans*, in the diamondback moth larva (*Plutella xylostella* (L.) (Lepidoptera: Plutellidae)) showed that co-infection (resulting in reproduction by both fungi) was relatively uncommon (around 20%) and tended to occur only when the species were introduced simultaneously. The last species to be introduced was most likely to benefit, and in some scenarios, overall mortality was lower in mixed compared to single infections (Sandoval-Aguilar et al., 2015). Thus, co-infecting species can clearly affect one another's fitness. However, questions such as how frequently co-challenge results in a mixed (co-) infection (at the individual level, i.e., with reproduction by both pathogens) and whether the first pathogen to establish always wins are unlikely to have simple answers.

The outcome of experiments with multiple pathogen species appears to be context- and species-dependent, and a more systematic approach, which addresses underlying mechanisms, is needed in order to begin to predict the outcome of mixed infections. When a single host is challenged with two pathogens, the pathogens are clearly competing for the same resource, so it is likely that one or both species will have reduced fitness compared to when they are infecting alone, unless they produce synergistic effects on host mortality. In trying to decide whether it will be beneficial to combine two or more entomopathogens for pest control, it will be useful to have a framework to indicate the direction that the outcome of the mixed infection is likely to take. One approach is to try

and understand the competitive mechanisms behind the interactions. For example, Staves and Knell (2010) studied the competitive interactions among different strains of a fungal pathogen, *Metarhizium anisopliae* (intraspecific competition), and between *M. anisopliae* and the entomopathogenic nematode *Steinernema feltiae* (interspecific competition), using the wax moth (*Galleria mellonella* (Lepidoptera: Pyralidae)) as their target host. They found that competition among the fungal strains depended on strain virulence (speed of kill in this case), with the more virulent strains being better competitors, although this also depended on dose. However, in competition with the nematode, less virulent strains of the fungus were more successful. They suggested that this difference is due to the nature of the competition, with the fungus engaging in indirect competition via host exploitation and the fungus and nematode engaging in direct competition involving fungal toxin production (interference competition). In another study using two previously undescribed species of *Steinernema* in the wax moth (*G. mellonella)*, Bashey et al. (2013) characterized the different strains of *Xenorhabdus* spp. bacteria carried by the nematodes and examined which produced bacteriocins that were capable of inhibiting the growth of another strain of the same bacterium. They found that there was no interference between *Xenorhabdus* strains and that the speed with which the host was killed was a predictor of competitive success. However, when there was interference via bacteriocins, this overcame any superiority in terms of killing speed. Thus, in some host-pathogen systems at least, it may be possible to predict the outcome of mixed infections, given sufficient information on the nature of the competition taking place.

Studies on mixed pathogen infections are moving away from a focus on host (pest) mortality and toward a more fundamental understanding of the processes and consequences of mixed infections, addressing these issues in a broad range of invertebrate hosts. For example, honeybees are infected by a wide range of pathogens, and studies are now investigating how they interact and what the consequences are for bee biology (e.g. Doublet et al., 2015; Klinger et al., 2015). A key focus here is the evolution of virulence and why pathogens evolve to have a particular level of virulence (here defined as harm to the host). One of the dominant models is the trade-off model (trade-off between virulence and transmission) (Alizon et al., 2009). Mixed infections are predicted to increase virulence, depending on the nature of the competitive interaction (Alizon et al., 2013). Experimental studies on the rodent malaria parasite, *Plasmodium chabaudi,* resulted in competition in mice that showed a strong relationship between the

within-host strain competition strength and virulence (de Roode et al 2005; Bell et al., 2006). However, other studies have also shown that within-host pathogen competition could cause a decrease in virulence over time. For example, in an experimental evolution study in which two strains of Bt - one pathogenic to the host (the diamondback moth, *P. xylostella*) and the other not - were passaged for several generations in hosts. In this setting, virulence in the mixed infection (compared to single passage of the pathogenic strain) decreased over time. The reduction in virulence was accompanied by an increased capacity to suppress the growth of competitors (antagonism) (Garbutt et al., 2011). These studies have relevance for the use of microbial control agents in situations where secondary cycling of the pathogen (pathogen being transmitted from an infected host to an uninfected host in the population) occurs (Hajek and Shapiro-Ilan, 2017), which is important for ongoing pest suppression within and between generations. Both the yield (production of transmissible stages) and the virulence of the pathogen that results from a mixed infection affect the outcome. However, empirical studies examining whether or how mixed infections alter virulence and transmission are rare. Generally, biodiversity is expected to improve natural pest control (Cardinale et al., 2006). There is support for this in studies with predators (e.g., Snyder et al., 2006). A lab experiment examining host mortality from an entomopathogen diversity perspective found that increasing pathogen species richness (combinations of *B. bassiana* and/or the nematodes *Heterorhabditis megidis, Steinernema carpocapsae*, and *S. feltiae*) increased mortality in the Colorado potato beetle (*Leptinotarsa decemlineata* (Coleoptera: Chrysomelidae)) and wax moth (*G. mellonella*), particularly when any nematode species were combined with *B. bassiana* (Jabbour et al., 2011). Although the study found potential evidence of complementarity or facilitation between nematode-fungus species, more experimental data are needed to determine the mechanisms underlying those interactions. At a slightly larger scale, a greenhouse experiment demonstrated that a combination of the nematode *Steinernema kraussei* and the fungus *M. anisopliae* resulted in an increase in the mortality of overwintering larvae of the black vine weevil (*Otiorhynchus sulcatus* F. (Coleoptera: Curculionidae)) (Ansari et al., 2010). Larger-scale population-level experiments on the impact of mixed pathogen applications on pest suppression or insect population dynamics are very rare. An exception is a recent study looking at the impact of combining *Bacillus thuringiensis* kurstaki (Btk) with an NPV application on cabbage moth (*Mamestra brassicae* (Lepidoptera: Noctuidae)) mortality, using small scale (1 m^2) field plots. Focusing on the consequences for the NPV, the study showed that co-inoculation resulted

in proportionally more cadavers with observable NPV replication (compared to the virus alone), even when the virus was applied 4 days after the Btk. However, secondary transmission of virus to a second generation of *M. brassicae* was lower, as co-exposure with Btk reduced the size of the cadavers and thus the amount of NPV inoculum produced (Hesketh and Hails, 2015). These few studies illustrate that there might be promise in using mixed pathogen applications for pest management. The study of mixed pathogen infections can also help us better understand the dynamics of newly introduced pathogens within a host population, whether they are invasive or introduced through pest management practices. A long-term field study of spongy moth (formerly gypsy moth (*Lymantria dispar* (Lepidoptera: Lymantriidae))) populations in the United States, where it is invasive, showed that a newly emerging specific fungal pathogen, *Entomophaga maimaiga*, was now a dominant pathogen, taking over from a species-specific baculovirus (*Lymantria dispar* multinucleopolyhedrovirus, LdMNPV) that had previously caused epizootics in outbreak populations. It also found that the fungus seems to outcompete parasitoids; the authors hypothesized that this was because the fungus developed more quickly, and both the fungus and the parasitoids needed to kill their host to complete their development (parasitoid) or produce transmissible stages (fungus). Virus and fungus were found to co-infect in the field, which supports the idea that they are only competing for resources (Hajek and van Nouhuys, 2016).

1.3. Host nutrition and within-host pathogen interactions

Another important element in host-pathogen and pathogen-pathogen interactions is the role of host nutrition. Resource availability, host plant quality along with their secondary chemicals, can influence the effect of many different pathogens. In single pathogen infections, limited resource availability (quantity), such as starvation and nutritional stress scenarios, have been shown to increase pathogen virulence, increasing host susceptibility to infection (Brown et al., 2000; Lord, 2010) and shortening the duration of pathogen infection (Furlong and Groden, 2003). Changes in nutritional availability can influence insect immune function, such as rapidly decreasing phenoloxidase activity in the mealworm beetle *Tenebrio molitor* (Siva-Jothy and Thompson, 2002). Similarly, high resource quality can increase host developmental rate or enable the host to increase resource allocation to immunity (Lee et al., 2002; kay et al 2014; Cotter and Al Shareefi 2022), affecting insect resistance to infection. Macronutrients, such as protein and

carbohydrates, and their overall balance in a diet have been shown to influence host susceptibility to infection. Indeed, higher levels of protein relative to carbohydrates in Lepidopteran larval diets increase host resistance to both bacteria and baculoviruses (McVean et al., 2002; Lee et al., 2006; Povey et al., 2009, 2014; Shikano and Cory, 2016). In addition, when given the choice, larvae that survived virus challenge increased their intake of protein-biased food after infection (Lee et al., 2006; Shikano and Cory, 2016).

Plants and their diverse physical and chemical defences against herbivores are a key component of insect pathogen interactions. The plant species (Cory and Ericsson, 2010; Cory and Deschodt, 2017; Shikano, 2017), the behaviour and movements of the insect around the plant (Peter and Shanower, 2001; Sarfraz et al., 2011), as well as the leaf surface chemistry (Peter and Shanower, 2001; Müller and Riederer, 2005; Jaronski, 2010) can affect pathogen persistence or likelihood of infection. Plant architecture played a major role in the persistence of NPV in the winter moth, *Opherophtera brumata* (Lepidoptera, Geometridae), on oak, Sitka spruce trees or heather plants, such that in the forested, shaded habitat, NPVs persisted longer than on heather, where no shading was provided by neighbouring plants (Raymond et al., 2005). Moreover, both *M. brassicae* NPV and *O. brumata* NPV applied on plant stems persisted longer than viruses applied to the leaves (Raymond et al., 2005). Similarly, fungal spore viability, germination capabilities, as well as persistence, have been shown to be affected by plant leaf surface chemistry (Müller and Riederer, 2005). Moreover, pathogens, such as bacteria and viruses, that need to be ingested to initiate infection, can interact directly with the plant material in the insect host midgut. The host plant can also affect the efficacy of pathogen infection through direct and indirect effects on the insect hosts themselves (Cory and Hoover, 2006; Cory and Deschodt, 2017). The complex interaction between plants, insects and their pathogens increases the variability in microbial control agent efficacy in the field. More broadly, the influence of insect nutrition on pathogen interactions is complex and is likely to have important consequences on host-pathogen and pathogen community dynamics as well as pathogen evolution in natural populations.

How variation in resources and host plants affect pathogen-pathogen interaction in invertebrates has received less attention. Exceptions are experiments on the infection of mosquito larvae, *Aedes aegypti* with two microsporidian parasites, *Vavraia culicis* and *Edhazardia aedis*, which showed that resource availability had an effect on both host mortality and potential pathogen transmission (Zilio and Koella, 2020). Similarly, Tritschler

et al., (2017) showed that providing the honey bee, *Apis mellifera*, with a protein supplement (pollen) increased host survival after infection by the deformed wing virus and a microsporidian pathogen, *Nosema ceranae*. Pollen-fed insects showed an increase in *N. ceranae* spore load, and there was a strong negative effect of the microsporidian on virus replication (Tritschler et al., 2017). However, the role of host nutrition on the outcome of multiple pathogen interactions within a single host and the consequences at the host population level remain largely unexplored.

1.4. Study system

1.4.1. Insects

The cabbage looper moth, *Trichoplusia ni* Hübner (Lepidoptera: Noctuidae), is a major pest along its migration route, from Mexico and California to Canada (Franklin et al., 2010; Erlandson, 2013) but occurs worldwide (Asia and Europe). Cabbage looper larval stages consist of 5 separate instars prior to pupation and the adult stage. The larvae are polyphagous herbivores at all instars, but they are particularly voracious during the final instars of their larval development. They can develop on more than 160 different host plant species (Sutherland and Greene, 1984), including all cruciferous plants, e.g., cabbage or broccoli, as well as a variety of greenhouse vegetable crops such as tomato and pepper, and other field crops such as potato, soybean and cotton. In Canada, during the summer, the *T. ni* life cycle from egg to adult takes approximately 24-33 days (temperature dependant). This rapid growth and ability to use a wide variety of host plants makes them particularly resilient as a pest species. However, their developmental time and fecundity is highly influenced by the host plant they feed on (Cameron et al., 2007; Coapio et al., 2017; Farias et al., 2022). Microbial control of *T. ni* populations is primarily accomplished by the use of *Bacillus thuringiensis* kurstaki (Btk) commercial insecticide; however, the development of resistant populations, especially in greenhouses in British Columbia (Janmaat and Myers, 2003), has reduced biological control options for this pest. Moreover, resistance of other lepidopteran pests to Bt has also been shown in the field (Tabashnik et al., 2013; Cory, 2017). *Trichoplusia ni* is an excellent candidate to explore the efficacy of other microbial control agents, as well as their interaction.

1.4.2. Entomopathogens

Baculovirus: TniSNPV

Baculoviruses are double-stranded DNA viruses that have been isolated from a large number of insects, particularly from Lepidoptera (Cory and Myers, 2003). Within the Baculoviridae, both nucleopolyhedroviruses (NPVs) and granulovirus (GVs) have received considerable research attention due to their obvious role in population dynamics in some species, as well as their potential as microbial control agents because of their high virulence (Lacey et al., 2015; Landwehr, 2021). Currently, *Autographa californica* multiple nucleopolyhedrovirus, AcMNPV, isolated from *T. ni* larvae, has been registered to control *T. ni* population in the field (Loopex FC®). Although not all species are equally susceptible, AcMPNV has been shown to have a broad host range, including across at least 25 lepidopteran families (Cory and Myers, 2003). However, field application of Loopex mainly targeted *T. ni* larvae (Franklin 2020). On the other hand, *Trichoplusia ni* nucleopolyhedrovirus (TniSNPV) has been shown to have a narrow host range, mainly restricted to *T. ni* larvae (Del Rincón-Castro and Ibarra, 1997). As with other baculoviruses, TniSNPV needs to be ingested and infections are initiated when the transmission stages, called occlusion bodies (OBs), disintegrate in the host midgut releasing multiple occlusion-derived virions, ODVs (Blissard and Theilmann, 2018). Infection is biphasic; infection within the host is propagated by non-occluded virus (budded virus) which is produced and spreads infection from the midgut epithelium to the tracheal system and then throughout the host tissues (Blissard and Theilmann, 2018). At the end of the infection cycle, the larger, proteinaceous occluded OB transmission stages are produced and are then released into the environment when the host dies and liquefies (Fig. 1.2) (Volkman, 1997). The proteinaceous matrix of the OB protects the virions and allows them to persist outside of the host, sometimes for considerable periods of time (if protected from UV irradiation) (Jaques 1967; Thompson et al., 1981).

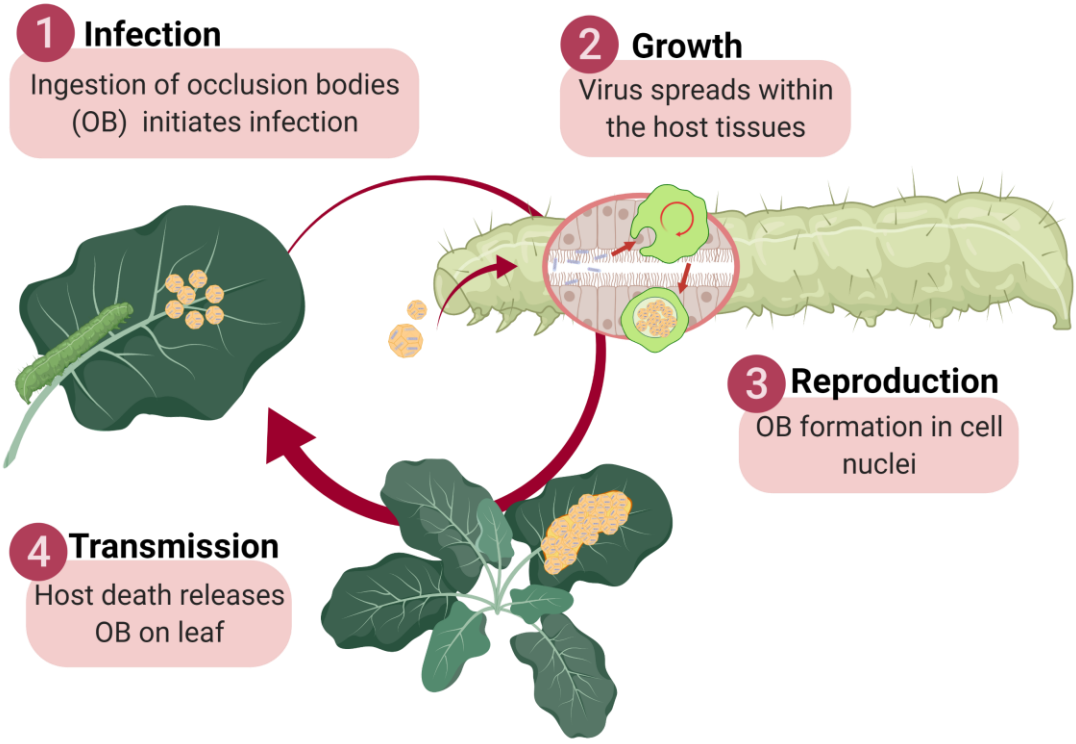

Figure 1.2. Life cycle of TniSNPV in *T. ni*.
Created with BioRender.com

Fungus: B. bassiana

The entomopathogenic fungus *Beauveria bassiana* is a generalist insect pathogen belonging to the order Hypocreales (Ascomycota) and was first discovered and studied in Italy in the 1830s (Zimmermann, 2007). Since then, *Beauveria bassiana* has been shown to be widely distributed all around the world. In Canada, *B. bassiana* has been isolated in more than 60 different insect species (Zimmermann, 2007), mainly Lepidoptera, but it is present in most insect Orders, as well as in Acari. The initiation of *B. bassiana* fungal infection occurs percutaneously when fungal spores adhere to the host cuticle and germinate. After breaking down through the different layers of the host cuticle, the fungus will invade the hemolymph, spreading through the host tissues as yeast-like blastospores (hyphal bodies) (Butt et al., 2016; Mascarin and Jaronski, 2016). After host death, fungal spores are produced on the insect cadavers (Fig. 1.3). Spore production, as well as dispersal, are highly dependent on temperature and humidity conditions (Hajek and St. Leger, 1994). The fungus is produced commercially, as Botanigard® ES, strain GHA, and

this product is widely used in greenhouses against aphids or whiteflies but has also been shown to be effective against *T. ni* (Cory and Ericsson, 2010).

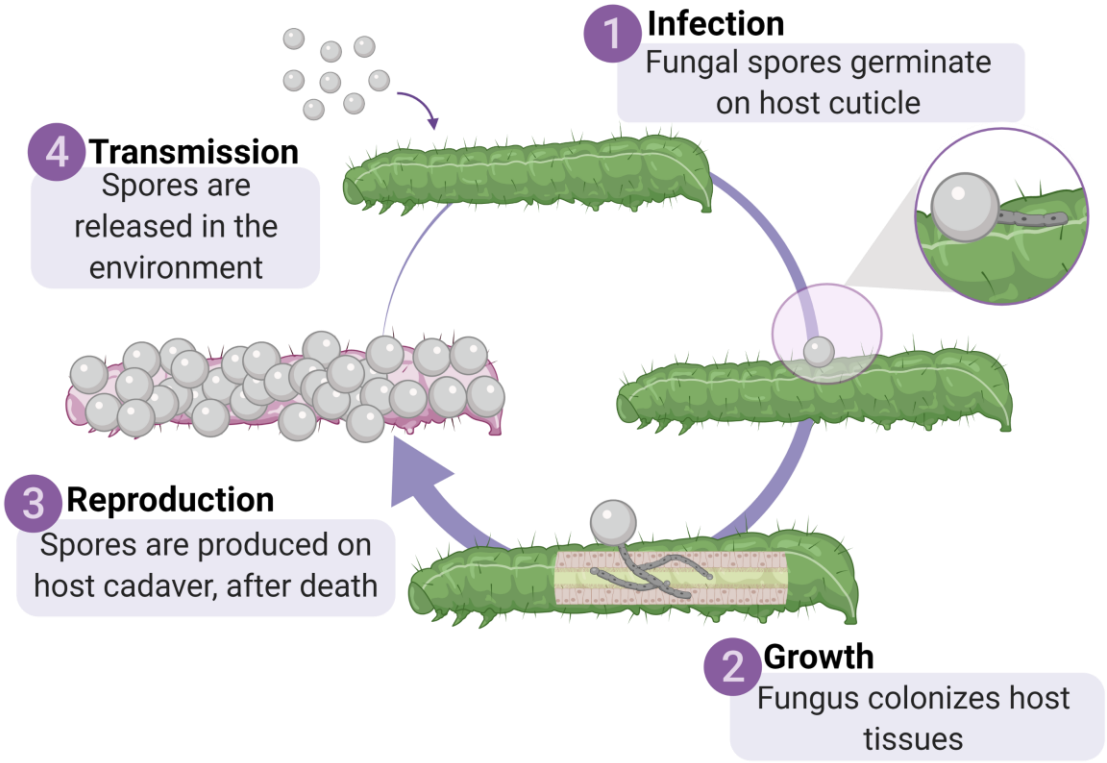

Figure 1.3. Life cycle of *B. bassiana* in *T. ni*.
Created with BioRender.com

1.5. Thesis objectives and chapters

In this thesis, I explore virus-fungus interactions under variable host nutritional scenarios using both artificial, more controlled resources (diet), and natural host plants. I investigated both host and pathogen fitness to better understand the complex interactions that modulate mixed infections and their possible impact on the efficacy of microbial insecticides and their implications for insect population dynamics.

In chapter 2, I explore the effects of host resource limitation on the outcome of mixed pathogen infections. In nature, access to resources can be limited or fluctuate over time; however, little is known about how, or even if, resource availability matters when multiple pathogens target the same host. I used diluted artificial diets to first examine how diet availability affected *T. ni* mortality in single and mixed infections. I then focused on pathogen fitness by investigating the production of pathogen transmission stages (yield).

In chapter 3, I study another aspect of insect nutritional ecology, macronutrient balance (diet quality). Insects use nutrients, such as protein and carbohydrates in different amounts throughout their development depending on their needs and the availability of those macronutrients. In this chapter, I explore the effect of protein-to-carbohydrate imbalance on host survival after single and mixed pathogen infections. Both host mortality and pathogen reproduction were investigated, in addition to the size of the virus OB and virus virulence after mixed infections.

In chapter 4, I examine the outcome of mixed pathogen interactions and pathogen priority effects using natural resources. Host plants are more complex than artificial diets and have the potential to directly or indirectly alter pathogen infection outcomes and competition within the host. Broccoli and cucumber leaves were provided to challenged *T.ni* larvae to determine whether plant identity could alter the outcome of virus-fungus co-infection.

In chapter 5, I investigate whether applying one or two pathogens in the field could increase pest mortality and whether the virus-fungus co-application outcome was affected by host plant species (broccoli vs tomato), as well as the timing of pathogen exposure.

Finally, in the conclusion chapter, I review the major findings of my thesis and discuss future research questions.

Chapter contribution

A modified version of Chapter 1 (Pathogen-pathogen Interactions) has been published: Cory, J. S. and Deschodt, P. (2017) The Biotic Environment, in: Hajek, A.E., Shapiro-Ilan, D.I. (Eds.), Ecology of Invertebrate Diseases. John Wiley & Sons, Ltd, Chichester, UK, pp. 187–212. https://doi.org/10.1002/9781119256106.ch6

1.6. References

Alizon, S., Hurford, A., Mideo, N., and Van Baalen, M. (2009). Virulence evolution and the trade-off hypothesis: history, current state of affairs and the future. *J. Evol. Biol.* 22, 245–259. doi:10.1111/j.1420-9101.2008.01658.x.

Alizon, S., de Roode, J. C., and Michalakis, Y. (2013). Multiple infections and the evolution of virulence. *Ecol. Lett.* 16, 556–567. doi:10.1111/ele.12076.

Alizon, S. (2008). Decreased overall virulence in coinfected hosts leads to the persistence of virulent parasites. *Am. Nat.* 172, E67-79. doi:10.1086/588077.

Ansari, M. A., Shah, F. A., and Butt, T. M. (2010). The entomopathogenic nematode *Steinernema kraussei* and *Metarhizium anisopliae* work synergistically in controlling overwintering larvae of the black vine weevil, *Otiorhynchus sulcatus,* in strawberry growbags. *Biocontrol Sci. Technol.* 20, 99–105. doi:10.1080/09583150903420031.

Araújo, J. P. M., and Hughes, D. P. (2016). “Diversity of entomopathogenic fungi,” in *Genetics and molecular biology of entomopathogenic fungi* Advances in Genetics. (Elsevier), 1–39. doi:10.1016/bs.adgen.2016.01.001.

Bartolomé, C., Buendía-Abad, M., Benito, M., Sobrino, B., Amigo, J., Carracedo, A., Martín-Hernández, R., Higes, M., and Maside, X. (2020). Longitudinal analysis on parasite diversity in honeybee colonies: new taxa, high frequency of mixed infections and seasonal patterns of variation. *Sci. Rep*. 10, 10454. doi:10.1038/s41598-020-67183-3.

Bashey, F., Hawlena, H., and Lively, C. M. (2013). Alternative paths to success in a parasite community: within-host competition can favor higher virulence or direct interference. *Evolution* 67, 900–907. doi:10.1111/j.1558-5646.2012.01825.x.

Bell, A. S., de Roode, J. C., Sim, D., and Read, A. F. (2006). Within-host competition in genetically diverse malaria infections: parasite virulence and competitive success. *Evolution* 60, 1358–1371. doi:10.1111/j.0014-3820.2006.tb01215.x.

Ben-Ami, F., Rigaud, T., and Ebert, D. (2011). The expression of virulence during double infections by different parasites with conflicting host exploitation and transmission strategies. *J. Evol. Biol.* 24, 1307–1316. doi:10.1111/j.1420-9101.2011.02264.x.

Ben-Ami, F., and Routtu, J. (2013). The expression and evolution of virulence in multiple infections: the role of specificity, relative virulence and relative dose. *BMC Evol. Biol.* 13, 97. doi:10.1186/1471-2148-13-97.

Blissard, G. W., and Rohrmann, G. F. (1990). Baculovirus diversity and molecular biology. *Annu. Rev. Entomol.* 35, 127–155. doi:10.1146/annurev.en.35.010190.001015.

Blissard, G. W., and Theilmann, D. A. (2018). Baculovirus Entry and Egress from Insect Cells. *Annu. Rev. Virol.* 5, 113–139. doi:10.1146/annurev-virology-092917-043356.

Brown, M. J. F., Loosli, R., and Schmid-Hempel, P. (2000). Condition-dependent expression of virulence in a trypanosome infecting bumblebees. *Oikos* 91, 421–427. doi:10.1034/j.1600-0706.2000.910302.x.

Butt, T. M., Coates, C. J., Dubovskiy, I. M., and Ratcliffe, N. A. (2016). Entomopathogenic Fungi: New Insights into Host-Pathogen Interactions. *Adv. Genet.* 94, 307–364. doi:10.1016/bs.adgen.2016.01.006.

Cameron, J. H., Isman, M. B., and Upadhyaya, M. K. (2007). *Trichoplusia ni* growth and preference on broccoli and eight common agricultural weeds. *Can. J. Plant Sci.* 87, 413–421. doi:10.4141/P06-032.

Cardinale, B. J., Srivastava, D. S., Duffy, J. E., Wright, J. P., Downing, A. L., Sankaran, M., and Jouseau, C. (2006). Effects of biodiversity on the functioning of trophic groups and ecosystems. *Nature* 443, 989–992. doi:10.1038/nature05202.

Choisy, M., and de Roode, J. C. (2010). Mixed infections and the evolution of virulence: effects of resource competition, parasite plasticity, and impaired host immunity. *Am. Nat.* 175, E105-18. doi:10.1086/651587.

Clay, P. A., and Rudolf, V. H. W. (2019). How parasite interaction strategies alter virulence evolution in multi-parasite communities. *Evolution* 73, 2189–2203. doi:10.1111/evo.13843.

Coapio, G. G., Cruz-López, L., Guerenstein, P., Malo, E. A., and Rojas, J. C. (2017). Oviposition preference and larval performance and behavior of *Trichoplusia ni* (Lepidoptera: Noctuidae) on host and nonhost plants. *Arthropod Plant Interact.* 12, 1–10. doi:10.1007/s11829-017-9566-9.

Cory, J. S., and Bishop, D. H. (1997). Use of baculoviruses as biological insecticides. *Mol. Biotechnol.* 7, 303–313. doi:10.1007/BF02740821.

Cory, J. S., and Deschodt, P. S. (2017). “The Biotic Environment,” in *Ecology of invertebrate diseases*, eds. A. E. Hajek and D. I. Shapiro-Ilan (Chichester, UK: John Wiley & Sons, Ltd), 187–212. doi:10.1002/9781119256106.ch6.

Cory, J. S., and Ericsson, J. D. (2010). Fungal entomopathogens in a tritrophic context. *Biocontrol* 55, 75–88. doi:10.1007/s10526-009-9247-4.

Cory, J. S., and Hoover, K. (2006). Plant-mediated effects in insect-pathogen interactions. *Trends Ecol. Evol.* 21, 278–286. doi:10.1016/j.tree.2006.02.005.

Cory, J. S., and Myers, J. H. (2003). The ecology and evolution of insect baculoviruses. *Annu. Rev. Ecol. Evol. Syst.* 34, 239–272. doi:10.1146/annurev.ecolsys.34.011802.132402.

Cory, J. S. (2017). Evolution of host resistance to insect pathogens. *Curr. Opin. Insect Sci.* 21, 54–59. doi:10.1016/j.cois.2017.04.008.

Cotter, S. C., and Al Shareefi, E. (2022). Nutritional ecology, infection and immune defence - exploring the mechanisms. *Curr. Opin. Insect Sci*. 49, xx-yy. doi:10.1016/j.cois.2021.12.002.

Cox, F. E. (2001). Concomitant infections, parasites and immune responses. *Parasitology* 122 Suppl, S23-38.

Del Rincón-Castro, Ma. C., and Ibarra, J. E. (1997). Genotypic divergence of three single nuclear polyhedrosis virus (SNPV) strains from the cabbage looper, Trichoplusia ni. *Biochem. Syst. Ecol.* 25, 287–295. doi:10.1016/S0305-1978(97)00002-1.

de Roode, J. C., Pansini, R., Cheesman, S. J., Helinski, M. E. H., Huijben, S., Wargo, A. R., Bell, A. S., Chan, B. H. K., Walliker, D., and Read, A. F. (2005). Virulence and competitive ability in genetically diverse malaria infections. *Proc Natl Acad Sci USA* 102, 7624–7628. doi:10.1073/pnas.0500078102.

Doublet, V., Natsopoulou, M. E., Zschiesche, L., and Paxton, R. J. (2015). Within-host competition among the honey bees pathogens *Nosema ceranae* and Deformed wing virus is asymmetric and to the disadvantage of the virus. *J. Invertebr. Pathol.* 124, 31–34. doi:10.1016/j.jip.2014.10.007.

Ebert, D., Zschokke-Rohringer, C. D., and Carius, H. J. (2000). Dose effects and density-dependent regulation of two microparasites of *Daphnia magna*. *Oecologia* 122, 200–209. doi:10.1007/PL00008847.

Erlandson, M. A. (2013). “Trichoplusia ni Hübner, cabbage looper (Lepidoptera: Noctuidae).,” in *Biological control programmes in Canada 2001-2012*, eds. P. G. Mason and D. R. Gillespie (Wallingford: CABI), 291–295. doi:10.1079/9781780642574.0291.

Farias, E. S., Farias, A. A., Santos, R. C., Santos, A. A., and Picanço, M. C. (2022). Forecasting the seasonal dynamics of *Trichoplusia ni* (Lep.: Noctuidae) on three Brassica crops through neural networks. *Int. J. Biometeorol.* doi:10.1007/s00484-022-02244-y.

Fellous, S., and Koella, J. C. (2009). Infectious dose affects the outcome of the within-host competition between parasites. *Am. Nat.* 173, E177-84. doi:10.1086/598490.

Franklin, M. (2020) Biocontrol products for control of insect pests and plant diseases: Practical solutions for today and the future in 62nd Annual Horticulture Growers’ Short Course. Eds: Todd Kabaluk and Lisa Frey

Franklin, M. T., Ritland, C. E., and Myers, J. H. (2010). Spatial and temporal changes in genetic structure of greenhouse and field populations of cabbage looper, Trichoplusia ni. *Mol. Ecol.* 19, 1122–1133. doi:10.1111/j.1365-294X.2010.04548.x.

Furlong, M. J., and Groden, E. (2003). Starvation induced stress and the susceptibility of the Colorado potato beetle, *Leptinotarsa decemlineata*, to infection by *Beauveria bassiana*. *Journal of invertebrate pathology* 83, 127–138. doi:10.1016/S0022-2011(03)00066-1.

Garbutt, J., Bonsall, M. B., Wright, D. J., and Raymond, B. (2011). Antagonistic competition moderates virulence in *Bacillus thuringiensis*. *Ecol. Lett.* 14, 765–772. doi:10.1111/j.1461-0248.2011.01638.x.

Georgievska, L., Hoover, K., van der Werf, W., Muñoz, D., Caballero, P., Cory, J. S., and Vlak, J. M. (2010). Dose dependency of time to death in single and mixed infections with a wildtype and egt deletion strain of *Helicoverpa armigera* nucleopolyhedrovirus. *J. Invertebr. Pathol.* 104, 44–50. doi:10.1016/j.jip.2010.01.008.

Hackett, K. J., Boore, A., Deming, C., Buckley, E., Camp, M., and Shapiro, M. (2000). *Helicoverpa armigera* granulovirus interference with progression of *H. zea* nucleopolyhedrovirus disease in *H. zea* larvae. *J. Invertebr. Pathol.* 75, 99–106. doi:10.1006/jipa.1999.4914.

Hajek, A. E., McManus, M. L., and Delalibera, I. (2007). A review of introductions of pathogens and nematodes for classical biological control of insects and mites. *Biological Control* 41, 1–13. doi:10.1016/j.biocontrol.2006.11.003.

Hajek, A. E., and Shapiro-Ilan, D. I. (2017). “General concepts in the ecology of invertebrate diseases,” in *Ecology of invertebrate diseases* (Chichester, UK: John Wiley & Sons, Ltd), 1–17. doi:10.1002/9781119256106.ch1.

Hajek, A. E., and St. Leger, R. J. (1994). Interactions between fungal pathogens and insect hosts. *Annu. Rev. Entomol.* 39, 293–322. doi:10.1146/annurev.en.39.010194.001453.

Hajek, A. E., and Tobin, P. C. (2011). Introduced pathogens follow the invasion front of a spreading alien host. *J. Anim. Ecol.* 80, 1217–1226. doi:10.1111/j.1365-2656.2011.01870.x.

Hajek, A. E., and van Nouhuys, S. (2016). Fatal diseases and parasitoids: from competition to facilitation in a shared host. *Proc. Biol. Sci.* 283. doi:10.1098/rspb.2016.0154.

Harper, J.D., 1986. Interactions between baculoviruses and other entomopathogens, chemical pesticides and parasitoids, in: Granados, R.R., Federici, B.A. (eds.), The Biology of Baculoviruses, Vol. 1. CRC Press, Boca Raton, FL, pp. 133–135.

Hesketh, H., and Hails, R. S. (2015). *Bacillus thuringiensis* impacts on primary and secondary baculovirus transmission dynamics in Lepidoptera. *J. Invertebr. Pathol.* 132, 171–181. doi:10.1016/j.jip.2015.09.008.

Ishii, T., Takatsuka, J., Nakai, M., and Kunimi, Y. (2002). Growth characteristics and competitive abilities of a nucleopolyhedrovirus and an entomopoxvirus in larvae of the smaller tea tortrix, *Adoxophyes honmai* (Lepidoptera: Tortricidae). *Biological Control* 23, 96–105. doi:10.1006/bcon.2001.0988.

Jabbour, R., Crowder, D. W., Aultman, E. A., and Snyder, W. E. (2011). Entomopathogen biodiversity increases host mortality. *Biological Control* 59, 277–283. doi:10.1016/j.biocontrol.2011.07.016.

Janmaat, A. F., and Myers, J. (2003). Rapid evolution and the cost of resistance to *Bacillus thuringiensis* in greenhouse populations of cabbage loopers, *Trichoplusia ni. Proc. Biol. Sci.* 270, 2263–2270. doi:10.1098/rspb.2003.2497.

Jaques, R.P. (1967) The persistence of a nuclear polyhedrosis virus in habitat of host insect *Trichoplusia ni.* I. Polyhedra deposited on foliage. *Can Entomol* 99(8):820–829

Jaronski, S. T. (2010). Ecological factors in the inundative use of fungal entomopathogens. *Biocontrol* 55, 159–185. doi:10.1007/s10526-009-9248-3.

Junglen, S., and Drosten, C. (2013). Virus discovery and recent insights into virus diversity in arthropods. *Curr. Opin. Microbiol.* 16, 507–513. doi:10.1016/j.mib.2013.06.005.

Kay, A. D., Bruning, A. J., van Alst, A., Abrahamson, T. T., Hughes, W. O. H., and Kaspari, M. (2014). A carbohydrate-rich diet increases social immunity in ants. *Proc. Biol. Sci*. 281, 20132374. doi:10.1098/rspb.2013.2374.

Klinger, E. G., Vojvodic, S., DeGrandi-Hoffman, G., Welker, D. L., and James, R. R. (2015). Mixed infections reveal virulence differences between host-specific bee pathogens. *J. Invertebr. Pathol.* 129, 28–35. doi:10.1016/j.jip.2015.05.003.

Lacey, L. A., Grzywacz, D., Shapiro-Ilan, D. I., Frutos, R., Brownbridge, M., and Goettel, M. S. (2015). Insect pathogens as biological control agents: Back to the future. *J. Invertebr. Pathol.* 132, 1–41. doi:10.1016/j.jip.2015.07.009.

Landwehr, A. (2021). Benefits of baculovirus use in IPM strategies for open field and protected vegetables. *Front. Sustain. Food Syst.* 4. doi:10.3389/fsufs.2020.593796.

Lee, K. P., Behmer, S. T., Simpson, S. J., and Raubenheimer, D. (2002). A geometric analysis of nutrient regulation in the generalist caterpillar Spodoptera littoralis (Boisduval). *J. Insect Physiol*. 48, 655–665. doi:10.1016/s0022-1910(02)00088-4.

Lee, K. P., Cory, J. S., Wilson, K., Raubenheimer, D., and Simpson, S. J. (2006). Flexible diet choice offsets protein costs of pathogen resistance in a caterpillar. *Proc. Biol. Sci.* 273, 823–829. doi:10.1098/rspb.2005.3385.

Lord, J. C. (2010). Dietary stress increases the susceptibility of *Tribolium castaneum* to *Beauveria bassiana*. *J. Econ. Entomol.* 103, 1542–1546. doi:10.1603/EC09311.

Malakar, R., Elkinton, J. S., Carroll, S. D., and Amico, V. D. (1999). Interactions between two gypsy moth (Lepidoptera: Lymantriidae) pathogens : Nucleopolyhedrovirus and *Entomophaga maimaiga* (Zygomycetes: Entomophthorales): Field studies and a simulation model. *Biol Control* 16, 189–198.

Mantzoukas, S., Milonas, P., Kontodimas, D., and Angelopoulos, K. (2013). Interaction between the entomopathogenic bacterium *Bacillus thuringiensis* subsp. kurstaki and two entomopathogenic fungi in bio-control of *Sesamia nonagrioides* (Lefebvre) (Lepidoptera: Noctuidae). *Ann. Microbiol.* 63, 1083–1091. doi:10.1007/s13213-012-0565-x.

Marchetto, K. M., and Power, A. G. (2018). Coinfection Timing Drives Host Population Dynamics through Changes in Virulence. *Am. Nat.* 191, 173–183. doi:10.1086/695316.

Mascarin, G. M., and Jaronski, S. T. (2016). The production and uses of *Beauveria bassiana* as a microbial insecticide. *World J. Microbiol. Biotechnol.* 32, 177. doi:10.1007/s11274-016-2131-3.

McVean, R. I., Sait, S. M., Thompson, D. J., and Begon, M. (2002). Effects of resource quality on the population dynamics of the Indian meal moth *Plodia interpunctella* and its granulovirus. *Oecologia* 131, 71–78. doi:10.1007/s00442-001-0862-8.

Mideo, N. (2009). Parasite adaptations to within-host competition. *Trends Parasitol.* 25, 261–268. doi:10.1016/j.pt.2009.03.001.

Müller, C., and Riederer, M. (2005). Plant surface properties in chemical ecology. *J. Chem. Ecol.* 31, 2621–2651. doi:10.1007/s10886-005-7617-7.

Nakai, M., Kumini, Y., (1998). Effects of the timing of entomopoxvirus administration to the smaller tea tortrix, *Adoxophyes sp*. (Lepidoptera: Tortricidae) on the survival of the endoparasitoid, *Ascogaster reticulatus* (Hymenoptera: Braconidae). *Biol. Contr*. 13, 63–69.

Peter, A. J., and Shanower, T. G. (2001). “Role of plant surface in resistance to insect herbivores,” in *Insects and plant defence dynamics*, ed. T. N. Ananthakrishnan (Enfield, NH, USA.: Science Publishers, Inc.), 107–132.

Povey, S., Cotter, S. C., Simpson, S. J., Lee, K. P., and Wilson, K. (2009). Can the protein costs of bacterial resistance be offset by altered feeding behaviour? *J. Anim. Ecol.* 78, 437–446. doi:10.1111/j.1365-2656.2008.01499.x.

Povey, S., Cotter, S. C., Simpson, S. J., and Wilson, K. (2014). Dynamics of macronutrient self-medication and illness-induced anorexia in virally infected insects. *J. Anim. Ecol.* 83, 245–255. doi:10.1111/1365-2656.12127.

Raymond, B., Hartley, S. E., Cory, J. S., and Hails, R. S. (2005). The role of food plant and pathogen-induced behaviour in the persistence of a nucleopolyhedrovirus. *J. Invertebr. Pathol.* 88, 49–57. doi:10.1016/j.jip.2004.09.005.

Rosenheim, J. A. (1995). Intraguild predation amoing biological- control agents: Theory and evidence. *Biol Control* 5, 303–335.

Sandoval-Aguilar, J. A., Guzmán-Franco, A. W., Pell, J. K., Clark, S. J., Alatorre-Rosas, R., Santillán-Galicia, Ma. T., and Valdovinos-Ponce, G. (2015). Dynamics of competition and co-infection between *Zoophthora radicans* and *Pandora blunckii* in *Plutella xylostella* larvae. *Fungal Ecol.* 17, 1–9. doi:10.1016/j.funeco.2015.04.003.

Sarfraz, R. M., Cervantes, V., and Myers, J. H. (2011). The effect of host plant species on performance and movement behaviour of the cabbage looper *Trichoplusia ni* and their potential influences on infection by *Autographa californica* multiple nucleopolyhedrovirus. *Agric. For. Entomol.* 13, 157–164. doi:10.1111/j.1461-9563.2010.00504.x.

Shanchez-Contreras, M., and Vlisidou, I. (2008). The diversity of insect-bacteria interactions and its applications for disease control. *Biotechnol. Genet. Eng. Rev.* 25, 203–243. doi:10.5661/bger-25-203.

Shikano, I., and Cory, J. S. (2016). Altered nutrient intake by baculovirus-challenged insects: Self-medication or compensatory feeding? *J. Invertebr. Pathol.* 139, 25–33. doi:10.1016/j.jip.2016.07.005.

Shikano, I. (2017). Evolutionary ecology of multitrophic interactions between plants, insect herbivores and entomopathogens. *J. Chem. Ecol.* 43, 586–598. doi:10.1007/s10886-017-0850-z.

Sih, A., Englund, G., Wooster, D., 1998. Emergent impacts of multiple predators on prey. *Trends Ecol. Evol*. 13, 350–355.

Siva-Jothy, M. T., and Thompson, J. J. W. (2002). Short-term nutrient deprivation affects immune function. *Physiol. Entomol.* 27, 206–212. doi:10.1046/j.1365-3032.2002.00286.x.

Snyder, W.E., Snyder, G.B., Finke, D.L., Straub, D.S. (2006). Predator diversity strengthens herbivore suppression. *Ecol. Lett*. 9, 789–796.

St. Leger, R. J., Wang, C., and Fang, W. (2011). New perspectives on insect pathogens. *Fungal Biol. Rev.* 25, 84–88. doi:10.1016/j.fbr.2011.04.005.

Staves, P. A., and Knell, R. J. (2010). Virulence and competitiveness: testing the relationship during inter- and intraspecific mixed infections. *Evolution* 64, 2643–2652. doi:10.1111/j.1558-5646.2010.00999.x.

Sutherland, D. W. S., and Greene, G. L. (1984). “Cultivated and wild host plants,” in *Suppression and management of cabbage looper populations*, eds. P. D. Lingren and G. L. Greene (Technical Bulletin No. 1684, United States Department of Agriculture.), 1–13.

Tabashnik, B. E., Brévault, T., and Carrière, Y. (2013). Insect resistance to Bt crops: lessons from the first billion acres. *Nat. Biotechnol.* 31, 510–521. doi:10.1038/nbt.2597.

Thompson, C. G., Scott, D.W., Wickman, B.E. (1981). Long-term persistence of the nuclear polyhedrosis virus of the Douglas-fir tussock moth, *Orgyia pseudotsugata* (Lepidoptera: Lymantriidae), in Forest soil. *Environ Entomol* 10(2):254–255

Tokarev, Y.S., Levchenko, M.V., Naumov, M.V., Senderskiy, I.V., Lednev, G.R. (2011). Interactions of two insect pathogens, *Paranosema locustae* (Protista: Microsporidia) and *Metarhizium acridum* (Fungi: Hypocreales), during a mixed infection of *Locusta migratoria* (Insects: Orthoptera) nymphs. *J. Invertebr. Pathol*. 106, 336–338.

Tritschler, M., Vollmann, J. J., Yañez, O., Chejanovsky, N., Crailsheim, K., and Neumann, P. (2017). Protein nutrition governs within-host race of honey bee pathogens. *Sci. Rep.* 7, 1–11. doi:10.1038/s41598-017-15358-w.

Trzebny, A., Slodkowicz-Kowalska, A., Becnel, J. J., Sanscrainte, N., and Dabert, M. (2020). A new method of metabarcoding Microsporidia and their hosts reveals high levels of microsporidian infections in mosquitoes (Culicidae). *Mol. Ecol. Resour.* 20, 1486–1504. doi:10.1111/1755-0998.13205.

Volkman, L. E. (1997). Nucleopolyhedrovirus interactions with their insect hosts. *Adv. Virus Res.* 48, 313–348. doi:10.1016/s0065-3527(08)60291-2.

Zilio, G., and Koella, J. C. (2020). Sequential co-infections drive parasite competition and the outcome of infection. *J. Anim. Ecol.* 89, 2367–2377. doi:10.1111/1365-2656.13302.

Zimmermann, G. (2007). Review on safety of the entomopathogenic fungi *Beauveria bassiana* and *Beauveria brongniartii*. *Biocontrol Sci. Technol.* 17, 553–596. doi:10.1080/09583150701309006.

Chapter 2.

Resource limitation has a limited impact on the outcome of virus-fungus co-infection in an insect host

2.1. Abstract

Infection by pathogens is strongly affected by the diet or condition of the prospective host. Studies that examine the impact of diet have mainly focussed on single pathogens; however, co-infections within a single host are thought to be common. Different pathogen groups might respond differently to resource availability and diverse infections could increase the costs of host defence, meaning the outcome of mixed infections under varying dietary regimes is likely to be hard to predict. We used the generalist cabbage looper, *Trichoplusia ni* and two of its pathogens, the DNA virus *T. ni* nucleopolyhedrovirus (TniSNPV) and the entomopathogenic fungus *Beauveria bassiana,* to examine how nutrient reduction affected the outcome of mixed pathogen infection. We challenged insects with a low or high effective dose of virus, alone or combined with a single dose of fungus. We manipulated food availability after pathogen challenge by diluting artificial diet with cellulose, a non-nutritious bulking agent, and examined its impact on host and pathogen fitness. Reducing diet quantity did not alter overall or pathogen-specific mortality. In all cases, TniSNPV-induced mortality was negatively affected by fungus challenge. Similarly, *B. bassiana*-induced mortality was negatively affected by TniSNPV challenge, but only at the higher virus dose. Dietary dilution mainly affected *B. bassiana* speed of kill when mixed with a high dose of TniSNPV, with an increase in the duration of fungal infection when cellulose was low (high quantity). One pathogen dominated the production of transmission stages in the cadavers and co-infection did not affect the yield of either pathogen. There was no evidence that co-infections were more costly to the survivors of pathogen challenge. In conclusion, dietary dilution did not determine the outcome of mixed pathogen infection, but it had more subtle effects, that differed between the two pathogens and could potentially alter pathogen recycling and host-pathogen dynamics.

Keywords: Baculovirus, *Beauveria bassiana*, host nutrition, mixed infection, pathogen replication, trade-offs

2.2. Introduction

For any organism, maintaining and mounting an immune response against invaders is energetically costly (Lochmiller and Deerenberg, 2000; Sandland and Minchella, 2003; Schmid-Hempel, 2005). From the host's perspective, limited resources can potentially lead to a decrease in lifespan, as well as an increase in susceptibility to pathogen or parasite infection (Furlong and Groden, 2003; Ayres and Schneider, 2009). In addition, the allocation of resources to defence mechanisms reduces their availability for other processes, including growth and reproduction (Sheldon and Verhulst, 1996; Moret and Schmid-Hempel, 2000; Ayres and Schneider, 2009; Graham et al., 2011; McKean and Lazzaro, 2011). From the pathogen's point of view, a decrease in nutrient availability or host condition could lower the host's resistance to infection, but also alter the capacity for the pathogen to replicate and produce transmission stages (Ponton et al., 2011; Cressler et al., 2014; Mouritsen and Andersen, 2017).

The impact of host nutrition on single pathogen species has received considerable attention and demonstrated that both reduction in food quantity and alteration of nutritional quality can affect host mortality and pathogen yield across a broad range of taxa (e.g. Bedhomme et al., 2004; Lee et al., 2006; Tseng and Myers, 2014; Budischak and Cressler, 2018). However, hosts are often challenged by multiple pathogen species, and thus an important question is how does variable nutrition affect co-infections? In mixed pathogen infections, co-infecting pathogens are predicted to interact both directly and indirectly within the host (i.e. direct interference with each other, exploitation of the same resources or indirect effects via the host immune response) (Mideo, 2009; Staves and Knell, 2010; Cressler et al., 2016). Focussing on studies that have only examined changes in the quantity (but not quality) of host food provided has shown its potential importance on the outcome of co-infections in vertebrates, invertebrates and plants (e.g. Lacroix et al., 2014; Duncan et al., 2015; Wale et al., 2017). In invertebrates, the outcome appears to be highly dependent on the study system (Fellous and Koella, 2010; Reyserhove et al., 2017; Zilio and Koella, 2020) as well as the timing of infection (Lohr et al., 2010; Zilio and Koella, 2020) and the relative pathogen dose (Fellous and Koella, 2009). The majority of studies in invertebrates that have investigated whether diet quantity alters the outcome of mixed infections have used freshwater invertebrates (mainly larval mosquitoes and waterfleas). These organisms have very different feeding mechanisms compared to

terrestrial invertebrates, and in the case of the waterfleas, experiments have tended to focus more on disease transmission as productive infections can extend into adulthood. For the mosquito *Aedes aegypti* infected with two microsporidian parasites (*Vavraia culicis* and *Edhazardia aedis*), halving larval food availability did not affect the outcome of co-infection or the time to death (Duncan et al., 2015; Zilio and Koella, 2020). In contrast, in the same host, Fellous and Koella (2010) found that co-infection with the microsporidian parasite *V. culicis* and the gregarine *Ascogregarina culicis*, depended on the interaction between food availability and infection treatment. It is currently difficult to draw general conclusions across multiple systems and this highlights the need to expand these studies to identify the role that different nutritional scenarios play in host-pathogen evolution and dynamics (Cotter and Al Shareefi, 2021; Duncan et al., 2015).

In this chapter, we investigate the effect of diet availability on mixed pathogen infection using a lepidopteran host, the cabbage looper, *Trichoplusia ni* and two of its pathogens, a *T. ni* specific nucleopolyhedrovirus (TniSNPV) and the generalist entomopathogenic fungus *Beauveria bassiana*. We first examined the effect of host condition and co-infection on host fitness (mortality and pupal weight as a potential cost of fighting off infection) and then analysed pathogen speed of kill and the number of transmission stages produced by each pathogen. We exposed the insects to two doses of the virus to examine how the outcome was influenced by the relative effective dose of each pathogen. Both pathogens require host death for horizontal transmission to occur; however, they differ in their host range, infection route and the symptoms caused in the infected host. TniSNPV transmission stages, called occlusion bodies (OBs), need to be ingested to initiate infection, whereas entomopathogenic fungi, such as *B. bassiana*, initiate infection when the fungal spores germinate on the host cuticle. The symptoms of the two pathogens are very distinct. Virus infection spreads from the mid-gut via the tracheoles to most organs of the larvae, resulting in a swollen, pale body. The transmission stages are not released until after host death, when the fragile integument breaks open to release millions of newly formed OBs (Cory, 2010). With fungal infection, *T. ni* cadavers are usually solid and purple-coloured and fungal spores are only produced after death under optimal humidity and temperature conditions (Meyling and Eilenberg, 2007). We define changes in quantity as the alteration in the amount of food available to the host, either in terms of the time when food is available or the concentration of the food, but where the quality of that food remains constant (same nutrients and ratios of those

components). Here, we diluted artificial diet using cellulose to limit the total amount of macronutrients available for *T. ni* larvae to consume, which would have resulted in increased feeding to gain similar nutrition. We hypothesized that an increase in dietary dilution (reduced diet quantity) would increase host mortality as the two pathogens have very different infection pathways and this should increase the cost of fighting the co-infection and the sublethal costs of survival. We also predicted that reduced host nutrition would result in earlier death and a reduction in the resources available for pathogen replication. In addition, as *B. bassiana* kills its host more rapidly than TniSNPV in single infections, we also predicted that TniSNPV would be at a disadvantage in mixed infections.

2.3. Materials and methods

2.3.1. Insects and pathogens

Trichoplusia ni eggs were obtained from Insect Production Services (Natural Resources Canada, Sault Ste Marie, ON). After hatch, the larvae were reared individually from the neonate stage and maintained at 25°C with a 16L:8D photoperiod on a wheat germ-based artificial diet, containing a protein to carbohydrate ratio of 1p:1.1c (Shikano and Cory, 2014). For the experiment, we used newly moulted 4th instar larvae.

Our focal pathogen was a species-specific baculovirus that was initially isolated from an infected *T. ni* larva collected in the Fraser Valley, British Columbia (Janmaat and Myers, 2005). The TniSNPV isolate was amplified in *T. ni* larvae and then semi-purified using multiple rounds of differential centrifugation. A *Beauveria bassiana* suspension was obtained by diluting the commercial product Botanigard® ES (initial concentration of $2.11*10^{10}$ spores/ml of *B. bassiana* GHA strain). We then estimated the concentration of transmission stages for both pathogens using an improved Neubauer haemocytometer (Hausser Scientific, depth 0.1mm) at 400x magnification. We counted four independent dilutions for each pathogen and took the average as the final concentration.

2.3.2. Pathogen challenge

A total of 70 newly moulted fourth instar larvae were randomly selected for each of the six pathogen treatments (two doses of TniSNPV, *B. bassiana* at a single dose, both pathogens together and no pathogens). Before pathogen challenge, each larva was

transferred individually into a 12-well plate. The insects were then exposed to either 100 or 1,000 TniSNPV OBs (LD30 and LD75 for 4th instar larvae respectively) by placing a 1 µl droplet of virus on a 3 x 2 mm plug of the standard rearing diet. Larvae that had not consumed the diet plug after 24 hrs were removed from the experiment. Larvae were infected with *B. bassiana* by placing a 1 µl droplet of fungal suspension containing $3*10^4$ spores (previously determined LD50) onto the dorsal abdomen of the larva. Larvae challenged with both pathogens were first placed into the 12-well plates containing the virus dose and immediately challenged with the fungus as described above. An additional 70 unchallenged control larvae were given 1µl of distilled water on a 3 x 2 mm diet plug and another 1 µl of distilled water on their dorsal abdomen.

2.3.3. Diet treatments

A total of 23 larvae from each of the six pathogen treatments (one unexposed, three single (virus low, virus high and fungus) and two mixed (virus low plus fungus, virus high plus fungus)) were transferred to individual 1oz SOLO® cups and randomly assigned to one of three diet quantity treatments. Diet quantity was altered by diluting the total amount of available nutrients (protein plus carbohydrate) with cellulose (a non-nutritive bulking agent) while keeping diet quality (macronutrient balance) the same. The concentration of total digestible protein and carbohydrate was diluted with one of three levels of cellulose: 25%, 35% or 40%; with 25% representing the amount of cellulose incorporated into the artificial diet of the stock *T. ni* colony. However, the nutritive macronutrient protein-to-carbohydrate ratio was kept at 1:1, close to their original artificial diet ratio. The rest of the diet components, including micronutrients and antimicrobials (Wesson's salt, cholesterol, ascorbic acid, sorbic acid, sodium alginate, vitamin wheat germ oil) (15% of dry ingredients) were kept constant. Dry ingredients were mixed and suspended at a 1:5 ratio in a 1.35% agar solution (modified from Shikano and Cory, 2014). We wanted to avoid selection and examine the potential impact on pupal weight as a proxy for fecundity, thus the larvae needed to be able to complete their development on each diet. We, therefore, avoided extreme starvation conditions which were likely to result in high larval mortality and pupation failure.

2.3.4. Insect Monitoring

Individual larvae were kept on the same diet, monitored daily and maintained at 24°C with a 16L:8D photoperiod until death or pupation occurred. Cause of death was determined visually as fatal infections caused by TniSNPV or *B. bassiana* are very distinct. NPV-infected larvae usually become flaccid and pale and finally lyse releasing millions of OBs. Fungal infection produces rigid, purple-coloured cadavers. To confirm fungal death and collect transmission stages (fungal spores), any cadavers which did not show the signs of viral infection were first surface sterilized, to reduce contamination, by dipping the cadavers in 1% sodium hypochlorite solution for 1 min. then rinsed twice in distilled water. Sterilized cadavers were then placed individually in a humidity chamber (1oz SOLO® cup containing a damp cotton wool) and kept at 24°C. All cadavers in humidity chambers were checked daily for sporulation. If no signs of sporulation occurred within 72 hours, the cadavers were then smeared and inspected under oil immersion on a light microscope (x1,000) for viral OBs or fungal hyphae. Larvae that survived pathogen challenge and successfully pupated were kept in separate individual cups and weighed 3 days after they formed pupae.

2.3.5. Pathogen yield

Larvae that died of viral infection were stored at -20°C after being carefully transferred into a 1.5 ml microtube. Only the cadavers that we were able to transfer whole were included in the viral yield analysis. Up to 10 cadavers per treatment were randomly selected, where possible, to estimate the number of OBs or spores produced per cadaver. To estimate the number of OBs, sterilized water was added to make the volume up to 1 ml. Cadavers were then macerated thoroughly with a micro-pestle for a minute and vortexed to release the OBs (Redman et al., 2016). A sub-sample of each cadaver was diluted by x100 or x1,000 and the number of OBs was estimated using an improved Neubauer haemocytometer (Hausser Scientific, depth 0.1mm) at 400x magnification. The total number of OBs was estimated four times independently for each cadaver and the average was used for the analysis. Sporulated cadavers were placed in a 1.5 ml microtube containing 0.5 ml of 0.01% Tween 80 and vigorously vortexed for 1 min, then macerated with a micro pestle for 1 min to dislodge the conidia (Inglis et al., 2012). To ensure that most conidia were removed from the cadaver surface, this step was repeated after adding another 0.5 ml of 0.01% Tween 80 before removing the larval cadaver from the tube.

Spores were stored at 4°C if not immediately used to estimate the number of spores. Fungal yield was estimated using the same method described for viral OBs.

2.3.6. Statistical analysis

The analysis of mortality and speed of kill was divided into three parts, (i) we first analysed overall larval mortality and speed of kill (as our focal pathogen species was TniSNPV, we excluded the *B. bassiana* single treatment from the overall analysis). (ii) To explore the impact of co-infection and diet on each pathogen individually we then analysed mortality and speed of kill focussing on insects which died of TniSNPV infection, and finally (iii) we analysed *B. bassiana* induced mortality and speed of kill. All mortality data were analysed using generalized linear models (GLM) with a binomial distribution and logit link function. For both overall mortality and TniSNPV-induced mortality the models included diet quantity (three levels) and virus dose (high or low) as ordinal, and infection treatment as a categorical variable (single or mixed) (Table 2.1a). In all statistical models, we initially included all interactions between fixed effects, and then simplified the models by removing non-significant interactions first and then any non-significant fixed effect (when not included in a significant interaction). For the fungal-induced mortality, only the amount of cellulose and virus dose (High, Low, No virus) as ordinal were included in the initial model (Table 1a). Where necessary, post-hoc comparisons between categorical and ordinal variables were made using the *glht* function (with the mcp="Tukey" specification) from the package *multcomp* (Hothorn et al., 2008) in R. Overall and virus-specific speed of kill were analysed using analysis of variance (ANOVA) with diet quantity (three levels) and virus dose (High, Low) as ordinal and infection treatment as categorical. Fungus-specific speed of kill was analysed using a similar model but only including diet quantity and virus dose (High, Low, No virus) as ordinal (Table 2.1b).

Viral and fungal yield were both analysed using general linear models. TniSNPV yield (numbers of OBs per insect) was square-root transformed to meet the assumption of normality. Diet quantity (three levels) and virus dose (High, Low) were included as ordinal variables and the infection treatment as categorical (single or mixed) (Table 2.1c). Speed of kill was included as a linear and quadratic covariate in the model. The total number of fungal spores harvested was log10-transformed to fit a normal distribution. Diet quantity, virus dose and speed of kill were included in the model as described above.

Table 2.1. Initial statistical models used to analyse (a) larval mortality, (b) speed of kill, (c) pathogen replication and (d) pupal weight. [a]Virus dose as ordinal (two levels: High, Low), [b]virus dose as ordinal (three levels: High, Low, No virus), [1]Only including pupal weight of uninfected larvae, [2]all pupal weight included (control and challenged larvae).

Response variable	Initial model formula	Statistical model
(a) Larval mortality		
Overall	~Quantity x virus dose[a] x single/mix	GLM (binomial)
Virus-induced	~ Quantity x virus dose[a] x single/mix	GLM (binomial)
Fungus induced	~ Quantity x virus dose[b]	GLM (binomial)
(b) Speed of kill		
Overall	~ Quantity x virus dose[a] x single/mix	ANOVA
Virus speed of kill	~ Quantity x virus dose[a] x single/mix	ANOVA
Fungus speed of kill	~ Quantity x virus dose[b]	ANOVA
(c) pathogen yield		
Virus yield	~ Quantity x virus dose[a] x single/ mix + speed of kill + (speed of kill)^2	GLM
Fungus yield	~ Quantity x virus dose[b] + speed of kill + (speed of kill)^2	GLM
(d) Pupal weight		
Pupal weight[1]	~ Quantity	ANOVA
Pupal weight[2]	~ Quantity x virus dose[b] x fungus	ANOVA

Finally, we looked at the larvae that survived pathogen challenge by analysing pupal weight. We first analysed the pupal weight of the control group only using an analysis of variance (ANOVA) to examine if dietary dilution alone had an impact on *T. ni* development. Only diet (three levels) was included in this first model. We then looked at both pupal weight from the control group and from the larvae that survived pathogen challenge in a second ANOVA, including diet quantity, virus dose (High, Low, No virus) as ordinal and fungus as categorical (Fungus or No fungus) variables (Table 2.1d). Pupal weight data were reflected (for a given pupa i, Reflected pupal weight$_i$= max(pupal weight) +1-pupal weight$_i$) and then log transformed to fit the assumption of normality. Tukey HSD comparisons were performed when significant differences among treatments were detected in the ANOVAs. All analyses were conducted in R-4.0.1.

2.4. Results

None of the unchallenged larvae died of pathogen infection thus they were not included in the mortality and speed of kill analyses. Interestingly, the outcome of pathogen infection (host death plus the production of transmission stages) in the mixed pathogen treatments resulted in one pathogen dominating the infection and thus cause of death was visually distinct and easy to establish in all cases.

2.4.1. Host mortality

Overall pathogen mortality

Overall mortality was about 21% higher in the mixed infections (76% total mortality) compared to the single virus treatments but was not affected by dietary dilution. Mortality was higher at the high virus dose (77%) compared to mortality in the low virus dose (63%), regardless of the infection treatment (Table 2.2a).

Table 2.2. Analysis of the effects of co-infection (Single/ mix) and virus dose (high or low) on (a) overall and (b) virus-specific mortality in *T. ni* larvae on diets containing different quantity diets (Cellulose) using generalized linear models with a binomial distribution (Type-III analysis-of-variance tables). Significant p values are highlighted in bold, and terms not included in the final model are italicized.

Analysis		Df	χ^2	P-value
Overall mortality	*Cellulose*	2	0.55	0.76
	Virus dose	1	5.79	0.02
	Single/ mix	1	4.59	0.03
	Cellulose x Virus dose	2	1.09	0.58
	Cellulose x Single/ mix	2	2.21	0.33
	Virus dose x Single/ mix	1	3.62	0.06
	Cellulose x Virus dose x Single/ mix	2	0.004	1.00
Virus -induced mortality	*Cellulose*	2	3.19	0.20
	Virus dose	1	28.33	<0.0001
	Single/ mix	1	19.13	<0.0001
	Cellulose x Virus dose	2	0.77	0.68
	Cellulose x Single/ mix	2	1.80	0.41
	Virus dose x Single/ mix	1	1.15	0.28
	Cellulose x Virus dose x Single/ mix	2	0.05	0.97

Virus-induced mortality

Dietary dilution had no impact on virus-induced mortality in single or co-infections. The combined viral mortality was 66% in the single infections but decreased to 38% when co-infected with fungus. When only virus-induced mortality was considered, it was much higher at the higher virus dose (68%) compared to the low dose (35%), regardless of treatment (Table 2.2b).

Fungus-induced mortality

Co-infection with the virus reduced fungal mortality by 67%, but only at the highest virus dose (virus dose: χ^2_2= 27.17, p<0.0001, Fig. 2.1). Dietary dilution had no effect on the level of fungal mortality in any of the treatments (cellulose*virus: χ^2_4= 2.21, p=0.70; cellulose: χ^2_2=2.18, p=0.34).

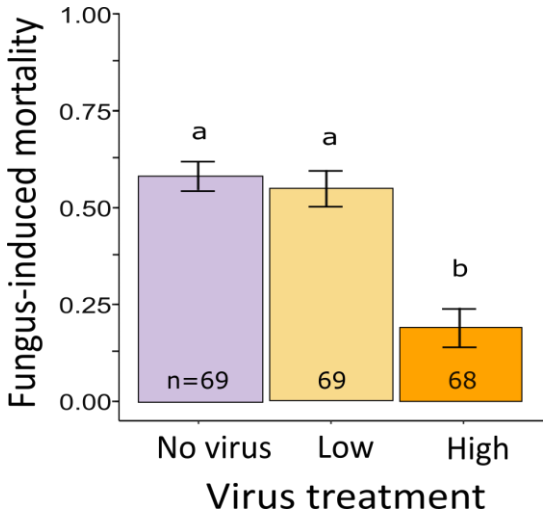

Figure 2.1. Fungus-induced mortality (mean ± s.e.) of 4th instar *T. ni* larvae challenged either with *B. bassiana* alone (No virus) or co-infected with TniSNPV at a low (100 OBs) or high (1,000 OBs) dose. Letters indicate significant differences at p<0.05 and numbers show the sample size.

2.4.2. Pathogen speed of kill

Overall speed of kill in single and mixed infections

As predicted, speed of kill was more rapid in the co-infections compared to virus alone (6.3 days ± 0.22 SEM compared to the respective single treatment 7.5 days ± 0.16 SEM), but only on the diet containing the least amount of cellulose (high quantity) (Fig.

2.2A) and only at the lower virus dose (Fig 2.2B, Table 2.3a). Similarly, larvae infected with the low virus dose died on average a day earlier (6.3 days total ±0.3 SEM) than the larvae in the virus high treatment regardless of whether it was a single or mixed infection, but only on low cellulose (high quantity) diet (Fig. 2.2C).

Table 2.3. Analysis of the effect of co-infection (single/mix) and virus dose (high or low) on the (a) overall and (b) virus-specific speed of kill in *T. ni* larvae on diets containing different amounts of cellulose (diet quantity). Significant p values are highlighted in bold, and terms not included in the final model are italicized.

Analysis		Sum of squares	df	F value	P-value
(a) Overall speed of kill	Cellulose	4.6	2	2.61	0.08
	Virus dose	0.6	1	0.73	0.39
	Single/ mix	14.4	1	16.37	**<0.0001**
	Cellulose x Virus dose	7.0	2	3.98	**0.02**
	Cellulose x Single/ mix	11.9	2	6.76	**0.001**
	Virus dose x Single/ mix	10.3	1	11.65	**<0.001**
	Cellulose x Virus dose x Single/ mix	3.0	2	1.74	0.18
(b) Virus speed of kill	*Cellulose*	3.3	2	2.90	0.06
	Virus dose	2.5	1	4.27	**0.04**
	Single/ mix	2.4	1	4.16	**0.04**
	Cellulose x Virus dose	1.3	2	1.22	0.30
	Cellulose x Single/ mix	3.1	2	2.78	0.07
	Virus dose x Single/ mix	0.3	1	0.48	0.49
	Cellulose x Virus dose x Single/ mix	0.7	2	0.66	0.52

Speed of kill by virus in single and mixed infections

The amount of cellulose in the diet did not affect the speed of kill of TniSNPV, although the significance was borderline (Table 2.3b) with virus-killed insects dying slower (around 7.5 days) on the low cellulose (high quantity) diet, and faster (around 7 days) on the medium quantity diet with high cellulose (low quantity) diet falling in between. However, virus-killed insects in the mixed infection treatments died on average 6.5h later than those from the single virus treatments (which took approximately 7.1 days). Larvae infected at the lower virus dose died approximately half a day later than those infected with the high dose (7.2 days ± 0.08 SEM), regardless of whether the insects were co-challenged with *B. bassiana*.

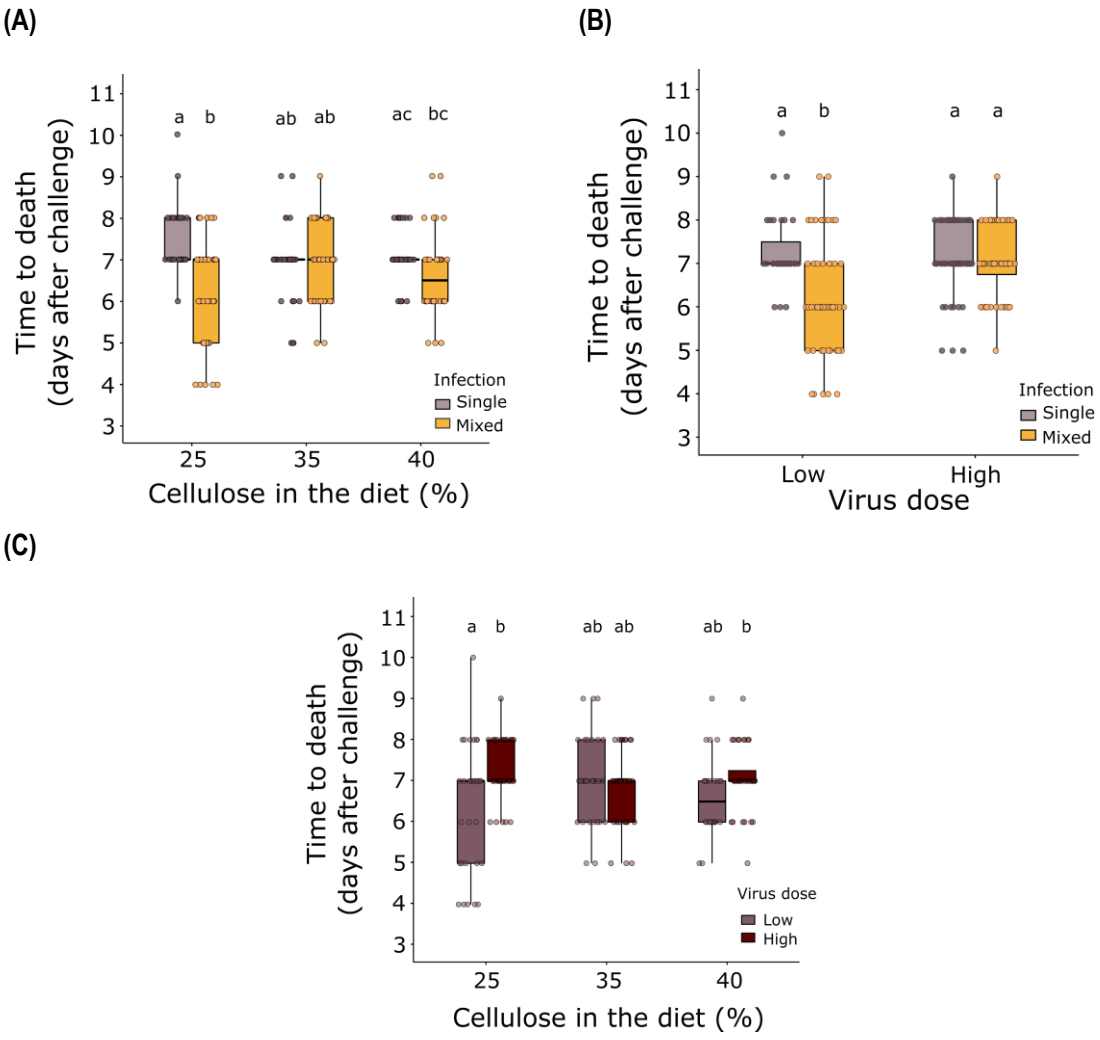

Figure 2.2. **Overall speed of kill of 4th instar *T. ni* larvae challenged with (A) TniSNPV either alone or co-infected with *B. bassiana*, on diets varying in levels of cellulose, (B) with TniSNPV at a low (100 OBs) or a high (1,000 OBs) dose, alone (Single) or co-infected with *B. bassiana* (Mixed), and (C) at a low or a high dose, on diets varying in levels of cellulose. Boxes indicate 25th and 75th percentiles, lines within boxes indicate medians, and whiskers includes values within 1.5 times the interquartile range. Letters indicate significant differences between treatments at p<0.05 (Tukey's HSD).**

Speed of kill by fungus in single and mixed infections

Beauveria bassiana speed of kill remained constant (around 5.6 days), regardless of diet quantity, when infecting larvae on its own. However, the speed of mixed infections was altered by diet quantity. On low cellulose (25% high quantity) diets, larvae killed by fungus took on average one day longer to die when co-infected with TniSNPV at the high dose (1,000 OBs) compared to the mixed low virus treatment, but on the middle 35% diet

the mixed low virus treatment was slower than the fungus alone (virus dose*cellulose: $F_{(4, 80)} = 5.45$, $p<0.001$; cellulose: $F_{(2, 80)} = 0.78$, $p=0.35$; virus dose: $F_{(2, 80)} = 4.68$, $p=0.01$; Fig. 3). On the poorest diet, there was no difference between the pathogen treatments, with all insects dying at the same speed as fungus alone (Fig. 2.3).

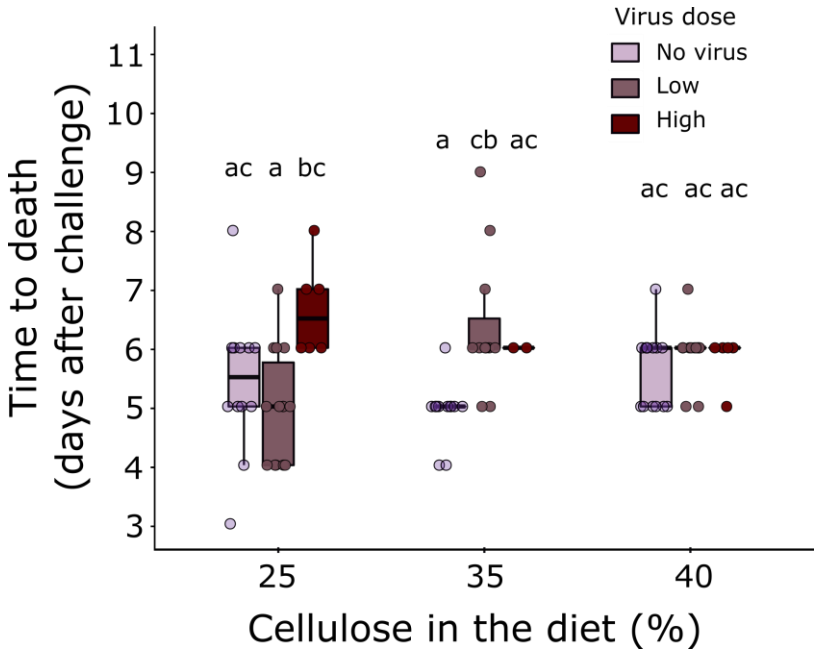

Figure 2.3. **Fungal speed of kill of 4th instar *T. ni* larvae challenged with *B. bassiana* alone (No virus) or co-infected with TniSNPV at low (100 OBs) or high (1,000 OBs) dose, on diets varying in the amount of cellulose. Boxes indicate 25th and 75th percentiles, lines within boxes indicate medians, and whiskers includes values within 1.5 times the interquartile range. Letters indicate significant differences between treatments at p<0.05 (Tukey's HSD).**

2.4.3. Production of pathogen transmission stages

Virus

On average, each virus-killed cadaver produced $3.3*10^9$ OBs and neither the diet nor co-challenge with the fungus had any impact on virus yield (cellulose*virus*sg/mix: $F_{(2, 77)}=0.44$, $p=0.65$; cellulose*sg/mix: $F_{(2, 79)}=1.925$, $p=0.15$; virus*sg/mix: $F_{(1, 79)}=0.10$, $p=0.76$; cellulose*virus: $F_{(1, 79)}=0.93$, $p=0.40$; sg/mix: $F_{(1, 84)}=0.14$, $p=0.71$; cellulose: $F_{(2, 84)}=2.16$, $p=0.12$;). The production of transmission stages was on average 11% higher ($1.4\text{-}6.9*10^9$ OBs) when larvae were challenged with a low dose of TniSNPV compared to a high dose (virus: $F_{(1, 87)}=4.93$, $p=0.03$). The total number of OBs per cadaver peaked at

7-8 days post-infection and declined with further increases in time to death (speed kill: $F_{(1, 87)}$=0.96, p=0.33; (speed kill)^2: $F_{(1, 87)}$=10.83, p=0.001, Fig. 2.4).

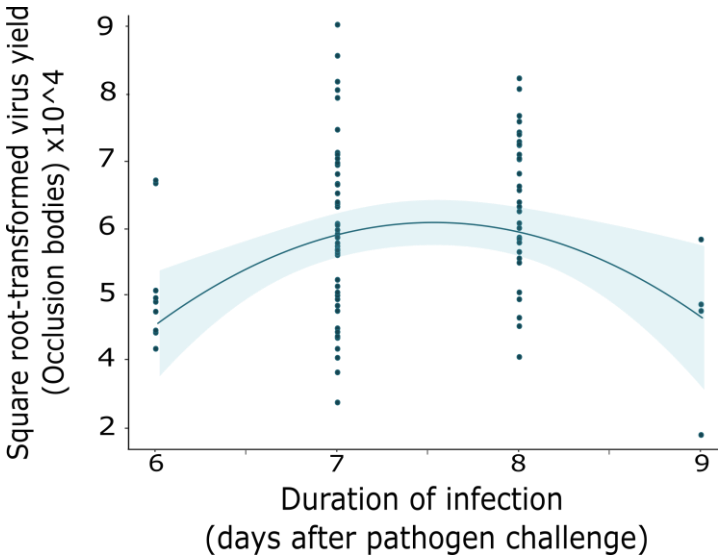

Figure 2.4. Virus yield and speed of kill trade-off. Lines show fitted statistical models with 95% confidence intervals and raw data points (n=140).

Fungus

Fungal cadavers produced on average $1.72*10^8$ spores and neither co-infection with virus nor diluting the diet with cellulose affected the number of spores produced by insects killed by *B. bassiana* (cellulose*virus dose: $F_{(4, 40)}$=0.41, p=0.80; cellulose: $F_{(2, 44)}$=1.16, p=0.32; virus dose: $F_{(2, 44)}$=0.83, p=0.44). The total number of spores produced on fungal cadavers was not related to speed of kill (speed kill: $F_{(1, 44)}$=0.98, p=0.33; (speed kill)^2: $F_{(1, 44)}$=0.07, p=0.79).

2.4.4. Pupal weight

The diet treatments alone, in the absence of pathogen infection, had no effect on the pupal weight of the unchallenged (control) larvae (cellulose: $F_{(2, 54)}$=2.24, p=0.12). However, when including the pupal weight from the control group and all the larvae that survived pathogen challenge in the single and mixed infections treatments, diluting the amount of food available reduced pupal weight (higher log-transformed reflected pupal weight), but only at the highest dilution level, and this was not influenced by infection treatment or virus dose (cellulose: $F_{(2, 162)}$= 8.98, p=0.0002; cellulose*fungus: $F_{(2, 152)}$= 0.86,

p=0.426; cellulose*virus: $F_{(4, 152)}$= 0.65, p=0.631; cellulose*virus*fungus: $F_{(4, 148)}$= 0.38, p=0.82; Fig. 2.5). Regardless of the diet or the virus treatment (control, virus single or mixed infections), larvae challenged with *B. bassiana* produced larger pupae than those that were not (fungus: $F_{(1, 162)}$= 4.96, p=0.027; virus*fungus: $F_{(2, 152)}$= 1.07, p=0.346; virus: $F_{(2, 160)}$= 1.01, p=0.366).

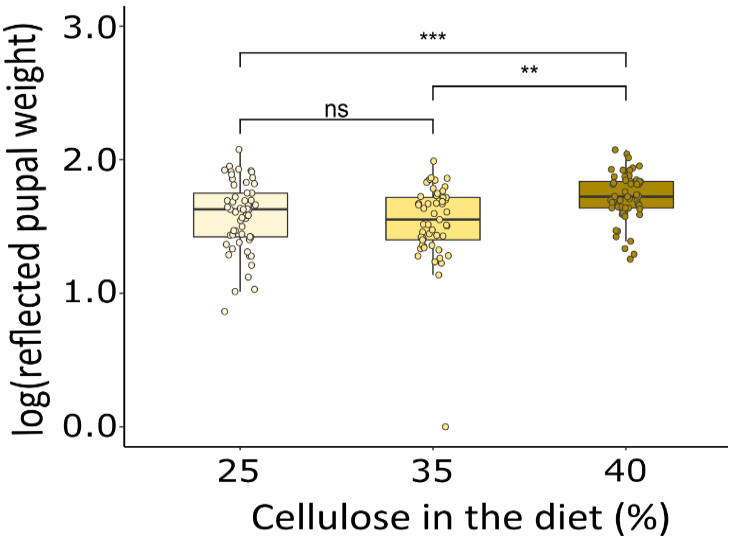

Figure 2.5. Effect of diet quantity on *T. ni* pupal weight (ns: non-significant; *: 0.05<p<0.01; **: 0.01<p<0.001; ***: p<0.001). Boxes indicate 25th and 75th percentiles, lines within boxes indicate medians, and whiskers includes values within 1.5 times the interquartile range.

2.5. Discussion

We expected that increasing the amount of cellulose in the diet would induce dietary stress and that this would be exacerbated when the host was challenged by two virulent pathogens with very different infection pathways, resulting in earlier or higher host mortality and a reduction in pathogen replication. However, we found that reducing the quantity of food available had no effect on overall or pathogen-specific mortality or the production of transmission stages in either mixed or single infections. Changing diet quantity did; however, have more subtle effects on speed of kill.

While extreme scenarios of food restriction, such as host starvation, after infection with parasites and pathogens, have resulted in obvious detrimental effects on host survival (Donegan and Lighthart, 1989; Furlong and Groden, 2003; Stucki et al., 2019), fecundity

(Valtonen et al., 2010), and development (Donegan and Lighthart, 1989; Pulkkinen and Ebert, 2004; Zhang et al., 2019), less extreme diet manipulations have tended to show no impact of reducing resources on host mortality, for example in worker bumblebees, *Bombus terrestris* (Sadd, 2011), the western tent caterpillar, *Malacosoma californicum* pluviale (Myers et al., 2011), and two species of lady beetle, *Adalia bipunctata* and *Hippodamia convergens* (Steele and Bjørnson, 2019), challenged with a trypanosome *Crithidia bombi*, *M. c.* pluviale nucleopolyhedrovirus, and two microsporidian species respectively. Similarly, while Lord (2010) studied host starvation, he also clearly demonstrated that fungus-induced mortality of the flour beetle larvae, *Tribolium castaneum*, increased linearly as the number of days between pathogen challenge and the period of food deprivation increased. While the time over which the diet was manipulated varied in each of these studies from pre- or post-pathogen challenge to over the whole lifespan, this does suggest that invertebrate hosts infected with single pathogens are able to compensate for any reduction in diet availability, as long as some food is available. This conclusion is supported by a taxonomically broad meta-analysis by Pike et al. (2019), who found no significant effect of changes in host nutritional quantity or quality on pathogen virulence, defined as host survival or mortality. Closer analysis found different patterns in vertebrate and invertebrate hosts, leading them to hypothesize that as invertebrate hosts lack an adaptive immune system, they would need less resources to fight pathogen infection, compared to the more complex vertebrates, and thus changes in resource quantity would be less likely to be seen at the host fitness level with pathogen challenge (Pike et al., 2019).

Our understanding of the impact of host resource availability on within-host pathogen competition is more limited. In our system, co-infection impacted both overall and pathogen-specific mortality but did not interact with changes in host resource quantity. Both the pathogens used in our experiment are obligate killers and the infections can develop very rapidly, giving only a relatively short window of time to fight off fatal infection. Thus, even small changes in nutrient availability might be expected to alter the outcome of infection, particularly under a double pathogen challenge. However, the *T. ni* larvae were fed *ad libitum*, and the results indicate that the larvae could mitigate any negative effect of reduced nutrient availability on disease resistance by increasing their food intake (Wheeler and Slansky, 1991; Lee et al., 2004). The diet treatments had a protein-to-carbohydrate ratio of 1:1, close to the optimum intake ratio for *T. ni* (Shikano and Cory,

2014), meaning that compensatory feeding, which can be costly on an unbalanced diet (Lee et al., 2006), would not have been limited by an excessive intake of one macronutrient over the other (Lee et al., 2004). However, when unchallenged and challenged treatments were combined (increasing the sample size), pupal weight declined at the highest level of cellulose (40%), irrespective of pathogen treatment. This could be a significant fitness cost, as pupal weight in female Lepidoptera is strongly linked to fecundity (Milks et al., 1998). This implies that the larvae were able to make up for the reduction in resources by increasing or prolonging their feeding, but that there is a level beyond which this is not possible. However, this does not appear to be trading-off against disease resistance, even under a two-pathogen challenge. Larvae challenged with *B. bassiana*, regardless of the infection treatment (single or mixed) produced heavier pupae, which could be the result of selection for larger larvae. However, NPV challenge did not affect pupal weight, although reduced female pupal mass and fecundity have been recorded in Lepidoptera as a result of surviving virus challenge (e.g. Milks et al., 1998; Myers et al., 2000).

Irrespective of the lack of an effect of diet quantity on co-infection and host mortality, the pathogens had a negative impact on each other in terms of pathogen-specific mortality, although this only occurred at the higher virus dose for fungal mortality. This is interesting as the outcome of each co-infection, in terms of symptoms and the production of transmission stages, was always dominated by one pathogen; there was a clear winner. This strongly suggests that any interaction between the two pathogens must have taken place very early in the infection process. The outcome of simultaneous baculovirus-entomopathogenic fungus infection is generally in favour of the fungus due to its more rapid speed of kill. However, the outcome is also highly dependent on the specific system studied, the infection dose and time of infection (Malakar et al., 1999; Pauli et al., 2018; Souza et al., 2019). Indeed, Souza et al. (2019) found that asynchronous infections of the fall armyworm larvae, *Spodoptera frugiperda*, with its nucleopolyhedrovirus and *Metarhizium riley* always resulted in a lower observed larval mortality than predicted using both single pathogen infection treatments (antagonistic interaction), whereas NPV-fungus interaction was only antagonistic when the fungus was introduced two days before the virus with the velvet bean caterpillar, *Anticarsia gemmatalis* This is similar to the results found by Malakar et al. (1999), where the shorter incubation time of the fungus *Entomophaga maimaiga* negatively affected NPV-induced mortality in the spongy moth larvae, *Lymantria dispar*. They also reported the presence of fungal hyphae in virus-killed

insects, although no spores were produced, which also suggests that both pathogens are able to undergo limited replication in the host until one comes to dominate in the production of transmission stages.

While host mortality was not affected by changes in diet quantity, infection duration was. Focussing on pathogen-specific speed of kill as this is what determines secondary transmission, TniSNPV took longer to kill its host in the co-infection treatments, regardless of the host diet. This suggests that either the virus access to necessary resources was limited due to competition with the fungus, or that the virus is slowing down the use of the host resource to keep the host alive to acquire the necessary resources for its own development (Choisy and de Roode, 2010). As virus yield did not differ between single and mixed treatments this does imply that the lengthened infection period was necessary to produce an optimum number of virus OBs. Baculoviruses usually express genes that manipulate the endocrine system of the host, delaying or preventing moulting, so there is a possible mechanism for this to occur (O'Reilly and Miller, 1989; Cory et al., 2004). The duration of fungal infections was affected by both diet and co-infection, with the most pronounced effects being seen on the higher quantity (low cellulose) diet. At the higher effective dose of TniSNPV, *B. bassiana* infections took longer, whereas on the medium diet, fungal infection took longer when co-infected with the lower virus dose, with these differences disappearing when the diet was most dilute (and all infections were as fast as fungus on its own). However, again, there was no difference in the production of transmission stages, which is not related to *B. bassiana* time to death, suggesting that the fungus was better at using resources (in competition with the virus) on the more dilute diet. Changes in the duration of infection are likely to impact the speed which secondary cycles are initiated in the wild, affecting the speed of transmission. Baculoviruses only infect the larval stage of their host, but changes in speed of kill will have added importance when pathogens can infect adult hosts where longevity could affect reproduction, both of which could have important consequences at the population level.

Surprisingly, we found no impact of reduced diet or co-infection on the yield of either pathogen, despite the clear effects of co-infection on pathogen-specific mortality. The question of how food availability and host condition affect pathogen yield is not clear. Tseng and Myers' (2014) study on *T. ni* showed a strong positive relationship between baculovirus yield and diet quantity (larger cadavers on high food treatments). However, in this experiment, larvae only had access to food for a specific period of time (4 to 5h for

the low food treatment), so the hosts were intermittently starved, which resulted in different sized larvae. Baculovirus yield is often strongly correlated with speed of kill, as more OBs can be produced if the host stays alive longer and grows larger (Cory and Myers, 2003; Georgievska et al., 2010), thus host weight is extremely important in this pathogen. Spore production in *B. bassiana* has been shown to increase with cadaver size as the surface area for spore production increases (Luz and Fargues, 1998), although the relationship plateaued in later instars (Woodring et al., 1995). In addition, high fungal doses kill the host too quickly for the fungus to fully colonize the host, negatively affecting sporulation (Woodring et al., 1995). In a different system, the mosquito *Aedes aegypti*, the authors showed a clear negative relationship between diet quantity and spore production in the microsporidian parasite, *Vavraia culicis* (Bedhomme et al., 2004); however, again the larvae were fed a set amount of food, limiting compensatory feeding behaviour. Another study looking at the mosquito *Aedes triseriatus* larvae infected with the gregarine parasite *Ascogregarina barreti*, showed that diet quantity had a lesser effect on parasite reproduction (count) within the host, but did significantly affect the size of the parasite, with larger parasites found in the treatments with the highest amount of food (Westby et al., 2019). Thus, this implies that the yield of these invertebrate pathogens is primarily related to cadaver size, and changes in the production of transmission stages will only result when the food supply is low enough to affect growth and development.

In conclusion, reducing food availability had no effect on the outcome of mixed (or single) pathogen infections in terms of host mortality or pathogen yield, although there were more subtle effects on speed of kill, which could affect the rate of pathogen recycling in the host population. This suggests that in circumstances where the hosts can compensate for a more dilute or reduced diet by eating more or for longer, the quantity of diet available has little effect on disease resistance in insects, although an extended development period is likely to have other costs in the field. It also suggests that different pathogen groups are affected differently by changes in dietary dilution; in this case, the fungus appeared to be more sensitive, potentially due to its high rate of exploitation of host resources. However, these effects were overridden by the relative effective dose of each pathogen, illustrating the subtle interplay between diet and disease. It is also interesting to note that diet reduction has also been shown to have transgenerational impacts on disease resistance (e.g. in baculoviruses, Shikano et al., 2015), indicating that a longer-term perspective is needed when studying dietary changes and disease. Understanding

the effect of changes in diet on disease outcome in co-infections is an important topic for epidemiology and pathogen dynamics and further studies are needed to better understand the relationship between diet, host disease resistance and within-host pathogen competition.

Chapter contributions

Pauline Deschodt and Dr. Jenny Cory conceived the idea and designed the experiment; Dr. Jenny Cory provided the funding and resources; Pauline collected the data, with the help of Olivia Walker and Tri Truong; Pauline and Dr. Jenny Cory analysed the data; Pauline drafted the manuscript; Pauline and Dr. Jenny Cory edited the manuscript.

Chapter 2 has been published in Ecology & Evolution: Deschodt, P. S., and Cory, J. S. (2022). Resource limitation has a limited impact on the outcome of virus–fungus co-infection in an insect host. *Ecol. Evol*. 12. doi:10.1002/ece3.8707.

2.6. References

Ayres, J. S., and Schneider, D. S. (2009). The role of anorexia in resistance and tolerance to infections in *Drosophila*. PLoS Biol. 7, e1000150. doi:10.1371/journal.pbio.1000150.

Bedhomme, S., Agnew, P., Sidobre, C., and Michalakis, Y. (2004). Virulence reaction norms across a food gradient. Proc. Biol. Sci. 271, 739–744. doi:10.1098/rspb.2003.2657.

Budischak, S. A., and Cressler, C. E. (2018). Fueling defense: effects of resources on the ecology and evolution of tolerance to parasite infection. Front. Immunol. 9, 2453. doi:10.3389/fimmu.2018.02453.

Choisy, M., and de Roode, J. C. (2010). Mixed infections and the evolution of virulence: effects of resource competition, parasite plasticity, and impaired host immunity. Am. Nat. 175, E105-18. doi:10.1086/651587.

Cory, J. S., Clarke, E. E., Brown, M. L., Hails, R. S., and O'Reilly, D. R. (2004). Microparasite manipulation of an insect: the influence of the egt gene on the interaction between a baculovirus and its lepidopteran host. Funct. Ecol. 18, 443–450. doi:10.1111/j.0269-8463.2004.00853.x.

Cory, J. S., and Myers, J. H. (2003). The ecology and evolution of insect baculoviruses. Annu. Rev. Ecol. Evol. Syst. 34, 239–272. doi:10.1146/annurev.ecolsys.34.011802.132402.

Cory, J. S. (2010). “The Ecology of Baculoviruses,” in Insect Virology, eds. S. Asgari and K. Johnson (Caister Academic Press, Norfolk), 411–427.

Cotter, S. C., and Al Shareefi, E. (2021). Nutritional ecology, infection and immune defence - exploring the mechanisms. Curr. Opin. Insect Sci. doi:10.1016/j.cois.2021.12.002.

Cressler, C. E., McLeod, D. V., Rozins, C., Van Den Hoogen, J., and Day, T. (2016). The adaptive evolution of virulence: a review of theoretical predictions and empirical tests. Parasitology 143, 915–930. doi:10.1017/S003118201500092X.

Cressler, C. E., Nelson, W. A., Day, T., and McCauley, E. (2014). Disentangling the interaction among host resources, the immune system and pathogens. Ecol. Lett. 17, 284–293. doi:10.1111/ele.12229.

Donegan, K., and Lighthart, B. (1989). Effect of several stress factors on the susceptibility of the predatory insect, *Chrysoperla carnea* (Neuroptera: Chrysopidae), to the fungal pathogen *Beauveria bassiana*. Journal of invertebrate pathology 54, 79–84. doi:10.1016/0022-2011(89)90143-2.

Duncan, A. B., Agnew, P., Noel, V., and Michalakis, Y. (2015). The consequences of co-infections for parasite transmission in the mosquito *Aedes aegypti.* J. Anim. Ecol. 84, 498–508. doi:10.1111/1365-2656.12302.

Fellous, S., and Koella, J. C. (2010). Cost of co-infection controlled by infectious dose combinations and food availability. Oecologia 162, 935–940. doi:10.1007/s00442-009-1535-2.

Fellous, S., and Koella, J. C. (2009). Infectious dose affects the outcome of the within-host competition between parasites. Am. Nat. 173, E177-84. doi:10.1086/598490.

Furlong, M. J., and Groden, E. (2003). Starvation induced stress and the susceptibility of the Colorado potato beetle, *Leptinotarsa decemlineata*, to infection by *Beauveria bassiana*. Journal of invertebrate pathology 83, 127–138. doi:10.1016/S0022-2011(03)00066-1.

Georgievska, L., Hoover, K., van der Werf, W., Muñoz, D., Caballero, P., Cory, J. S., and Vlak, J. M. (2010). Dose dependency of time to death in single and mixed infections with a wildtype and egt deletion strain of *Helicoverpa armigera* nucleopolyhedrovirus. J. Invertebr. Pathol. 104, 44–50. doi:10.1016/j.jip.2010.01.008.

Graham, A. L., Shuker, D. M., Pollitt, L. C., Auld, S. K. J. R., Wilson, A. J., and Little, T. J. (2011). Fitness consequences of immune responses: strengthening the empirical framework for ecoimmunology. Funct. Ecol. 25, 5–17. doi:10.1111/j.1365-2435.2010.01777.x.

Hothorn, T., Bretz, F.and Westfall, P. (2008). Simultaneous inference in general parametric models. Biometrical Journal 50(3), 346-363.

Inglis, G. D., Enkerli, J., and Goettel, M. S. (2012). “Laboratory techniques used for entomopathogenic fungi,” in Manual of techniques in invertebrate pathology (Elsevier), 189–253. doi:10.1016/B978-0-12-386899-2.00007-5.

Janmaat, A. F., and Myers, J. H. (2005). The cost of resistance to *Bacillus thuringiensis* varies with the host plant of *Trichoplusia ni*. Proc. Biol. Sci. 272, 1031–1038. doi:10.1098/rspb.2004.3040.

Lacroix, C., Seabloom, E. W., and Borer, E. T. (2014). Environmental nutrient supply alters prevalence and weakens competitive interactions among coinfecting viruses. New Phytol, 424–433.

Lee, K. P., Cory, J. S., Wilson, K., Raubenheimer, D., and Simpson, S. J. (2006). Flexible diet choice offsets protein costs of pathogen resistance in a caterpillar. Proc. Biol. Sci. 273, 823–829. doi:10.1098/rspb.2005.3385.

Lee, K. P., Raubenheimer, D., and Simpson, S. J. (2004). The effects of nutritional imbalance on compensatory feeding for cellulose-mediated dietary dilution in a generalist caterpillar. Physiol. Entomol. 29, 108–117. doi:10.1111/j.0307-6962.2004.00371.x.

Lochmiller, R. L., and Deerenberg, C. (2000). Trade-offs in evolutionary immunology: just what is the cost of immunity? Oikos 88, 87–98. doi:10.1034/j.1600-0706.2000.880110.x.

Lohr, J. N., Yin, M., and Wolinska, J. (2010). Prior residency does not always pay off-co-infections in *Daphnia*. Parasitology 137, 1493–1500. doi:10.1017/S0031182010000296.

Lord, J. C. (2010). Dietary stress increases the susceptibility of *Tribolium castaneum* to *Beauveria bassiana*. J. Econ. Entomol. 103, 1542–1546. doi:10.1603/EC09311.

Luz, C., and Fargues, J. (1998). Factors affecting conidial production of *Beauveria bassiana* from fungus-killed cadavers of *Rhodnius prolixus*. J. Invertebr. Pathol. 72, 97–103. doi:10.1006/jipa.1998.4774.

Malakar, R., Elkinton, J. S., Hajek, A. E., and Burand, J. P. (1999). Within-host interactions of *Lymantria dispar* (Lepidoptera: Lymantriidae) nucleopolyhedrosis virus and *Entomophaga maimaiga* (Zygomycetes: entomophthorales). J. Invertebr. Pathol. 73, 91–100. doi:10.1006/jipa.1998.4806.

McKean, K. A., and Lazzaro, B. (2011). “The costs of immunity and the evolution of immunological defense mechanisms,” in Mechanisms of Life History Evolution: The Genetics and Physiology of Life History Traits and Trade-Offs, eds. T. Flatt and A. Heyland (Oxford University Press), 299–310. doi:10.1093/acprof:oso/9780199568765.003.0023.

Meyling, N. V., and Eilenberg, J. (2007). Ecology of the entomopathogenic fungi *Beauveria bassiana* and *Metarhizium anisopliae* in temperate agroecosystems: Potential for conservation biological control. Biological Control 43, 145–155. doi:10.1016/j.biocontrol.2007.07.007.

Mideo, N. (2009). Parasite adaptations to within-host competition. Trends Parasitol. 25, 261–268. doi:10.1016/j.pt.2009.03.001.

Milks, M. L., Burnstyn, I., and Myers, J. H. (1998). Influence of larval age on the lethal and sublethal effects of the nucleopolyhedrovirus of *Trichoplusia ni* in the cabbage looper. Biological Control 12, 119–126. doi:10.1006/bcon.1998.0616.

Moret, Y., and Schmid-Hempel, P. (2000). Survival for immunity: the price of immune system activation for bumblebee workers. Science 290, 1166–1168. doi:10.1126/science.290.5494.1166.

Mouritsen, K. N., and Andersen, C. (2017). Worms at war: interspecific parasite competition and host resources alter trematode colony structure and fitness. Parasitology 144, 1530–1542. doi:10.1017/S003118201700107X.

Myers, J. H., Cory, J. S., Ericsson, J. D., and Tseng, M. L. (2011). The effect of food limitation on immunity factors and disease resistance in the western tent caterpillar. Oecologia 167, 647–655. doi:10.1007/s00442-011-2023-z.

Myers, J. H., Malakar, R., and Cory, J. S. (2000). Sublethal nucleopolyhedrovirus infection effects on female pupal weight, egg mass size, and vertical transmission in gypsy moth (Lepidoptera: Lymantriidae). Environ. Entomol. 29, 1268–1272. doi:10.1603/0046-225X-29.6.1268.

O’Reilly, D. R., and Miller, L. K. (1989). A baculovirus blocks insect molting by producing ecdysteroid UDP-glucosyl transferase. Science 245, 1110–1112. doi:10.1126/science.2505387.

Pauli, G., Moura Mascarin, G., Eilenberg, J., and Delalibera Júnior, I. (2018). Within-host competition between two entomopathogenic fungi and a granulovirus in *Diatraea saccharalis* (Lepidoptera: Crambidae). Insects 9. doi:10.3390/insects9020064.

Pike, V. L., Lythgoe, K. A., and King, K. C. (2019). On the diverse and opposing effects of nutrition on pathogen virulence. Proc. Biol. Sci. 286, 20191220. doi:10.1098/rspb.2019.1220.

Ponton, F., Lalubin, F., Fromont, C., Wilson, K., Behm, C., and Simpson, S. J. (2011). Hosts use altered macronutrient intake to circumvent parasite-induced reduction in fecundity. Int. J. Parasitol. 41, 43–50. doi:10.1016/j.ijpara.2010.06.007.

Pulkkinen, K., and Ebert, D. (2004). Host starvation decreases parasite load and mean host size in experimental populations. Ecology 85, 823–833. doi:10.1890/03-0185.

Redman, E. M., Wilson, K., and Cory, J. S. (2016). Trade-offs and mixed infections in an obligate-killing insect pathogen. J. Anim. Ecol. 85, 1200–1209. doi:10.1111/1365-2656.12547.

Reyserhove, L., Muylaert, K., Vanoverberghe, I., and Decaestecker, E. (2017). Synergistic effects of dual parasitism in *Daphnia magna* under nutrient limitation. Belg. J. Zool. 147, 61–76. doi:10.26496/bjz.2017.5.

Sadd, B. M. (2011). Food-environment mediates the outcome of specific interactions between a bumblebee and its trypanosome parasite. Evolution 65, 2995–3001. doi:10.1111/j.1558-5646.2011.01345.x.

Sandland, G. J., and Minchella, D. J. (2003). Costs of immune defense: an enigma wrapped in an environmental cloak? Trends Parasitol. 19, 571–574. doi:10.1016/j.pt.2003.10.006.

Schmid-Hempel, P. (2005). Evolutionary ecology of insect immune defenses. Annu. Rev. Entomol. 50, 529–551. doi:10.1146/annurev.ento.50.071803.130420.

Sheldon, B. C., and Verhulst, S. (1996). Ecological immunology: costly parasite defences and trade-offs in evolutionary ecology. Trends Ecol. Evol. 11, 317–321. doi:10.1016/0169-5347(96)10039-2.

Shikano, I., and Cory, J. S. (2014). Dietary mechanism behind the costs associated with resistance to Bacillus thuringiensis in the cabbage looper, *Trichoplusia ni*. PLoS ONE 9, e105864. doi:10.1371/journal.pone.0105864.

Shikano, I., Oak, M. C., Halpert-Scanderbeg, O., and Cory, J. S. (2015). Trade-offs between transgenerational transfer of nutritional stress tolerance and immune priming. Funct. Ecol. 29, 1156–1164. doi:10.1111/1365-2435.12422.

Souza, M. L., Sanches, M. M., Souza, D. A. de, Faria, M., Espinel-Correal, C., Sihler, W., and Lopes, R. B. (2019). Within-host interactions of *Metarhizium rileyi* strains and nucleopolyhedroviruses in *Spodoptera frugiperda* and *Anticarsia gemmatalis* (Lepidoptera: Noctuidae). J. Invertebr. Pathol. 162, 10–18. doi:10.1016/j.jip.2019.01.006.

Staves, P. A., and Knell, R. J. (2010). Virulence and competitiveness: testing the relationship during inter- and intraspecific mixed infections. Evolution 64, 2643–2652. doi:10.1111/j.1558-5646.2010.00999.x.

Steele, T., and Bjørnson, S. (2019). Effects of microsporidiosis and food availability on the two-spotted lady beetle, *Adalia bipunctata* L., and convergent lady beetle, *Hippodamia convergens* Guérin-Méneville. J. Invertebr. Pathol. 161, 7–13. doi:10.1016/j.jip.2018.12.007.

Stucki, D., Freitak, D., Bos, N., and Sundström, L. (2019). Stress responses upon starvation and exposure to bacteria in the ant *Formica exsecta*. PeerJ 7, e6428. doi:10.7717/peerj.6428.

Tseng, M., and Myers, J. H. (2014). The relationship between parasite fitness and host condition in an insect--virus system. PLoS ONE 9, e106401. doi:10.1371/journal.pone.0106401.

Valtonen, T. M., Kleino, A., Rämet, M., and Rantala, M. J. (2010). Starvation reveals maintenance cost of humoral immunity. Evol. Biol. 37, 49–57. doi:10.1007/s11692-009-9078-3.

Wale, N., Sim, D. G., and Read, A. F. (2017). A nutrient mediates intraspecific competition between rodent malaria parasites in vivo. Proc. Biol. Sci. 284. doi:10.1098/rspb.2017.1067.

Westby, K. M., Sweetman, B. M., Adalsteinsson, S. A., Biro, E. G., and Medley, K. A. (2019). Host food quality and quantity differentially affect *Ascogregarina barretti* parasite burden, development and within-host competition in the mosquito *Aedes triseriatus*. Parasitology 146, 1665–1672. doi:10.1017/S0031182019000994.

Wheeler, G. S., and Slansky, F. (1991). Compensatory responses of the fall armyworm (*Spodoptera frugiperda*) when fed water- and cellulose-diluted diets. Physiol. Entomol. 16, 361–374. doi:10.1111/j.1365-3032.1991.tb00574.x.

Woodring, J. L., Kaya, H. K., and Kerwin, J. L. (1995). *Lagenidium giganteum* in *Culex tarsalis* larvae: production of infective propagules. J. Invertebr. Pathol. 66, 25–32. doi:10.1006/jipa.1995.1056.

Zhang, D.-W., Xiao, Z.-J., Zeng, B.-P., Li, K., and Tang, Y.-L. (2019). Insect behavior and physiological adaptation mechanisms under starvation stress. Front. Physiol. 10, 163. doi:10.3389/fphys.2019.00163.

Zilio, G., and Koella, J. C. (2020). Sequential co-infections drive parasite competition and the outcome of infection. J. Anim. Ecol. 89, 2367–2377. doi:10.1111/1365-2656.13302.

Chapter 3.

Diet quality has complex, dose-dependent effects on pathogen fitness in virus-fungus co-infections

3.1. Abstract

Co-infection of a single host by multiple pathogens is common and is predicted to affect pathogen virulence and transmission, thereby influencing host-pathogen dynamics. What determines the outcome when pathogens compete is poorly understood and we have limited information on how environmental factors influence these interactions. We investigated the effect of host nutrition on co-infections and whether it was affected by pathogen dose in the cabbage looper *Trichoplusia ni*, using a DNA virus, *T. ni* nucleopolyhedrovirus (TniSNPV), and an entomopathogenic fungus, *Beauveria bassiana*. We manipulated protein-to-carbohydrate (P:C) ratio (diet quality) after pathogen challenge and examined its impact on host mortality, the relative success of each pathogen and virus fitness. Changes in diet quality did not affect overall mortality. The two pathogens were affected differently by changes in diet quality (P:C ratio). Virus-induced mortality (in single or mixed treatments) was not affected; however, more protein in the diet resulted in later death and a reduction in the number of virus transmission stages produced when the dose was high. Fungal-induced mortality decreased with increasing carbohydrate in the diet but there was no change in speed of kill or yield. Regardless of diet quality, TniSNPV induced mortality was negatively affected by the fungus challenge at both virus doses. Similarly, *B. bassiana* induced mortality was negatively affected by TniSNPV challenge, but only at the higher virus dose. Virus virulence was affected by the interaction between co-infection, diet quality and virus dose. There was no difference in mortality at the lower virus dose; however, virus from high dose infections tended to be less virulent when larvae were previously fed a high protein diet. The resulting OB size from the different infections also varied but only in the high dose treatments. OBs tended to be larger in the higher protein treatments at the higher virus dose but were strongly influenced by whether the infection was single or mixed. We clearly show that diet quality can alter the outcome of single and mixed infections both in terms of the relative success of each pathogen and pathogen virulence. This demonstrates that pathogen competition is dependent on the environment and that one pathogen can influence the epidemiology of the other.

Keywords: Baculovirus, *Beauveria bassiana*, co-infection, diet quality, TniSNPV, trade-offs, *Trichoplusia ni, virulence*

3.2. Introduction

Hosts are commonly challenged by multiple pathogen or parasite species (Read and Taylor, 2001; Hajek and Tobin, 2011; Abdullah et al., 2017; Venter et al., 2020). For the host, mounting an immune response against a pathogen is highly demanding in terms of both energy and essential amino acid precursors (Sandland and Minchella, 2003; Schmid-Hempel, 2005; Simpson and Raubenheimer, 2011). Being challenged by multiple pathogen or parasite species could further increase the burden on host resources, in addition to potentially resulting in trade-offs with other core activities (Sheldon and Verhulst, 1996; Sandland and Minchella, 2003; Alizon et al., 2009). The role of host nutrition in pathogen infections has primarily been investigated in single host-single pathogen systems (reviewed by Huber et al., 2012; Budischak and Cressler, 2018; Pike et al., 2019; Cotter and Al Shareefi, 2022). Yet, how changes in diet might alter the outcome of co-infections has received far less attention and is likely to be hard to predict if infections induced by different pathogen or parasite types respond differently to varying host nutritional status. Host nutrition can vary in terms of diet composition (diet quality) or availability (quantity), or both together, as well as when the host is exposed to dietary changes (before, during or after infection, or continuously). Experimental studies on host nutritional status and co-infections cover very different pathogen groups and host species (e.g. mice-helminths and resource limitation (Budischak et al., 2015); plants-viruses and diet quality (Lacroix et al., 2014); invertebrate-pathogens and diet quantity or quality (Vale et al., 2013; Tritschler et al., 2017; Zilio and Koella, 2020)). As a result, it is difficult to identify clear patterns that would be necessary to better understand within-host pathogen competition.

The strength and outcome of interspecific co-infections are likely to depend on numerous factors, including the host and pathogen species (Thomas et al., 2003; Syller, 2012; Cory and Deschodt, 2017), pathogen virulence (Hughes et al., 2004; Ben-Ami et al., 2011; Lange et al., 2014; Venter et al., 2022), relative dose (Ebert et al., 2000; Fellous and Koella, 2009; Phuoc et al., 2009; Shameena et al., 2021), timing of infection (Lohr et al., 2010; Wakil et al., 2017; Ramsay and Rohr, 2021), and resources (Cressler et al.,

2014; Budischak et al., 2015; Tritschler et al., 2017). Moreover, co-infecting pathogens can exhibit a variety of interactions ranging from direct or indirect competition to cooperation, which are predicted to have different impacts on both host and parasite fitness (Mideo, 2009; Cressler et al., 2016; Pike et al., 2019; Rovenolt and Tate, 2022; Venter et al., 2022). Different pathogen groups are likely to have different infection pathways, resource needs, replication rates or transmission mechanisms making any outcome on the host or pathogen fitness hard to predict. Alterations in host nutrition can impact both host weight and condition, including immunity, as well as pathogen acquisition (through changes in feeding rate). Hosts feeding on better quality resources usually have greater fitness, such as higher weights and increased reproduction (Awmack and Leather, 2002; Grindstaff et al., 2005; Hall et al., 2009; Simpson and Raubenheimer, 2011; Lange et al., 2014). Hence higher host condition due to better nutrition has been shown to improve survival in single (Cotter et al., 2011; Zhang et al., 2012; Palermo et al., 2013) and mixed pathogen infections (Gorsich et al., 2014; Budischak et al., 2015; Roberts and Longdon, 2021). In particular, the amount of protein, relative to carbohydrate, in the host diet has been shown to play a key role in host survival when challenged with multiple pathogens. For example, increasing protein in the diet enhances mouse survival in helminths-microparasite co-infections (Budischak et al., 2015) and protein supplement increases the survival of the honey bee, *Apis mellifera*, when co-infected with a microsporidian, *Nosema ceranae*, and the deformed wing virus-B (Tritschler et al., 2017). However, more experimental data are needed to understand how variation in resources affects host fitness when challenged by multiple pathogens.

In theory, within-host competition is expected to select for an increase in host exploitation by competing pathogens, resulting in an increase in pathogen virulence (Choisy and de Roode, 2010; Alizon et al., 2013; Clay and Rudolf, 2019). However, changes in resource quality or quantity can have major impacts on pathogen within-host interaction and hence virulence. Indeed, increased within-host resources can be beneficial for pathogen replication (Johnson et al., 2007; Vale et al., 2013; Cressler et al., 2014; Tseng and Myers, 2014) or limit the negative effect of pathogen competition (Lacroix et al., 2014; Budischak et al., 2015), while low or bad quality food can exacerbate competition between pathogens, lower the reproduction of the non-dominant pathogen and cause faster exhaustion of resources (Smith, 2007; Cressler et al., 2014; Abdullah et al., 2017). Moreover, within-host competition can be detrimental for competing pathogens

themselves if they aren't able to complete their life cycle, particularly in the case of pathogens that need to kill their host to release or produce their transmission stages (obligate killers). Earlier death caused by a dominant competitor is likely to have a major impact on the epidemiology of a slower-killing pathogen species (Malakar et al., 1999; Chouvenc et al., 2012). Nevertheless, trying to better understand how co-infections are influenced by the environment is crucial if we want to be able to manage disease outbreaks in natural populations

In this chapter, we examine the impact of diet quality on the outcome of co-infection with two insect pathogens, a host-specific baculovirus and a generalist entomopathogenic fungus, *Beauveria bassiana*. It is well established that host diet can alter the likelihood of baculovirus infection, both through direct interactions in the midgut, and indirectly through changes in host feeding behaviour, development, morphology, and immunity (Lee et al., 2006; Plymale et al., 2008; Povey et al., 2014; Shikano and Cory, 2016; Shikano, 2017; Chen et al., 2018). Even changes in diet after virus challenge can alter the outcome of infection: lepidopteran larvae fed a higher protein diet after virus challenge have a higher chance of survival (Lee et al., 2006; Povey et al., 2014; Shikano and Cory, 2016). Unlike baculoviruses, entomopathogenic fungi enter their host through the cuticle (Shah et al., 2005; Mascarin and Jaronski, 2016). There is less information on the impact of nutrition on fungal infection, although effects could be exerted both directly, via plant surface interactions on the transmission stages (spores), and indirectly by changes in host condition and behaviour, as with the baculoviruses (Cory and Ericsson, 2010). The Australian plague locust (*Chortoicetes terminifera*) had a higher chance of survival post-infection on high carbohydrate diets when challenged with the fungus *Metarhizium acridum* (Graham et al., 2014). Conversely, Srygley & Jaronski (2018) found the opposite trend in Mormon crickets (*Anabrus simplex*) challenged with *B. bassiana*. These studies suggest that pathogen groups might not respond in the same manner to variation in diet, highlighting the need for more experimental data on the effect of host nutritional status on the outcome of co-infection.

Using the cabbage looper, *Trichoplusia ni* Hübner (Lepidoptera: Noctuidae) as the host, we challenged larvae with our focal pathogen, the baculovirus, *Trichoplusia ni* single nucleopolyhedrovirus (TniSNPV), and examined the impact of co-infection with the entomopathogenic fungus *B. bassiana* on host and pathogen fitness. After pathogen challenge, the larvae were reared on one of three artificial diets differing in their quality

(protein-to-carbohydrate ratios). To examine whether the response was dose-dependent, we challenged the larvae with two virus doses (with or without fungus). (i) We first investigated whether diet quality altered overall larval mortality and speed of kill following single and mixed infections. (ii) We then examined how co-infection, diet and virus dose affected the mortality caused by each pathogen individually, defined by the production of the appropriate transmission stages. (iii) We also explored whether co-infection increased potential sublethal effects in the larvae that survived pathogen challenge, by comparing pupal weights, which in (female) Lepidoptera are strongly linked to fecundity. (iv) Lastly, we examined whether co-infection altered the production of pathogen transmission stages and whether virus OBs differed in virulence.

3.3. Materials and Methods

3.3.1. Insects and pathogens

Trichoplusia ni eggs were obtained from Insect Production Services (Natural Resources Canada, Sault Ste Marie, ON). After hatch, *T. ni* larvae were maintained at 25°C with a 16L:8D photoperiod on a wheat germ-based artificial diet, containing a 1:1.1 (P:C) ratio (Shikano and Cory, 2014). Newly moulted 4th instar larvae used in the experiment were reared individually from the neonate stage.

TniSNPV was initially isolated from an infected *T. ni* larva collected from a greenhouse in the Fraser Valley, British Columbia (Janmaat and Myers, 2005). The virus was amplified in *T. ni* larvae and semi-purified by multiple rounds of differential centrifugation. *Beauveria bassiana* suspensions were obtained by serial dilutions from the original commercial product, BotaniGardES (initial concentration of 2.11×10^{10} spores/ml). Numbers of both transmission stages, virus occlusion bodies (OBs) and fungal spores, were estimated at 400x magnification using an improved Neubauer haemocytometer (Hausser Scientific^{TM}, depth 0.1mm).

3.3.2. Pathogen challenge

Newly moulted fourth instar larvae were challenged with either a single pathogen, TniSNPV or *B. bassiana*, or a combination of the two. For each pathogen treatment, 70 larvae were randomly selected and transferred individually into 12-well plates. As the virus

must be ingested to initiate infection, larvae were exposed to either 100 or 1,000 TniSNPV OBs (low and high dose respectively) by pipetting a 1µl droplet of virus suspension onto a 3 x 2 mm plug of the standard rearing diet. Preliminary experiments showed that 100 and 1,000 OBs killed approximately 30% and 75% of fourth instar larvae respectively. Larvae that did not eat the diet plug in 24 hrs were removed from the experiment. To mimic natural fungal infection, larvae were challenged with *B. bassiana* by placing a 1 µl droplet containing $3x10^4$ spores onto their dorsal abdomen (previously determined LD50). Larvae exposed to both pathogens were challenged with fungus immediately after being placed in the wells with the virus dose. The experiment therefore had a total of six pathogen treatments (unchallenged control, three single pathogens (virus low, virus high and fungus) and two co-infection treatments (virus low plus fungus, virus high plus fungus)). Unchallenged control larvae were treated with 1 µl of distilled water placed on their dorsal abdomen and on a 3 x 2 mm diet plug.

3.3.3. Diet quality experiment

Diets

Twenty-three larvae from each of the six pathogen exposures were randomly assigned to one of three diet qualities in individual 1oz SOLO® cups (i.e. 18 treatments). The diets contained a total of 60% (dry mass) of digestible macronutrients, protein (casein) and carbohydrate (sucrose), in different ratios: 20% protein (P) with 40% carbohydrate (C) (20:40), 30:30 or 40:20. All other dry ingredients were the same; 15% of micronutrients and antimicrobials (Wesson's salt, cholesterol, ascorbic acid, sorbic acid, sodium alginate, vitamin wheat germ oil) and 25% cellulose. Dry ingredients were mixed and suspended at a 1:5 ratio in a 1.35% agar solution (modified from Shikano and Cory, 2014). In a choice experiment, Shikano & Cory (2014) showed that the final intake target for *T. ni* larvae was around 1.3:1 (P:C), suggesting that the larvae preferred a more balanced diet when not infected. The range of macronutrient ratios was also chosen to reflect what *T. ni* larvae could encounter in the wild (Shikano and Cory, 2015). We therefore avoided extreme P:C ratios which, while likely to exaggerate any differences, were also likely to result in high larval mortality and pupation failure.

Insect monitoring

Individual larvae were maintained at 24°C with a 16L:8D photoperiod and monitored daily until death or pupation. The symptoms caused by each pathogen are visually distinct and easily identifiable. Larvae that die of baculovirus infection are swollen and pale with a soft tegument, which easily ruptures to release millions of milky OBs. Larvae that succumb to *B. bassiana* challenge are purple-coloured and rigid. These cadavers were first surface sterilized by dipping in 1% sodium hypochlorite solution for 1 min, followed by two rinses in distilled water, to remove any contaminating external pathogens. Then to confirm fungal death, cadavers were placed in individual humidity chambers (1oz SOLO® cup with damp cotton wool) and kept at 24°C to allow the fungus to sporulate. The fungus produces obvious conidia, which form a white cloud on the surface of the cadaver. Any unclear deaths were initially treated as fungal cadavers and checked for sporulation. Survivors were weighed 3 days after they formed pupae.

3.3.4. Pathogen fitness

Pathogen yield

When possible, up to 10 cadavers per treatment for each pathogen-diet quality combination were randomly selected to estimate the number of OBs or spores produced. For the viral yield analysis, only the cadavers that we were able to transfer whole were included. Sterilized water was added to each cadaver to make the volume up to 1 ml in a 1.5 ml microtube. Cadavers were then mixed thoroughly with a micro-pestle for a minute and vortexed to release the OBs. A sub-sample of each cadaver was diluted by x100 or x1,000 to estimate the number of OBs produced using a 0.1mm deep improved Neubauer haemocytometer and a light microscope at x400. The total number of OBs was estimated four times independently for each cadaver and we took the average as our final count. To estimate the number of transmission stages produced in the fungal infections, sporulated cadavers were placed in a 1.5 ml microtube containing 0.5 ml of 0.01% Tween 80, around seven days after sporulation, and vigorously vortexed for 1 min, then macerated with a micro pestle for 1 min to enhance dislodgement of conidia. This step was repeated after adding another 0.5 ml of 0.01% Tween 80 before removing the body. Microtubes containing spores were then stored at 4°C until further analysis. Fungal yield was estimated using the same method described for viral OBs.

Size of viral transmission stage

It is possible that the size of the transmission stage changes as a result of co-infection or altered diet quality, therefore we estimated OB volume. We randomly selected four cadavers per virus treatment (12 treatments total: diet quality (3) * virus dose (2) * infection treatment (2)) along with the original TniSNPV suspension used in the experiment. The four cadavers were pooled by mixing 500 µl of each suspension. The samples were prepared on scanning electron microscope stubs by drop-casting the concentrated suspensions of OBs, followed by 2 hrs of air-drying. They were then sputter-coated with a conductive layer of iridium at a nominal thickness of 15 nm (15.49 nm by thickness monitor), using a Leica EM ACE600 coater. Imaging was carried out at room temperature using an FEI (Field Electron and Ion Company, now part of Thermo Fisher Scientific) Nova Nano scanning electron microscope 430, with the incident beam at 5 keV. The diameter (d) of individual OBs was measured directly from the photographs using ImageJ. As OBs are approximately spherical in shape, OB volume (V) was estimated using the following formula: $V=(\pi d^3)/6$. When possible, at least 20 different OBs were randomly selected for each treatment.

Virus virulence

To examine whether co-challenge or host nutrition affected the virulence of the baculovirus after one generation, we carried out a dose-response assay. To enable comparison between OB size and virus virulence, the same four cadavers selected to measure OB size were used. Yield for all the selected cadavers was previously estimated for virus yield data analysis. Each cadaver suspension was then serially diluted individually to obtain the chosen virus concentrations for the bioassay (10, 50, 100, 500 OBs/µl). For each of the dilutions, we then combined 0.25 ml from each of the four cadaver suspensions and mixed thoroughly. A total of 1,100 naïve and newly moulted fourth instar *T. ni* larvae, obtained from Insect Production Services (see above), were weighed, then transferred individually into 12-well plates and challenged with one of the four virus doses (10, 50, 100, 500 OBs). Twenty larvae were inoculated per dose, per treatment (12 treatments total: diet quality (3) * virus dose (2) * infection treatment (2)). Control larvae were inoculated with 1µl of distilled water. Larvae that did not eat the diet plug after 24 hrs were removed from the experiment. The day after inoculation, each larva was individually transferred into a 1oz cup and reared at 25°C with a 16L:8D photoperiod on normal colony stock, wheat germ-based, artificial diet. Larvae were monitored daily until death or

pupation. Any unclear deaths were smeared and examined under oil immersion on a light microscope (x1,000) to look for OBs (Giemsa stain).

3.3.5. Statistical analysis

Host mortality

We first wanted to know whether overall mortality in single and mixed infections was altered by diet quality and whether this was modulated by virus dose. Mortality was analysed using a generalized linear model (GLM) with a binomial distribution and logit link function. In our models, diet was included as an ordinal variable (three levels of protein). In addition to diet, pathogen treatment was included in the model as categorical (three single and two mixed treatments) (Table 3.1a). No unchallenged control larvae died of either pathogen; thus, control larvae were not included in mortality analyses. The few larvae for which a cause of death could not be assigned were also not included in the analysis.

We then repeated the GLM analysis focusing on the mortality only caused by either the virus or the fungus (cause of death, defined by the production of transmission stages, was unambiguous in all cases). Virus-induced mortality was analysed including protein level and virus dose as ordinal variables as well as the infection treatment (mixed or single) as a categorical variable (Table 3.1a). For the fungal mortality analysis, we omitted the single virus treatments and only included diet (protein as ordinal) and virus dose as ordinal (high, low and no virus) in our model (Table 3.1a). In this case, “no virus” represented a challenge with the fungus alone.

Duration of infection

Pathogen speed of kill (time from pathogen challenge to death) was examined using an Analysis of Variance (ANOVA). For the overall speed of kill, the initial model included protein (ordinal) along with the pathogen treatment as categorical (three single and two mixed treatments) (Table 3.1a). Then, for the virus-specific speed of kill the initial model included protein and virus dose as ordinal (high or low) as well as infection treatment as categorical (mixed or single) variables, excluding *B. bassiana* single infection and only considering larvae that died of virus infection. Similarly, fungus speed of kill was

analysed including protein and virus dose (high, low and no virus) as ordinal variables (Table 3.1b).

Sublethal effects

Pupal weights of the survivors were analysed by ANOVA. Initially, we assessed the underlying impact of the diet treatments alone on pupal weight (i.e. in the absence of pathogen challenge) by comparing the unchallenged control larvae, with diet treatment as ordinal (three levels) (Table 3.1c). Then, we examined whether surviving a challenge with a single pathogen or a co-infection altered this relationship, indicating a possible sublethal effect of fighting off the pathogen. In this analysis diet and virus dose were classified as ordinal (three levels) and fungus treatment as a categorical (no fungus or fungus) variable (Table 3.1c).

Yield and virus virulence

Production of virus and fungus transmission stages were both analysed using general linear models. Virus yield (numbers of OBs per insect) was $\log_{10}$-transformed to meet the assumption of normality. The infection treatment was included as categorical (mixed or single); diet (levels of protein) and virus dose (high or low) were included as ordinal (Table 3.1d). Speed of kill was then included as a linear and quadratic covariate (Table 3.1d). The total number of fungal spores harvested was square root transformed to fit the normal distribution. Virus dose and protein in the diet and fungal speed of kill (linear and quadratic covariate) were included in the model as described above (Table 3.1d).

OB volume was $\log_{10}$-transformed and analysed by ANOVA. We first compared the initial virus suspension to the OBs produced in all treatments. Then, to determine if co-infection and diet influenced OB size, we ran a second model excluding the initial suspension and including the infection treatment (mixed or single) as categorical variable as well as virus dose and protein in the diet as ordinal explanatory variables (Table 3.1e).

Finally, viral mortality in the passage assay was analysed using a GLM with a binomial distribution and logit link function, corrected for overdispersion. The original infection treatment (mixed or single) was included as categorical, diet treatment (three levels of protein), and previous virus dose were included as ordinal (high or low) with the

bioassay virus dose (log_{10}-transformed) included as a covariate (Table 3.1f). The LC50 values with standard errors were then extracted from the final GLM model

For each model, all the interactions between fixed effects were included in the initial model and removed if not significant following model simplification procedures. All analyses were conducted in R-4.0.1, the package *emmeans* (formerly known as *lsmeans;* Lenth, 2020) was used to carry out post hoc comparisons among groups after fitting a GLM model.

Table 3.1. Statistical models used to analyse (a) larval mortality (overall or pathogen-specific), (b) speed of kill (overall or pathogen-specific), (c) pathogen yield (virus and fungus), (d) OB size, and (e) mortality form the passage experiment. [i]Control larvae only, [ii]all pupal weight included (control and challenged larvae), [*]OB size model excluding the initial suspension data.

Response variable	Initial model formula	Statistical model
(a) Larval mortality		
Overall	~Diet x pathogen treatment	GLM (binomial)
Virus-induced	~Diet x virus dose x single/mix	GLM (binomial)
Fungus-induced	~Diet x virus dose	GLM (binomial)
(b) Speed of kill		
Overall	~Diet x pathogen treatment	ANOVA
Virus speed of kill	~Diet x virus dose x single/mix	ANOVA
Fungus speed of kill	~Diet x virus dose	ANOVA
(c) Pupal weight		
Pupal weight[i]	~Diet	ANOVA
Pupal weight[ii]	~Diet x virus dose x fungus	ANOVA
(c) Pathogen yield		
Virus yield	~Diet x virus dose x single/mix + speed kill + $(\text{speed kill})^2$	Linear model
Fungus yield	~Diet x single/mix+ speed kill + $(\text{speed kill})^2$	Linear model
(d) OB size		
OB volume	~ Initial/passaged	ANOVA
OB volume[*]	~ Diet x virus dose x single/mix	ANOVA
(e) Virulence assay		
Mortality	~previous diet x previous virus x previous single/mix x log10(dose)	GLM (binomial)

3.4. Results

3.4.1. Host mortality

Overall pathogen mortality

Overall larval mortality was significantly higher in the mixed treatments compared to mortality in the single fungus treatment, but not the single virus treatments (Pathogen treatment: χ^2_4=22.29. p<0.001; Protein x pathogen treatment: χ^2_8=11.47, p=0.18; Fig. 3.1A). However, mortality in the low virus dose co-infection treatment was of borderline significance and 38% higher than when larvae were challenged with TniSNPV at low dose alone (contrast: virus low - virus low + fungus: p=0.08). The amount of protein in the diet provided to the larvae post-pathogen challenge did not affect overall mortality in any of the infection treatments (Protein: χ^2_2=0.18. p=0.92).

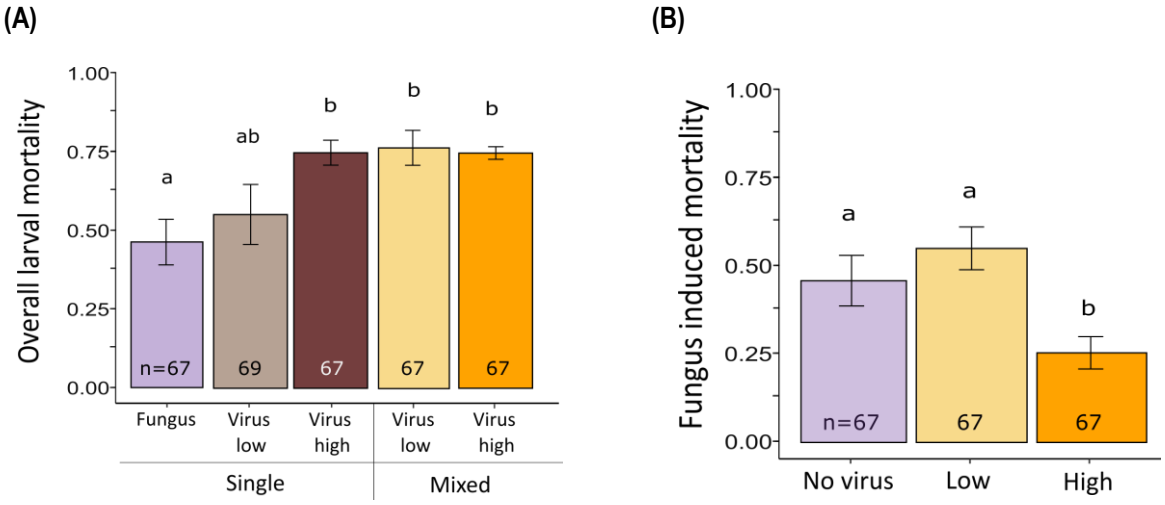

Figure 3.1. Larval mortality of fourth instar *T. ni* larvae (mean ± s.e.), (A) overall mortality after challenge with either *B.bassiana*, a low (100 OBs) or high (1,000 OBs) dose of TniSNPV alone (single) or co-infected with *B. bassiana* (mixed); and (B) fungal-induced mortality of larvae singly challenged with *B. bassiana* (no virus) or co-challenged with *B. bassiana* and TniSNPV (low or high dose). Letters indicate significant differences between treatments at p<0.05.

Virus-induced mortality

Co-infection with the fungus reduced virus-induced mortality by almost half compared to the single dose treatments, 34% and 65% respectively regardless of the virus dose (Table 3.2a). Changing protein levels did not significantly alter virus mortality (Table 3.2a). As expected, virus-induced mortality was significantly higher at the higher dose, 63% and 37% for high and low respectively.

Table 3.2. Analysis of the effect of diet quality (amount of protein), co-infection (single/mix) and virus dose (high or low) on (a) virus-induced the mortality, data analysed using generalized linear models (Type-III analysis-of-variance tables). and (b) virus-specific speed of kill. Significant p values are highlighted in bold and terms not included in the final model are italicized.

Response	Explanatory variables	df	χ^2	p value
(a) Virus-induced mortality	*Protein*	2	2.81	0.25
	Virus dose	1	17.21	**<0.0001**
	Single/mix	1	25.96	**<0.0001**
	Protein x virus dose	2	0.62	0.73
	Protein x single/mix	2	0.70	0.71
	Virus dose x single/mix	1	0.71	0.40
	Protein x virus dose x single/mix	2	3.81	0.15
		df	**F-value**	**p value**
(b) Virus speed of kill	Protein	*2, 129*	2.70	0.07
	Virus dose	*1, 129*	1.52	0.22
	Single/mix	*1, 128*	2.44	0.12
	Protein x virus dose	*2, 129*	3.73	**0.03**
	Protein x single/ mix	*2, 125*	0.05	0.95
	Virus dose x single/ mix	*1, 125*	0.27	0.60
	Protein x virus dose x single/ mix	*2, 123*	1.29	0.28

Fungus-induced mortality

Co-infection with the virus significantly reduced fungal mortality by 51%, but only at the highest virus dose (Virus dose: χ^2_2=13.60. p<0.001). Fungal mortality also increased with increasing protein in the diet and co-infection with the virus did not affect this (Protein: χ^2_2=5.91. p=0.05; Protein x virus dose: χ^2_4=0.48, p=0.98. Fig. 3.1B).

3.4.2. Pathogen speed of kill

Overall speed of kill

Larvae that were co-challenged with TniSNPV and *B. bassiana* died more rapidly than the larvae in their respective single virus treatments and larvae challenged with *B. bassiana* alone died faster than in any other treatments (Pathogen treatment: $F_{(4, 215)}$=35.09, p<0.0001; Protein x pathogen treatment: $F_{(8, 208)}$=1.47, p=0.17; Fig. 3.2A). Larvae simultaneously challenged with the low virus dose and fungus died faster than the larvae in the mixed virus high treatment. Diet quality did not affect the overall speed of kill (Protein: $F_{(2, 213)}$=0.80, p=0.45).

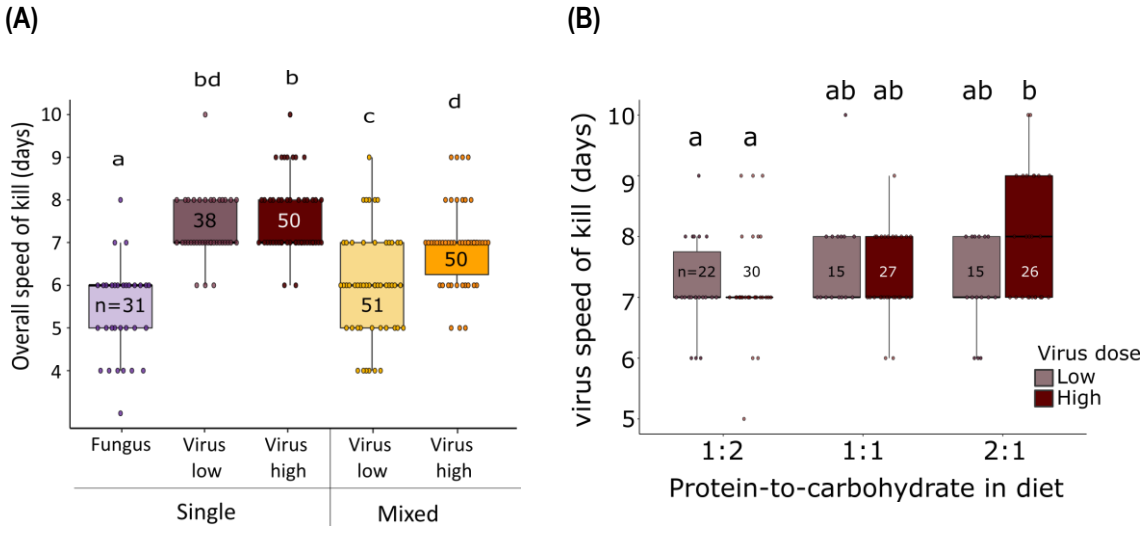

Figure 3.2. Pathogen speed of kill, fourth instar *T. ni* larvae. (A) Overall speed of kill of larvae challenged with either *B.bassiana*, a Low (100 OBs) or High (1,000 OBs) dose of TniSNPV alone (single) or co-infected with *B. bassiana* (mixed), and (B) virus-specific speed of kill of larvae challenged with either a low (100 OBs) or high (1,000 OBs) dose of TniSNPV on different quality diets (P:C ratios). Boxes indicate 25th and 75th percentiles, lines within boxes indicate medians, and whiskers includes values within 1.5 times the interquartile range. Letters indicate significant differences between treatments at p<0.05 (Tukey's HSD).

TniSNPV speed of kill

Co-infection with *B. bassiana* did not affect TniSNPV speed of kill (Table 3.2b). Virus-killed insects took on average one day longer to die on the highest levels of protein

when challenged with a high dose of TniSNPV, irrespective of the infection treatment (Single/Mixed), but there was no effect of protein on the duration of viral infection for larvae challenged at low virus dose (Table 3.2b, Fig. 3.2B).

Fungus speed of kill

Co-infection with TniSNPV at the high dose lengthened the duration of fungal infection by approximately one day (total 6.4 days) compared to the fungus alone and the mixed virus low treatment, regardless of the protein levels in the diet (Virus dose: $F_{(2, 82)}$=5.51, p=0.006; Protein: $F_{(2, 80)}$=1.19, p=0.31; Protein x Virus dose: $F_{(4, 76)}$=1.78, p=0.14).

3.4.3. Pupal weight

In the absence of infection, larvae reared on the highest protein diet produced pupae that were on average 7% lighter than those from the other two diets (protein: $F_{(2, 53)}$=5.46, p=0.007) and pathogen challenge did not alter this (Table 3.3, Fig. 3.4A). Pupae from larvae that were challenged with the fungus alone were heavier than the unchallenged control pupae, but virus challenge had no effect (Table 3.3; Fig. 3.4B).

Table 3.3. Analysis of the effect of diet quality (protein level), virus dose (high or low) and fungus (absent or present) on *T. ni* pupal weight. Significant p values are highlighted in bold, and terms not included in the final model are italicized.

		df	F-value	p value
Pupal weight	Protein	2, 163	15.74	**<0.0001**
	Virus dose	2, 163	2.65	0.07
	Fungus	1, 163	7.67	**0.006**
	Protein x virus dose	4, 157	0.33	0.86
	Protein x fungus	2, 157	0.27	0.77
	Virus dose x fungus	2, 163	3.86	**0.02**
	Protein x virus dose x fungus	4, 153	0.89	0.47

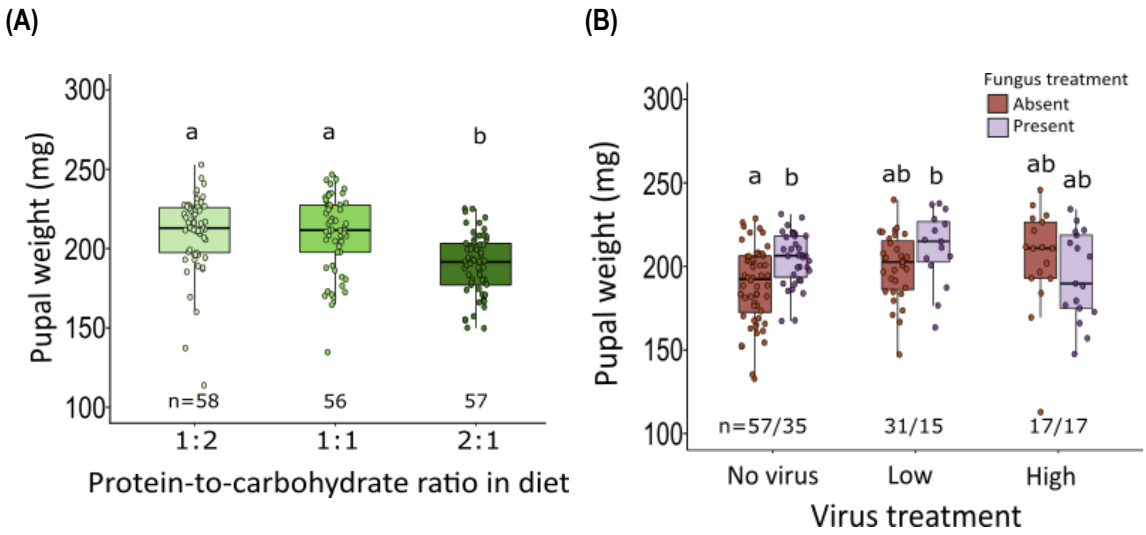

Figure 3.3. Effect of (A) change in dietary protein levels and (B) pathogen infection (single and mixed) and virus dose on *T. ni* pupal weight. Boxes indicate 25th and 75th percentiles, lines within boxes indicate medians, and whiskers includes values within 1.5 times the interquartile range. Letters indicate significant differences between treatments at p<0.05 (Tukey's HSD).

3.4.4. Pathogen yield

Virus yield

The number of transmission stages produced after TniSNPV infection decreased at the highest protein level, but only significantly so at the higher virus dose, such that there was a 60% decrease in virus yield compared to the low protein diet (Table 3.4a, Fig. 3.4A). Co-challenge with the fungus had no effect on the total number of OBs produced in each cadaver. Viral yield also had a non-linear relationship with speed of kill with the production of OBs peaking 7-8 days after challenge (Table 3.4a, Fig. 3.4B).

Fungus yield

Co-infection with the virus increased the production of fungal spores by 35%, compared to a single infection but only at the lower virus dose (Virus dose: $F_{(2, 41)}$=4.23, p=0.02; Virus dose x protein: $F_{(4, 35)}$=0.41, p=0.80; Fig. 3.5). Neither the level of protein in the diet nor the time it took for *B. bassiana* to kill its host influenced spore production (Protein: $F_{(2, 39)}$=0.55, p=0.58; speed of kill: $F_{(1, 39)}$=0.13, p=0.72; Speed of kill^2: $F_{(1, 39)}$=0.98, p=0.33).

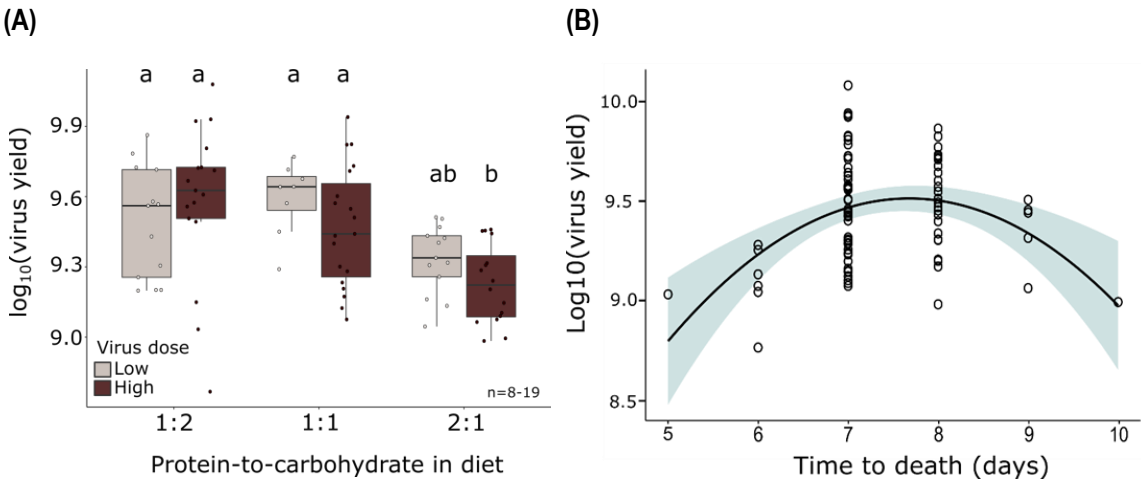

Figure 3.4. **Virus yield of fourth instar *T. ni* larvae (A) challenged with either a low (100 OBs) or a high (1,000 OBs) dose of TniSNPV on different quality diets (P:C ratios), boxes indicate 25th and 75th percentiles, lines within boxes indicate medians, and whiskers includes values within 1.5 times the interquartile range. Letters indicate significant differences between treatments at p<0.05 (Tukey's HSD). (B) Virus yield and speed of kill trade-off, lines show fitted statistical models with 95% confidence intervals and raw data points.**

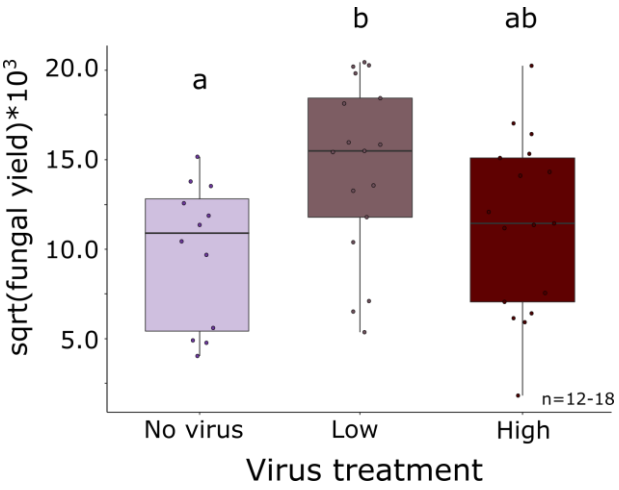

Figure 3.5. **Fungal yield (square-root transformed) of fourth instar *T. ni* larvae challenged with either fungus alone (no virus) or co-challenged with *B. bassiana* and a low (100 OBs) or a high (1,000 OBs) dose of TniSNPV. Boxes indicate 25th and 75th percentiles, lines within boxes indicate medians, and whiskers includes values within 1.5 times the interquartile range. Letters indicate significant differences between treatments at p<0.05 (Tukey's HSD).**

3.4.5. Viral transmission stages and virulence

Size of virus transmission stages

The OBs harvested from cadavers in the experiment were 1.7 times larger than the OBs from the initial suspension used to challenge *T. ni* larvae, 0.67 μm^3 (± 0.02 SEM) and 0.39 μm^3 (± 0.03 SEM) respectively ($F_{(1, 402)}$=85.09, p<0.0001). Analysis of OB volume, after excluding the initial suspension from the analysis, showed that OBs were larger when larvae were fed higher protein diets (2:1 P:C ratio) but only in single infections; there was no significant difference in size for OBs from mixed infected cadavers (Table 3.4b, Fig. 3.6A). Similarly, OB volume increased with increasing protein in the diet, but only at high virus dose (Table 3.4b, Fig. 3.6B). Co-infection with *B. bassiana* significantly reduced OB size at the high virus dose, such that OBs from the single challenge treatment were on average 1.5 times bigger than OBs from the mixed infection treatment (table 3.4b, Fig. 3.6C). No difference was observed at low virus dose.

Table 3.4. Analysis of the effect of diet quality (level of protein), co-infection (Single/mix) and virus dose (high or low) on (a) viral yield and (b) OB size. Significant p values are highlighted in bold and terms not included in the final model are italicized.

Pathogen fitness		Df	F-value	p value
(a) Virus yield	Protein	2, 78	13.93	**<0.0001**
	Virus dose	1, 78	0.70	0.40
	Single/mix	1, 77	0.59	0.44
	Protein x virus dose	2, 78	3.26	**0.04**
	Protein x single/mix	2, 75	1.86	0.16
	Virus dose x single/mix	1, 75	1.55	0.22
	Protein x virus dose x single/mix	2, 72	1.08	0.35
	Speed of kill	1, 78	5.58	**0.02**
	Speed of kill^2	1, 78	16.00	**0.0001**
(b) OB volume	Protein	2, 291	4.37	**0.01**
	Virus dose	1, 291	3.70	0.06
	Single/mix	1, 291	3.32	0.07
	Protein x virus dose	2, 291	6.95	**0.001**
	Protein x single/ mix	2, 291	12.41	**<0.0001**
	Virus dose x single/ mix	1, 291	9.50	**0.002**
	Protein x virus dose x single/ mix	2, 289	2.09	0.13

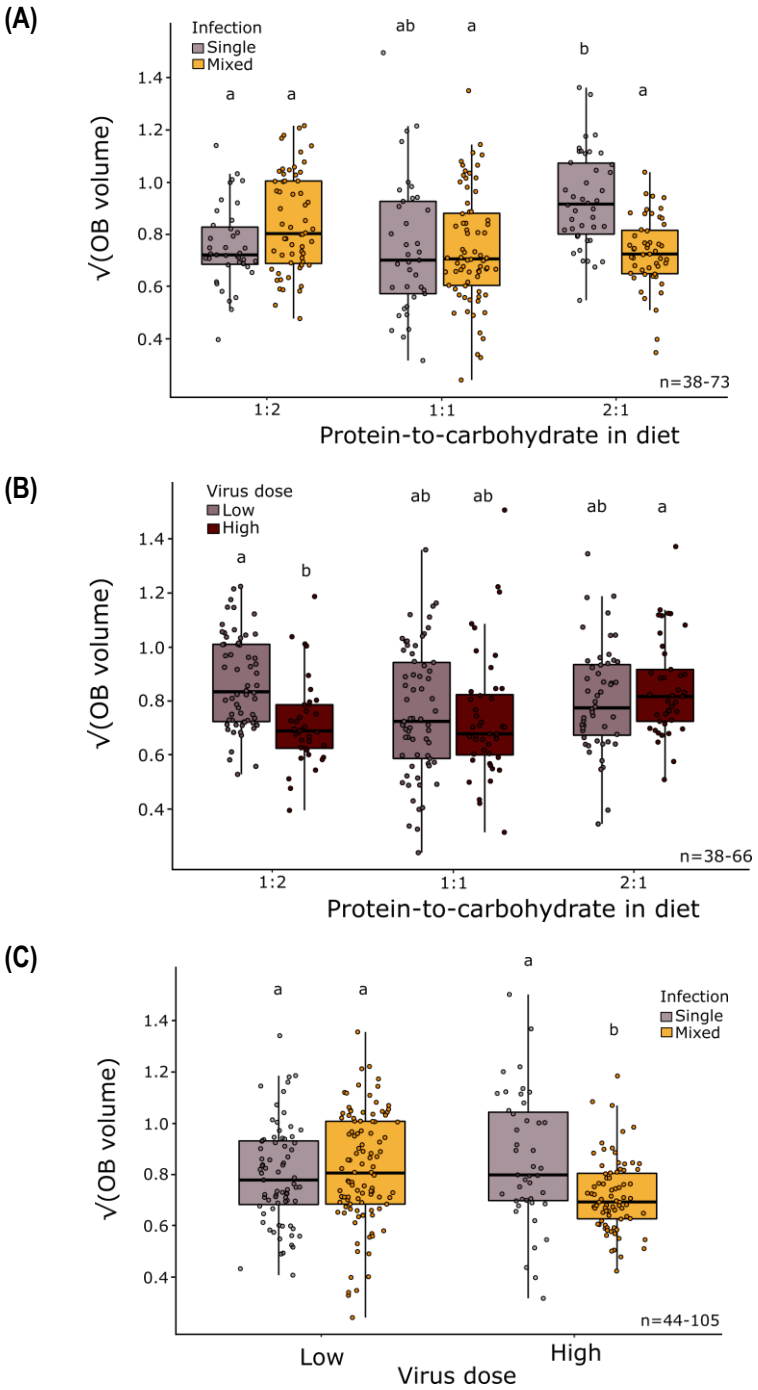

Figure 3.6. **Effect of diet quality (P:C ratios) on occlusion body size (area in μm^2) for *T. ni* larvae challenged with (i) a low (100 OBs) or (ii) a high (1,000 OBs) dose of TniSNPV either alone (Single) or co-challenged with *B. bassiana* (Mixed). Boxes indicate 25th and 75th percentiles, lines within boxes indicate medians, and whiskers includes values within 1.5 times the interquartile range. Letters indicate significant differences between treatments at p<0.05 (Tukey's HSD).**

Virus virulence

We collected transmission stages from the larvae that succumbed to viral infection in the nutrition experiment and used them to challenge naïve 4th instar *T. ni* larvae to examine whether there were differences in virus virulence. None of the control larvae died of viral infection and overall, less than 1.5% of the total larvae died of unknown causes or handling issues, consequently larvae that did not die of viral infection were removed from the analysis. OBs collected from cadavers infected with the low virus dose, whether single or mixed, produced the same mortality at all protein levels (Table 3.5; Fig. 3.8). However, when OBs were collected from cadavers infected with a high viral dose there were differences in virulence. OBs harvested from insects challenged with TniSNPV alone were significantly less virulent (46%) when the infected larvae were reared on high protein diet (2:1 P:C) compared to both the balanced and low protein diets (Table 3.5; Fig. 3.8). In the mixed infection with the higher virus dose, OBs were less virulent when larvae were fed a balanced diet (1:1 P:C), significantly so compared to lower protein levels.

If the viruses from the different treatments are simply compared in terms of their LD50, TniSNPV virulence increased after passage in 4th instar *T. ni* larvae such that most LD50s were at least 50% lower than initial suspension (Table 3.6).

Table 3.5. Analysis of the effects of co-infection, virus dose and diets varying in protein-to-carbohydrate (P:C) ratios on the virulence of TniSNPV OBs in term of mortality of fourth instar *T. ni* larvae. Data analysed using generalized linear models (Type-III analysis-of-variance tables). Significant p values are highlighted in bold and terms not included in the final model are italicized.

		Df	F-value	p value
Larval mortality	Log_{10}(virus dose)	1	310.52	**<0.0001**
	Previous protein: PP	2	9.86	**0.0004**
	Previous virus dose (low/ high): PV	1	1.97	0.17
	Previous infection (single/mixed): PI	1	0.67	0.42
	PP x PV	2	10.31	**0.0003**
	PP x PI	2	2.07	0.14
	PV x PI	2	1.07	0.31
	PP x PV x PI	2	11.90	**0.0001**

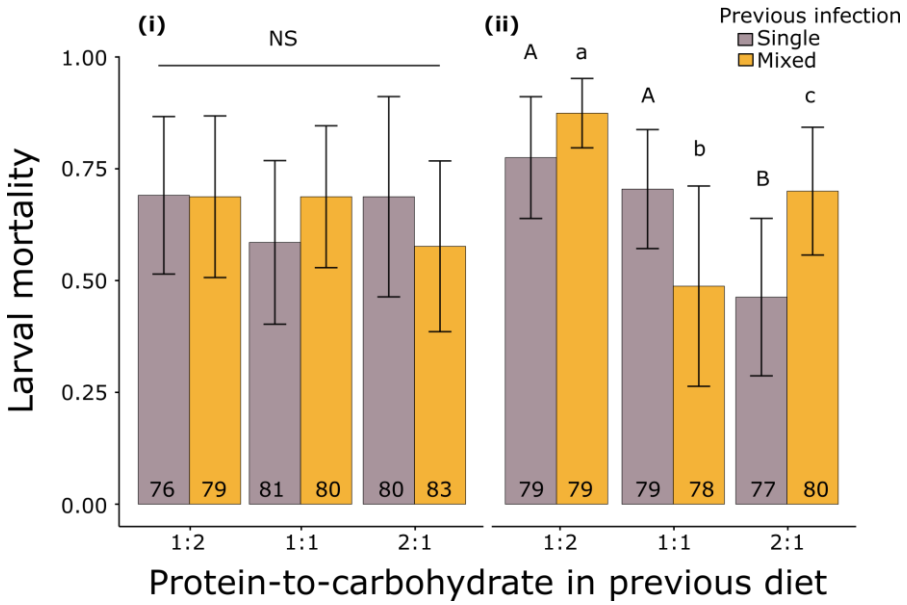

Figure 3.7. **Mortality (mean ± s.e) of fourth instar *T. ni* larvae challenged with OBs collected from cadavers fed on different quality diets (P:C ratio) and challenged with either (i) a low or (ii) high dose of TniSNPV alone (Single) or co-challenged with *B. bassiana* (Mixed). Letters indicate significant differences at p<0.05 (contrasts).**

Table 3.6. **Mean lethal dose (LD) 50s ± standard errors of TniSNPV initial stock suspension and TniSNPV after one passage through fourth instar *T. ni* larvae fed diets varying in their protein-to-carbohydrate (P:C) ratios after being singly challenge with TniSNPV, at low or high dose, or co-challenged with TniSNPV and *B.bassiana*.**

Previous virus dose	Previous infection	Previous diet (P:C)	LD50 (OBs/larvae)
Initial suspension	N/A	N/A	~100
Low	Single	1:2	23.17 (±1.87)
	Mixed	1:2	21.23 (±.13)
	Single	1:1	40.57 (±1.93)
	Mixed	1:1	21.34 (±2.05)
	Single	2:1	29.87 (±1.75)
	Mixed	2:1	42.82 (±1.75)
High	Single	1:2	13.72 (±1.96)
	Mixed	1:2	7.15 (±2.50)
	Single	1:1	17.96 (±2.27)
	Mixed	1:1	72.39 (±1.40)
	Single	2:1	75.35 (±2.00)
	Mixed	2:1	19.16 (±2.13)

3.5. Discussion

Our study clearly showed that not only is host nutrition extremely important in determining the outcome of pathogen challenge but also that the impact of diet varies with the pathogen type. The result of co-infection was complex and dependent on both the relative dose (impact) of the pathogens and the diet of the host post-infection. Diet quality (change in P:C ratio) altered the relative success of each pathogen and/or the duration of infection. In addition, TniSNPV occlusion body production, size and virulence were all affected by a complex interaction between diet quality, virus dose and co-infection status.

3.5.1. Single pathogens

Unexpectedly, changes in diet quality after challenge did not affect baculovirus mortality. This is in contrast with other studies that have found higher survival in insects challenged with the baculovirus and provided with high protein/low carbohydrate diets (Lee et al., 2006; Shikano and Cory, 2016). The most likely explanation for this is that we excluded extreme P:C ratios (which would likely result in unsuccessful pupation) as we wanted to examine sub-lethal effects. There is a trend for increased survival in the higher protein diets underlying the data, although this did not reach significance (Appendix, Fig. A1). Information on the impact of nutrition on fungal challenge in insects is sparse and contradictory. We found that *T. ni* larvae challenged with *B. bassiana* had better survival on a higher carbohydrate/lower protein diet; this is the first study on Lepidoptera. This agrees with Graham *et al.'s* (2014) work on *Metarhizum acridum* infection in the Australian plague locust, *Chortoicetes terminifera*, although this experiment also showed that a high carbohydrate/low protein diet reduced the levels of several measures of constitutive immunocompetence, which might have been expected to have made the hosts more vulnerable to fungal infection. The authors suggested that the fungus could be more effective at utilizing protein for growth than the host is in using nutrients for immune defence. This would support the pathogen priority model (Cressler et al., 2014), which suggests that an increase in resources (energy) is firstly used by the pathogen, depleting resources available for the host, thereby increasing pathogen proliferation and suppressing the host immune response. In addition, studies on artificial media have shown that protein increased fungal growth rate and mycelial development (Barnes et al., 1975; Safavi et al., 2007; Behle et al., 2022), but these studies do not take into account the

complexity of interactions between the host, resources and the pathogen. Contrary to Graham *et al.'s* (2014) results, a study on fungal infection in Mormon crickets, *Anabrus simplex*, showed increased survival after exposure to *B. bassiana* on higher protein diets (Srygley and Jaronski, 2018). However, one difference between the two studies is that the Mormon crickets (Srygley and Jaronski, 2018) were fed on the diets prior to infection, as well as after, so the hosts are likely to have differed in both weight and condition prior to infection, making the specific effects of diet alone on infection hard to untangle. While increasing protein relative to carbohydrate improves host survival in a number of studies (e.g. lepidopteran larvae - baculovirus (McVean et al., 2002; Lee et al., 2006; Shikano and Cory, 2016); the African armyworm, *Spodoptera exempta*, - bacterium, *Bacillus subtilis* (Povey et al., 2009); bumblebee, *Bombus terrestris*,- microsporidia, *Nosema ceranae* (Gómez-Moracho et al., 2021); fungal infections - *Daphnia dentifera* (Hall et al., 2009)), the exceptions to the rule in the locust, *C. terminifera* - fungus, *M. acridium* system (Graham et al., 2014); the adult fruit fly, *Bactrocera tryoni*, - bacterium, *Serratia marcescens* (Dinh et al., 2019); and ant workers, *Ectatomma ruidum* - fungus, *M. anisopliae* systems (Kay et al., 2014)) highlight the gaps in our understanding of the effect of nutrition in invertebrates.

3.5.2. Co-infection and virulence

Changes in diet quality after pathogen challenge did not alter the overall mortality in the co-infections, and mortality was not always greater in mixed compared to single infections. The duration of infection in the co-infection treatments fell in between the speed of kill of the single infections of the two pathogens; slower than the fastest pathogen (*B. bassiana*) but faster than TniSNPV infections. Thus, where the virus dominated the mixed infections at the higher dose, the average speed of kill was slower, reflecting its slower speed of action compared to the fungus.

One of the interesting results of this study is that the outcome of the mixed pathogen challenge was dominated by one pathogen type (in terms of producing final transmission stages) and there were very few obvious mixed infections. However, there was still clear evidence for an interaction between the two pathogens. Virus-induced mortality was halved in the co-infections compared to the single infections, whereas fungal mortality was only reduced when co-challenged with a high virus dose. This illustrates the importance of the relative effective dose of each pathogen and suggests that the fungus

was the stronger competitor, likely in part due to its more rapid speed of kill. The duration of virus-induced deaths was not altered by co-infection, although they did tend to lengthen when there was more protein in the diet. Conversely, fungal infections were slower when larvae were co-challenged with TniSNPV, but only at the high virus dose. Hence, competition between the two pathogens was likely to be stronger at this dose. Baculoviruses and fungi invade by different routes (mid-gut versus cuticle); thus, any interaction is likely to be indirect, through competition for resources or via a shared host-induced immune response, or possibly by a combination of the two, e.g. the fungus limiting the host's capacity to fight off the virus by using protein. The fungus response to varying protein levels did not change when co-infected with the virus, which supports the suggestion that the fungus is interacting directly with the protein available. This indicates that there is a delicate balance between nutrition and the risk of dying from infection which depends on pathogen identity. Studies on baculovirus-fungal interactions in Lepidoptera have generally found that the interaction was antagonistic (in terms of host mortality), although timing of exposure had inconsistent effects on whether the outcome was additive or antagonistic and also altered which was the dominant pathogen (Richter and Fuxa, 1984; Souza et al., 2019). Pauli *et al.* (2018) reported differing outcomes depending on the fungal species: *Metarhizium anisopliae* co-challenged with *Diatraea saccharalis* granulovirus (DsGV - a type of baculovirus) resulted in additive effects, with lethal concentration (LC_{50}) in the mixed infection similar to the LC_{50} expected from each pathogen alone, whereas *B. bassiana* plus DsGV showed antagonistic interaction, as the LC_{50} observed in the mixed infection was found to be smaller than expected. Knowing how different pathogen groups interact with each other at different doses, will enable us to disentangle within-host pathogen interactions and determine pathogen dominance in a variety of situations (host conditions).

The few studies that have investigated whether co-infections in invertebrates are influenced by nutrition have focused mainly on aquatic, filter-feeding species; mosquitoes (Fellous and Koella, 2010, 2009; Westby et al., 2019; Zilio and Koella, 2020) and waterfleas, particularly *Daphnia magna* (Lange et al., 2014; Reyserhove et al., 2017). Moreover, the focus of each of these studies varies from host fitness only (Fellous and Koella, 2010; Reyserhove et al., 2017) to predominantly parasite fitness (Fellous and Koella, 2009; Westby et al., 2019) and transmission within the host population (Lange et al., 2014; Zilio and Koella, 2020). In a non-aquatic study, Tritschler *et al.* (2017) recorded

the impact of additional protein (pollen) on microsporidian-virus co-infections in worker bees (*Apis mellifera*) and found that feeding the bees pollen decreased viral infection. Although in this example the deformed wing virus infections were naturally occurring and thus not controlled. Unfortunately, direct comparisons with our results are limited. While all the studies presented above agree that host nutritional status plays a key role in multiple parasite and pathogen interactions, the large differences between studies in term of the period of time the hosts were reared on the different diets, as well as the scale and variables measured (centred on the host or pathogens) emphasize the need for more comparative studies on the complexity of interactions between host resources and the outcome of within-host pathogen competition.

3.5.3. Sublethal effects

The unchallenged larvae showed that the high protein-low carbohydrate diet produced smaller pupae (which would likely develop into less fecund adults (Greenberg et al., 2001)). In our study, pupal weight was only negatively affected by the diet that contained the highest level of protein and lowest level of carbohydrate, indicating a detrimental effect of over-ingesting protein (Lee et al., 2008; Cotter et al., 2011) or the inability for the larvae to reach the optimal intake of carbohydrate (Ojeda-Avila et al., 2003). However, to determine any trade-offs or detrimental effect of ingesting an imbalanced diet, it would be necessary to measure the intake of each nutrient over the period of the experiment (Raubenheimer and Simpson, 1997). Pathogen challenge is likely to increase the demand on resources and this could have costs if the nutrients are limited (Ponton et al., 2011). We hypothesized that challenge with multiple pathogens would show increased costs for surviving hosts compared to a single pathogen challenge (Shapiro, 2000; Han et al., 2015; Wakil et al., 2017; Valverde-Garcia et al., 2019), particularly as TniSNPV and *B. bassiana* have such a different life cycle. We found no evidence that host diet quality interacted with single or mixed pathogen challenge to influence pupal weight. Overall, the survivors of pathogen challenge tended to be heavier, particularly so for those challenged with fungus. This is not unexpected as pathogen challenge is likely to select for larger individuals which are often more resistant to pathogen challenge. However, changes in diet quality seemed to be the main driver here, with pupal weight decreasing as the amount of protein increased. In our study, we did not separate male and female pupae, which might have obscured treatment differences. It might also

be more informative to examine the consequences of single versus mixed pathogen infections and the influences of diet quality after the pupal stage, for example, egg number, egg size and quality, egg viability and offspring development.

3.5.4. Pathogen yield

The production of transmission stages is the key to pathogen success. Interestingly, even though our data indicated that there were strong interactions between the two pathogens, it had no effect on virus yield. The production of virus OBs reached a similar level of replication before killing the host, regardless of the dose or co-infection. However, virus yield did decline at high protein levels at the high dose, possibly due to poorer growth (as reflected in pupal weight). Changes in diet quality did not affect *B. bassiana* yield, which also suggests that the fungus is likely to be using macronutrients for its own growth before the host can use the resources to combat infection and protein was not limited for the fungus to grow. The production of fungal spores was not related to time to death, although it would have been interesting to measure other factors such as cadaver size, as increase in size has previously been correlated to increase in spore production in single fungal infections (Luz and Fargues, 1998; Roy et al., 2006). Nonetheless, *B. bassiana* yield did increase when co-infecting with TniSNPV, especially at the low virus dose. The lack of a direct effect of co-infection on virus or fungus yield when co-infected with TniSNPV at high dose suggests that competition between the two pathogens is happening in the early stages of infection. This is also validated by the presence of only one type of transmission stage (either OBs or spores) in each individual cadavers in the co-infection treatments, meaning that a single pathogen quickly won the battle inside the host, limiting the development of the other competitor. Another possibility is that since both pathogens produce their transmission stages either late in the infection cycle (TniSNPV) or after death (*B. bassiana*), earlier stages of infection might be more important in terms of host invasion than productivity. Similar to our results, Pauli *et al.,* (2018) found that in mixed infections of the sugarcane borer larvae, *Diatraea saccharalis,* with its granulovirus and either *B. bassiana* or *Metarhizium anisopliae*, only one pathogen was able to produce transmission stages in individual cadavers. However, they did not find differences in either *B. bassiana* or *M. anisopliae* yield between mixed and single infections (Pauli et al., 2018): virus yield was not recorded. Malakar *et al.* (1999) showed the presence of fungal hyphae in virus dead cadaver in mixed infection of the spongy moth larvae, *Lymantria dispar*, with

its specific nucleopolyhedrovirus (LdNPV) and fungal pathogen *Entomophaga maimaiga*. They found a decrease in the production of LdNPV OBs/mg of body weight when larvae were challenged 10 days prior to the fungal pathogen, *E. maimaiga*, compared to single LdNPV treatment, mainly related to the faster speed of kill of the fungus (Malakar et al., 1999). However, spore production was not measured in the study and while a few cadavers contained both viral OBs and fungal hyphae, no connection was made between presence of fungal hyphae in larval cadavers and potential variation in OB yield. In the same host, LdNPV OB production was significantly lower when the microsporidian *Nosema* was inoculated 6 days prior to virus infection (Bauer et al., 1998). The decrease in LdNPV yield was mainly correlated with the decrease in the time to death when *Nosema* was inoculated first. Interestingly, *Nosema* spore production was reduced more when both pathogens were inoculated synchronously than when *Nosema* was inoculated first (Bauer et al., 1998). Baculovirus and fungus replication are generally highly correlated with the duration of infection (O'Reilly et al., 1998; Hesketh and Hails, 2015) and cadaver size respectively (Luz and Fargues, 1998; Roy et al., 2006). Hence decreases in host longevity or growth in co-infections can dramatically impair pathogen yield (Bauer et al., 1998; Duncan et al., 2015).

3.5.5. Size and virulence of TniSNPV transmission particles

The number of transmission stages released into the environment is crucial for future infection; however, their quality is also important. Indeed, the size of the transmission stage, which might affect infectivity, could be altered in mixed pathogen infections (Westby et al., 2019). This is particularly feasible in nucleopolyhedroviruses (NPVs) because each OB transmission stage contains many virus particles, each of which is capable of initiating infection. Thus, the virus particles can be repackaged in larger or smaller numbers (Kolodny-Hirsch and Van Beek, 1997; Hamm and Styer, 1985), potentially changing the size and number of OBs available. Our results show that the TniSNPV OB increased considerably in size as a result of the experiment, compared to the OBs from the initial stock. The pattern from the passage experiment was less clear but did indicate that changes in OB size were mainly associated with the higher, potentially more competitive, virus dose. Indeed, high virus dose infections produced larger OBs when larvae were fed high protein diets, as well as when larvae were challenged with TniSNPV alone. Although more data are needed to speculate on the impact of OB size

and the consequences on pathogen transmission, our results illustrate that pathogen fitness can not only be altered through the total number of transmission stages produced but also through other physical characteristics and traits.

The size of the viral OBs could affect several crucial features of the infection process, including virulence, and in the case of baculoviruses, their persistence in the environment outside the host. Here we looked at whether diet and co-infection altered virulence and whether this could be linked to OB size. The larger OBs produced at the end of a single passage in 4th instar *T. ni* larvae showed an increase in virulence (lower LD_{50}) compared to our initial suspension (Table 3.6). This is supported by serial passage studies that found that baculovirus virulence increased with increasing OB size, which was associated with an increase in the number of virions within each OB (Tompkins et al., 1981; Hamm and Styer, 1985). However, OB size was not necessarily related to increased virulence in our bioassay. In agreement with our OB size data, we found no difference in virus mortality from any low dose treatments. Whereas, at the high virus dose, larval mortality was affected by both co-infection and diet quality. Indeed, virus virulence (host mortality) decreased with increasing protein in the diet after single virus infection at high dose, despite the larger size of OBs found after infection of TniSNPV at high dose on high protein diet. This suggests that in addition to decreasing the total number of OBs produced on high protein diets, the host response to pathogen infection also negatively affected the viability or infectivity of the OBs. However, OBs produced in co-infected larvae, were less virulent when larvae were fed a balanced diet. While we expected that mixed infections would lead to an increase in pathogen virulence, particularly in the context of resource competition (Alizon et al., 2013; Choisy and de Roode, 2010), this was only found after infection of TniSNPV at high dose on high protein diet. Experimental studies on virulence evolution in the context of within-host competition have focused on the interactions between different strains of the same parasite species (Read and Taylor, 2001; Hughes et al., 2004; Gower and Webster, 2005), rather than different species. While strains are more likely to interact within the host in similar ways and require similar resources, the difference in life cycle, resources needed and host response between two different pathogen groups makes it more complex and harder to interpret. Similarly, theoretical models on pathogen virulence evolution after within-host competition (Clay and Rudolf, 2019; Day, 2002; Choisy and de Roode, 2010) usually look at long-term pathogen competition and maintenance of virulence. Here, we demonstrate the potential complexity

of within-host pathogen competition and its impact on pathogen fitness and showed that multiple traits can also be impacted by host nutrition.

In summary, we show that the outcome of co-infections can be strongly impacted by environmental factors. However, this is dependent on the effective dose of each pathogen. Nutritional variation has the potential to affect the resulting pathogen-specific mortality, the duration of infection and pathogen yield. The results indicate that within-host pathogen competition combined with host nutrition can have major impacts on pathogen fitness which are likely to affect host-pathogen dynamics and evolution at the population level. Further studies are needed to investigate how immune responses change in different combinations of diet and pathogens, and more importantly, demonstrate that these actually impact disease resistance. Given the variation in the host-parasite systems studied, it is challenging to draw broad general conclusions about the impact of dietary variation on co-infections at this stage, but it is clear that the influence of nutrient quality can potentially have complex and far-reaching outcomes.

Chapter contributions

Pauline Deschodt and Dr. Jenny Cory conceived the idea and designed the experiments; Pauline, Alana Breitkreutz and Olivia Walker collected the data for the diet quality bioassay; Pauline and Jess Hercus collected the data for the virulence evolution bioassay. The viral OB imaging were realized at the 4D LABS by Dr. Xin Zhang. Dr. Jenny Cory provided funding and materials, Pauline and Dr. Jenny Cory analysed the data; Pauline drafted the chapter; Pauline and Dr. Jenny Cory edited the chapter.

3.6. References

Abdullah, A. S., Moffat, C. S., Lopez-Ruiz, F. J., Gibberd, M. R., Hamblin, J., and Zerihun, A. (2017). Host-multi-pathogen warfare: pathogen interactions in co-infected plants. *Front. Plant Sci.* 8, 1806. doi:10.3389/fpls.2017.01806.

Alizon, S., Hurford, A., Mideo, N., and Van Baalen, M. (2009). Virulence evolution and the trade-off hypothesis: history, current state of affairs and the future. *J. Evol. Biol.* 22, 245–259. doi:10.1111/j.1420-9101.2008.01658.x.

Alizon, S., de Roode, J. C., and Michalakis, Y. (2013). Multiple infections and the evolution of virulence. *Ecol. Lett.* 16, 556–567. doi:10.1111/ele.12076.

Awmack, C. S., and Leather, S. R. (2002). Host plant quality and fecundity in herbivorous insects. *Annu. Rev. Entomol.* 47, 817–844. doi:10.1146/annurev.ento.47.091201.145300.

Barnes, G. L., Boethel, D. J., Eikenbary, R. D., Criswell, J. T., and Gentry, C. R. (1975). Growth and sporulation of *Metarrhizium anisopliae* and *Beauveria bassiana* on media containing various peptone sources. *Journal of invertebrate pathology* 25, 301–305. doi:10.1016/0022-2011(75)90086-5.

Bauer, L. S., Miller, D. L., Maddox, J. V., and McManus, M. L. (1998). Interactions between a Nosema sp. (Microspora: nosematidae) and nuclear polyhedrosis virus infecting the gypsy moth, Lymantria dispar (Lepidoptera: Lymantriidae). *J. Invertebr. Pathol.* 72, 147–153. doi:10.1006/jipa.1998.4773.

Behle, R. W., Wu, S., Toews, M. D., Duffield, K. R., and Shapiro-Ilan, D. I. (2022). Comparing production and efficacy of *Cordyceps javanica* with *Cordyceps fumosorosea*. *J. Econ. Entomol.* XX, 1–7. doi:10.1093/jee/toac002.

Ben-Ami, F., Rigaud, T., and Ebert, D. (2011). The expression of virulence during double infections by different parasites with conflicting host exploitation and transmission strategies. *J. Evol. Biol.* 24, 1307–1316. doi:10.1111/j.1420-9101.2011.02264.x.

Budischak, S. A., and Cressler, C. E. (2018). Fueling defense: effects of resources on the ecology and evolution of tolerance to parasite infection. *Front. Immunol.* 9, 2453. doi:10.3389/fimmu.2018.02453.

Budischak, S. A., Sakamoto, K., Megow, L. C., Cummings, K. R., Urban, J. F., and Ezenwa, V. O. (2015). Resource limitation alters the consequences of co-infection for both hosts and parasites. *Int. J. Parasitol.* 45, 455–463. doi:10.1016/j.ijpara.2015.02.005.

Chen, E., Kolosov, D., O'Donnell, M. J., Erlandson, M. A., McNeil, J. N., and Donly, C. (2018). The effect of diet on midgut and resulting changes in infectiousness of AcMNPV baculovirus in the cabbage looper, *Trichoplusia ni*. *Front. Physiol.* 9, 1348. doi:10.3389/fphys.2018.01348.

Choisy, M., and de Roode, J. C. (2010). Mixed infections and the evolution of virulence: effects of resource competition, parasite plasticity, and impaired host immunity. *Am. Nat.* 175, E105-18. doi:10.1086/651587.

Chouvenc, T., Efstathion, C. A., Elliott, M. L., and Su, N.-Y. (2012). Resource competition between two fungal parasites in subterranean termites. *Naturwissenschaften* 99, 949–958. doi:10.1007/s00114-012-0977-2.

Clay, P. A., and Rudolf, V. H. W. (2019). How parasite interaction strategies alter virulence evolution in multi-parasite communities. *Evolution* 73, 2189–2203. doi:10.1111/evo.13843.

Cory, J. S., and Deschodt, P. S. (2017). “The Biotic Environment,” in *Ecology of invertebrate diseases*, eds. A. E. Hajek and D. I. Shapiro-Ilan (Chichester, UK: John Wiley & Sons, Ltd), 187–212. doi:10.1002/9781119256106.ch6.

Cory, J. S., and Ericsson, J. D. (2010). Fungal entomopathogens in a tritrophic context. *Biocontrol* 55, 75–88. doi:10.1007/s10526-009-9247-4.

Cotter, S. C., and Al Shareefi, E. (2022). Nutritional ecology, infection and immune defence - exploring the mechanisms. *Curr. Opin. Insect Sci.* 49, xx-yy. doi:10.1016/j.cois.2021.12.002.

Cotter, S. C., Simpson, S. J., Raubenheimer, D., and Wilson, K. (2011). Macronutrient balance mediates trade-offs between immune function and life history traits. *Funct. Ecol.* 25, 186–198. doi:10.1111/j.1365-2435.2010.01766.x.

Cressler, C. E., McLeod, D. V., Rozins, C., Van Den Hoogen, J., and Day, T. (2016). The adaptive evolution of virulence: a review of theoretical predictions and empirical tests. *Parasitology* 143, 915–930. doi:10.1017/S003118201500092X.

Cressler, C. E., Nelson, W. A., Day, T., and McCauley, E. (2014). Disentangling the interaction among host resources, the immune system and pathogens. *Ecol. Lett.* 17, 284–293. doi:10.1111/ele.12229.

Day, T. (2002). On the evolution of virulence and the relationship between various measures of mortality. *Proc. Biol. Sci.* 269, 1317–1323. doi:10.1098/rspb.2002.2021.

Dinh, H., Mendez, V., Tabrizi, S. T., and Ponton, F. (2019). Macronutrients and infection in fruit flies. *Insect Biochem. Mol. Biol.* 110, 98–104. doi:10.1016/j.ibmb.2019.05.002.

Duncan, A. B., Agnew, P., Noel, V., and Michalakis, Y. (2015). The consequences of co-infections for parasite transmission in the mosquito *Aedes aegypti*. *J. Anim. Ecol.* 84, 498–508. doi:10.1111/1365-2656.12302.

Ebert, D., Zschokke-Rohringer, C. D., and Carius, H. J. (2000). Dose effects and density-dependent regulation of two microparasites of *Daphnia magna*. *Oecologia* 122, 200–209. doi:10.1007/PL00008847.

Fellous, S., and Koella, J. C. (2010). Cost of co-infection controlled by infectious dose combinations and food availability. *Oecologia* 162, 935–940. doi:10.1007/s00442-009-1535-2.

Fellous, S., and Koella, J. C. (2009). Infectious dose affects the outcome of the within-host competition between parasites. *Am. Nat.* 173, E177-84. doi:10.1086/598490.

Gómez-Moracho, T., Durand, T., Pasquaretta, C., Heeb, P., and Lihoreau, M. (2021). Artificial diets modulate infection rates by *Nosema ceranae* in bumblebees. *Microorganisms* 9. doi:10.3390/microorganisms9010158.

Gorsich, E. E., Ezenwa, V. O., and Jolles, A. E. (2014). Nematode-coccidia parasite co-infections in African buffalo: Epidemiology and associations with host condition and pregnancy. *Int. J. Parasitol. Parasites Wildl.* 3, 124–134. doi:10.1016/j.ijppaw.2014.05.003.

Gower, C. M., and Webster, J. P. (2005). Intraspecific competition and the evolution of virulence in a parasitic trematode. *Evolution* 59, 544–553. doi:10.1111/j.0014-3820.2005.tb01014.x.

Graham, R. I., Deacutis, J. M., Pulpitel, T., Ponton, F., Simpson, S. J., and Wilson, K. (2014). Locusts increase carbohydrate consumption to protect against a fungal biopesticide. *J. Insect Physiol.* 69, 27–34. doi:10.1016/j.jinsphys.2014.05.015.

Greenberg, S. M., Sappington, T. W., Legaspi, B. C., Liu, T. X., and Sétamou, M. (2001). Feeding and life history of *Spodoptera exigua* (Lepidoptera: Noctuidae) on different host plants. *AN* 94, 566–575. doi:10.1603/0013-8746(2001)094[0566:FALHOS]2.0.CO;2.

Grindstaff, J. L., Demas, G. E., and Ketterson, E. D. (2005). Diet quality affects egg size and number but does not reduce maternal antibody transmission in Japanese quail Coturnix japonica. *J. Anim. Ecology* 74, 1051–1058. doi:10.1111/j.1365-2656.2005.01002.x.

Hajek, A. E., and Tobin, P. C. (2011). Introduced pathogens follow the invasion front of a spreading alien host. *J. Anim. Ecol.* 80, 1217–1226. doi:10.1111/j.1365-2656.2011.01870.x.

Hall, S. R., Knight, C. J., Becker, C. R., Duffy, M. A., Tessier, A. J., and Cáceres, C. E. (2009). Quality matters: resource quality for hosts and the timing of epidemics. *Ecol. Lett.* 12, 118–128. doi:10.1111/j.1461-0248.2008.01264.x.

Hamm, J. J., and Styer, E. L. (1985). Comparative pathology of isolates of *Spodoptera frugiperda n*uclear polyhedrosis virus in *S. frugiperda* and *S. exigua*. *Journal of General Virology* 66, 1249–1261. doi:10.1099/0022-1317-66-6-1249.

Han, G., Li, C., Liu, Q., and Xu, J. (2015). Synergistic Effect of Combining Plutella xylostella Granulovirus and Bacillus thuringiensis at Sublethal Dosages on Controlling of Diamondback Moth (Lepidoptera: Plutellidae). *J. Econ. Entomol.* 108, 2184–2191. doi:10.1093/jee/tov182.

Hesketh, H., and Hails, R. S. (2015). *Bacillus thuringiensis* impacts on primary and secondary baculovirus transmission dynamics in Lepidoptera. *J. Invertebr. Pathol.* 132, 171–181. doi:10.1016/j.jip.2015.09.008.

Huber, D., Römheld, V., and Weinmann, M. (2012). “Relationship between Nutrition, Plant Diseases and Pests,” in *Marschner’s mineral nutrition of higher plants* (Elsevier), 283–298. doi:10.1016/B978-0-12-384905-2.00010-8.

Hughes, W. O. H., Petersen, K. S., Ugelvig, L. V., Pedersen, D., Thomsen, L., Poulsen, M., and Boomsma, J. J. (2004). Density-dependence and within-host competition in a semelparous parasite of leaf-cutting ants. *BMC Evol. Biol.* 4, 45. doi:10.1186/1471-2148-4-45.

Janmaat, A. F., and Myers, J. H. (2005). The cost of resistance to *Bacillus thuringiensis* varies with the host plant of *Trichoplusia ni*. *Proc. Biol. Sci.* 272, 1031–1038. doi:10.1098/rspb.2004.3040.

Johnson, P. T. J., Chase, J. M., Dosch, K. L., Hartson, R. B., Gross, J. A., Larson, D. J., Sutherland, D. R., and Carpenter, S. R. (2007). Aquatic eutrophication promotes pathogenic infection in amphibians. *Proc Natl Acad Sci USA* 104, 15781–15786. doi:10.1073/pnas.0707763104.

Kay, A. D., Bruning, A. J., van Alst, A., Abrahamson, T. T., Hughes, W. O. H., and Kaspari, M. (2014). A carbohydrate-rich diet increases social immunity in ants. *Proc. Biol. Sci.* 281, 20132374. doi:10.1098/rspb.2013.2374.

Kolodny-Hirsch, D. M., and Van Beek, N. A. M. (1997). Selection of a morphological variant of *Autographa californica* nuclear polyhedrosis virus with increased virulence following serial passage in *Plutella xylostella*. *Journal of invertebrate pathology* 69, 205–211. doi:10.1006/jipa.1997.4659.

Lacroix, C., Seabloom, E. W., and Borer, E. T. (2014). Environmental nutrient supply alters prevalence and weakens competitive interactions among coinfecting viruses. *New Phytol*, 424–433.

Lange, B., Reuter, M., Ebert, D., Muylaert, K., and Decaestecker, E. (2014). Diet quality determines interspecific parasite interactions in host populations. *Ecol. Evol.* 4, 3093–3102. doi:10.1002/ece3.1167.

Lee, K. P., Cory, J. S., Wilson, K., Raubenheimer, D., and Simpson, S. J. (2006). Flexible diet choice offsets protein costs of pathogen resistance in a caterpillar. *Proc. Biol. Sci.* 273, 823–829. doi:10.1098/rspb.2005.3385.

Lee, K. P., Simpson, S. J., and Wilson, K. (2008). Dietary protein-quality influences melanization and immune function in an insect. *Funct. Ecol.* 22, 1052–1061. doi:10.1111/j.1365-2435.2008.01459.x.

Lenth, R. V. (2020). Emmeans: estimated marginal means, aka least-squares means. R package version 1.5.3. https://CRAN.R-project.org/package=emmeans

Lohr, J. N., Yin, M., and Wolinska, J. (2010). Prior residency does not always pay off-co-infections in *Daphnia*. *Parasitology* 137, 1493–1500. doi:10.1017/S0031182010000296.

Luz, C., and Fargues, J. (1998). Factors affecting conidial production of *Beauveria bassiana* from fungus-killed cadavers of *Rhodnius prolixus*. *J. Invertebr. Pathol.* 72, 97–103. doi:10.1006/jipa.1998.4774.

Malakar, R., Elkinton, J. S., Hajek, A. E., and Burand, J. P. (1999). Within-host interactions of *Lymantria dispar* (Lepidoptera: Lymantriidae) nucleopolyhedrosis virus and *Entomophaga maimaiga* (Zygomycetes: entomophthorales). *J. Invertebr. Pathol.* 73, 91–100. doi:10.1006/jipa.1998.4806.

Mascarin, G. M., and Jaronski, S. T. (2016). The production and uses of *Beauveria bassiana* as a microbial insecticide. *World J. Microbiol. Biotechnol.* 32, 177. doi:10.1007/s11274-016-2131-3.

McVean, R. I., Sait, S. M., Thompson, D. J., and Begon, M. (2002). Effects of resource quality on the population dynamics of the Indian meal moth *Plodia interpunctella* and its granulovirus. *Oecologia* 131, 71–78. doi:10.1007/s00442-001-0862-8.

Mideo, N. (2009). Parasite adaptations to within-host competition. *Trends Parasitol.* 25, 261–268. doi:10.1016/j.pt.2009.03.001.

O'Reilly, D. R., Hails, R. S., and Kelly, T. J. (1998). The impact of host developmental status on baculovirus replication. *J. Invertebr. Pathol.* 72, 269–275. doi:10.1006/jipa.1998.4785.

Ojeda-Avila, T., Arthur Woods, H., and Raguso, R. A. (2003). Effects of dietary variation on growth, composition, and maturation of *Manduca sexta* (Sphingidae: Lepidoptera). *J. Insect Physiol.* 49, 293–306. doi:10.1016/S0022-1910(03)00003-9.

Palermo, T., Rawat, R., Weiser, S. D., and Kadiyala, S. (2013). Food access and diet quality are associated with quality of life outcomes among HIV-infected individuals in Uganda. *PLoS ONE* 8, e62353. doi:10.1371/journal.pone.0062353.

Pauli, G., Moura Mascarin, G., Eilenberg, J., and Delalibera Júnior, I. (2018). Within-host competition between two entomopathogenic fungi and a granulovirus in *Diatraea saccharalis* (Lepidoptera: Crambidae). *Insects* 9. doi:10.3390/insects9020064.

Phuoc, L. H., Corteel, M., Thanh, N. C., Nauwynck, H., Pensaert, M., Alday-Sanz, V., den Broeck, W. V., Sorgeloos, P., and Bossier, P. (2009). Effect of dose and challenge routes of *Vibrio* spp. on co-infection with white spot syndrome virus in *Penaeus vannamei*. *Aquaculture* 290, 61–68. doi:10.1016/j.aquaculture.2009.02.004.

Pike, V. L., Lythgoe, K. A., and King, K. C. (2019). On the diverse and opposing effects of nutrition on pathogen virulence. *Proc. Biol. Sci.* 286, 20191220. doi:10.1098/rspb.2019.1220.

Plymale, R., Grove, M. J., Cox-Foster, D., Ostiguy, N., and Hoover, K. (2008). Plant-mediated alteration of the peritrophic matrix and baculovirus infection in lepidopteran larvae. *J. Insect Physiol.* 54, 737–749. doi:10.1016/j.jinsphys.2008.02.005.

Ponton, F., Lalubin, F., Fromont, C., Wilson, K., Behm, C., and Simpson, S. J. (2011). Hosts use altered macronutrient intake to circumvent parasite-induced reduction in fecundity. *Int. J. Parasitol.* 41, 43–50. doi:10.1016/j.ijpara.2010.06.007.

Povey, S., Cotter, S. C., Simpson, S. J., Lee, K. P., and Wilson, K. (2009). Can the protein costs of bacterial resistance be offset by altered feeding behaviour? *J. Anim. Ecol.* 78, 437–446. doi:10.1111/j.1365-2656.2008.01499.x.

Povey, S., Cotter, S. C., Simpson, S. J., and Wilson, K. (2014). Dynamics of macronutrient self-medication and illness-induced anorexia in virally infected insects. *J. Anim. Ecol.* 83, 245–255. doi:10.1111/1365-2656.12127.

Ramsay, C., and Rohr, J. R. (2021). The application of community ecology theory to co-infections in wildlife hosts. *Ecology* 102, e03253. doi:10.1002/ecy.3253.

Raubenheimer, D., and Simpson, S. J. (1997). Integrative models of nutrient balancing: application to insects and vertebrates. *Nutr. Res. Rev.* 10, 151–179. doi:10.1079/NRR19970009.

Read, A. F., and Taylor, L. H. (2001). The ecology of genetically diverse infections. *Science* 292, 1099–1102. doi:10.1126/science.1059410.

Reyserhove, L., Muylaert, K., Vanoverberghe, I., and Decaestecker, E. (2017). Synergistic effects of dual parasitism in *Daphnia magna* under nutrient limitation. *Belg. J. Zool.* 147, 61–76. doi:10.26496/bjz.2017.5.

Richter, A. R., and Fuxa, J. R. (1984). Pathogen–pathogen and pathogen–insecticide interactions in velvetbean caterpillar (Lepidoptera: Noctuidae). *J. Econ. Entomol.* 77, 1559–1564. doi:10.1093/jee/77.6.1559.

Roberts, K. E., and Longdon, B. (2021). Viral susceptibility across host species is largely independent of dietary protein to carbohydrate ratios. *J. Evol. Biol.* 34, 746–756. doi:10.1111/jeb.13773.

Rovenolt, F. H., and Tate, A. T. (2022). The impact of coinfection dynamics on host competition and coexistence. *Am. Nat.* 199, 91–107. doi:10.1086/717180.

Roy, H. E., Steinkraus, D. C., Eilenberg, J., Hajek, A. E., and Pell, J. K. (2006). Bizarre interactions and endgames: entomopathogenic fungi and their arthropod hosts. *Annu. Rev. Entomol.* 51, 331–357. doi:10.1146/annurev.ento.51.110104.150941.

Safavi, S. A., Shah, F. A., Pakdel, A. K., Reza Rasoulian, G., Bandani, A. R., and Butt, T. M. (2007). Effect of nutrition on growth and virulence of the entomopathogenic fungus *Beauveria bassiana*. *FEMS Microbiol. Lett.* 270, 116–123. doi:10.1111/j.1574-6968.2007.00666.x.

Sandland, G. J., and Minchella, D. J. (2003). Costs of immune defense: an enigma wrapped in an environmental cloak? *Trends Parasitol.* 19, 571–574. doi:10.1016/j.pt.2003.10.006.

Schmid-Hempel, P. (2005). Evolutionary ecology of insect immune defenses. *Annu. Rev. Entomol.* 50, 529–551. doi:10.1146/annurev.ento.50.071803.130420.

Shah, F. A., Wang, C. S., and Butt, T. M. (2005). Nutrition influences growth and virulence of the insect-pathogenic fungus *Metarhizium anisopliae*. *FEMS Microbiol. Lett.* 251, 259–266. doi:10.1016/j.femsle.2005.08.010.

Shameena, S. S., Kumar, K., Kumar, S., Kumari, P., Krishnan, R., Karmakar, S., Sanath Kumar, H., Rajendran, K. V., and Raman, R. P. (2021). Dose-dependent co-infection of *Argulus* sp. and *Aeromonas hydrophila* in goldfish (*Carassius auratus*) modulates innate immune response and antioxidative stress enzymes. *Fish Shellfish Immunol.* 114, 199–206. doi:10.1016/j.fsi.2021.04.026.

Shapiro, M. (2000). Effect of two granulosis viruses on the activity of the gypsy moth (Lepidoptera: Lymantriidae) nuclear polyhedrosis virus. *J. Econ. Entomol.* 93, 1633–1637. doi:10.1603/0022-0493-93.6.1633.

Sheldon, B. C., and Verhulst, S. (1996). Ecological immunology: costly parasite defences and trade-offs in evolutionary ecology. *Trends Ecol. Evol.* 11, 317–321. doi:10.1016/0169-5347(96)10039-2.

Shikano, I., and Cory, J. S. (2016). Altered nutrient intake by baculovirus-challenged insects: Self-medication or compensatory feeding? *J. Invertebr. Pathol.* 139, 25–33. doi:10.1016/j.jip.2016.07.005.

Shikano, I., and Cory, J. S. (2014). Genetic resistance to *Bacillus thuringiensis* alters feeding behaviour in the cabbage looper, *Trichoplusia ni*. *PLoS ONE* 9, e85709. doi:10.1371/journal.pone.0085709.

Shikano, I., and Cory, J. S. (2015). Impact of environmental variation on host performance differs with pathogen identity: Implications for host-pathogen interactions in a changing climate. *Sci. Rep.* 5, 1–10. doi:10.1038/srep15351.

Shikano, I. (2017). Evolutionary ecology of multitrophic interactions between plants, insect herbivores and entomopathogens. *J. Chem. Ecol.* 43, 586–598. doi:10.1007/s10886-017-0850-z.

Simpson, S. J., and Raubenheimer, D. (2011). The nature of nutrition: a unifying framework. *Aust. J. Zool.* 59, 350. doi:10.1071/ZO11068.

Smith, V. (2007). Host resource supplies influence the dynamics and outcome of infectious disease. *Integr. Comp. Biol.* 47, 310–316. doi:10.1093/icb/icm006.

Souza, M. L., Sanches, M. M., Souza, D. A. de, Faria, M., Espinel-Correal, C., Sihler, W., and Lopes, R. B. (2019). Within-host interactions of *Metarhizium rileyi* strains and nucleopolyhedroviruses in *Spodoptera frugiperda* and *Anticarsia gemmatalis* (Lepidoptera: Noctuidae). *J. Invertebr. Pathol.* 162, 10–18. doi:10.1016/j.jip.2019.01.006.

Srygley, R. B., and Jaronski, S. T. (2018). Protein deficiency lowers resistance of Mormon crickets to the pathogenic fungus *Beauveria bassiana*. *J. Insect Physiol.* 105, 40–45. doi:10.1016/j.jinsphys.2018.01.005.

Syller, J. (2012). Facilitative and antagonistic interactions between plant viruses in mixed infections. *Mol. Plant Pathol.* 13, 204–216. doi:10.1111/j.1364-3703.2011.00734.x.

Thomas, M. B., Watson, E. L., and Valverde-Garcia, P. (2003). Mixed infections and insect-pathogen interactions. *Ecol. Lett.* 6, 183–188. doi:10.1046/j.1461-0248.2003.00414.x.

Tompkins, G. J., Vaughn, J. L., Adams, J. R., and Reichelderfer, C. F. (1981). Effects of Propagating Autographa californica Nuclear Polyhedrosis Virus and Its Trichoplusia ni Variant in Different Hosts 12. *Environ. Entomol.* 10, 801–806. doi:10.1093/ee/10.5.801.

Tritschler, M., Vollmann, J. J., Yañez, O., Chejanovsky, N., Crailsheim, K., and Neumann, P. (2017). Protein nutrition governs within-host race of honey bee pathogens. *Sci. Rep.* 7, 1–11. doi:10.1038/s41598-017-15358-w.

Tseng, M., and Myers, J. H. (2014). The relationship between parasite fitness and host condition in an insect--virus system. *PLoS ONE* 9, e106401. doi:10.1371/journal.pone.0106401.

Vale, P. F., Choisy, M., and Little, T. J. (2013). Host nutrition alters the variance in parasite transmission potential. *Biol. Lett.* 9, 20121145. doi:10.1098/rsbl.2012.1145.

Valverde-Garcia, P., Santiago-Álvarez, C., Thomas, M. B., Maranhao, E. A. A., Garrido-Jurado, I., and Quesada-Moraga, E. (2019). Sublethal effects of mixed fungal infections on the Moroccan locust, *Dociostaurus maroccanus. J. Invertebr. Pathol.* 161, 61–69. doi:10.1016/j.jip.2018.12.009.

Venter, F., Matthews, K. R., and Silvester, E. (2022). Parasite co-infection: an ecological, molecular and experimental perspective. *Proc. Biol. Sci.* 289, 20212155. doi:10.1098/rspb.2021.2155.

Wakil, W., Yasin, M., and Shapiro-Ilan, D. (2017). Effects of single and combined applications of entomopathogenic fungi and nematodes against *Rhynchophorus ferrugineus* (Olivier). *Sci. Rep.* 7, 1–11. doi:10.1038/s41598-017-05615-3.

Westby, K. M., Sweetman, B. M., Adalsteinsson, S. A., Biro, E. G., and Medley, K. A. (2019). Host food quality and quantity differentially affect *Ascogregarina barretti* parasite burden, development and within-host competition in the mosquito *Aedes triseriatus*. *Parasitology* 146, 1665–1672. doi:10.1017/S0031182019000994.

Zhang, J., Friman, V.-P., Laakso, J., and Mappes, J. (2012). Interactive effects between diet and genotypes of host and pathogen define the severity of infection. *Ecol. Evol.* 2, 2347–2356. doi:10.1002/ece3.356.

Zilio, G., and Koella, J. C. (2020). Sequential co-infections drive parasite competition and the outcome of infection. *J. Anim. Ecol.* 89, 2367–2377. doi:10.1111/1365-2656.13302.

Chapter 4.

Plant identity alters the outcome of mixed infections in an insect herbivore

4.1. Abstract

Host plant quality is a key driver of the success of insect herbivores and for generalists, host plant selection can have a significant impact on growth and survival. The influence of host plant identity extends to other trophic levels, including the interaction of the insect with pathogens. The impact of different plant species has been shown to alter host mortality after exposure to a range of insect pathogens. However, insects can be infected by multiple pathogens simultaneously and little is known about how plant identity affects pathogen competition. We used the cabbage looper, *Trichoplusia ni*, two of its pathogens, a specialist baculovirus, TniSNPV, and the generalist entomopathogenic fungus, *Beauveria bassiana*, and two host plants, broccoli and cucumber to investigate the effect of host plant identity after pathogen challenge. In addition, we explored the effect of timing of infection by challenging *T. ni* larvae with both pathogens synchronously or inoculating with one pathogen 48 hours prior to the second infection. Interestingly, the outcome of mixed infection was considerably variable depending on the timing of pathogen introduction and which plant the insects were fed on. While, overall survival rates were generally higher on cucumber, survival rates on broccoli were different for all three mixed pathogen treatments on broccoli. Indeed, delaying the exposure to the fungus in mixed infections on broccoli decreased survival by the largest degree and showed a synergistic interaction whereas the opposite asynchronous treatment showed an antagonistic interaction between the two pathogens. In terms of pathogen-specific mortality, the fungus was more affected by host plant than the virus and differences tended to be exacerbated on the cucumber. On broccoli, virus clearly had a negative effect on fungus mortality if the insect was exposed to virus second, but the impact of the fungus on the virus was less clear-cut. This is the first study to demonstrate the impact of plant identity in moderating priority effects and determining the outcome of mixed pathogen infections in insects.

Keywords: *Beauveria bassiana,* co-infection, host plant identity, mixed pathogen infection, nucleopolyhedrovirus, priority effects, tritrophic interactions, *Trichoplusia ni*.

4.2. Introduction

Plants are the main drivers of insect herbivore performance (Simpson et al., 2015; Wetzel et al., 2016). Generalist herbivores can feed on a variety of host plants that differ in their nutrient composition, as well as their various defence mechanisms against herbivory. The effect of the different nutritional components of plant tissues on herbivore growth (e.g. Jansen and Stamp, 1997; Tikkanen et al., 2000), fecundity (e.g. Awmack and Leather, 2002; Saeed et al., 2010) and survival (e.g. Saeed et al., 2010; Wetzel et al., 2016) have been thoroughly investigated. In addition, plants have the ability to influence tritrophic interactions, including those between insect herbivores and their pathogens (Cory and Hoover, 2006). Diversity in plant structural architecture, secondary metabolites and other defensive compounds can directly or indirectly alter pathogen viability or infectivity (Duffey et al., 1995; Hoover et al., 1998b). This can potentially occur through modification of resource-costly immune responses (Lochmiller and Deerenberg, 2000; Schmid-Hempel, 2005), in addition to altering herbivore development and behaviour (Awmack and Leather, 2002; Saeed et al., 2010), which can increase exposure to pathogens (Cory and Hoover, 2006; Cory and Ericsson, 2010). Thus a herbivore's diet can play a critical role in its ability to survive pathogen challenge (Smith et al., 2005; Simpson et al., 2015; Pike et al., 2019). However, different insect pathogen groups can differ in their response to their host's diet (Cory and Hoover, 2006; Shikano, 2017) and yet, most studies have focussed on single pathogen-single plant interactions. Insects and other hosts are highly likely to be exposed to multiple pathogens or parasites in the wild (Stirnadel and Ebert, 1997; Johnson and Buller, 2011; Bartolomé et al., 2020), but the impact of different host plants on mixed pathogen infections and how this might affect the dynamics of both the hosts and the pathogens have received little attention.

Plant defence mechanisms against insect herbivores are diverse and complex (Chen, 2008; Aljbory and Chen, 2018) and many of these could potentially affect pathogen infection. For example, when lepidopteran larvae were fed on cotton or tomato foliage, the synthesis of phenolic compounds was activated, resulting in decreased susceptibility to both *Heliothis virescens* and *Helicoverpa zea* larvae to baculovirus infections (lower mortality) compared to larvae fed non-damaged leaves (Hoover et al., 1998a). Interactions

between plants and insect pathogens are also likely to depend on the type of pathogen and its route of infection. Orally ingested pathogens, such as bacteria (e.g. Janmaat et al., 2014; Jafary et al., 2016) and baculoviruses (e.g. Raymond et al., 2002; Ji et al., 2016) are likely to be consumed while the host is feeding on the plant, thus pathogen transmission stages will be in direct contact with plant material in the host midgut (Shikano, 2017). Whereas entomopathogenic fungi mainly enter their hosts through contact with the cuticle and can be affected by plants through variations in germination success (Poprawski et al., 2000) or thickening of the insect cuticle (Lee et al., 2008; Shikano, 2017). For example, α-tomatine, a glycoalkaloid defensive compound produced by tomato plants, negatively affected germination of the fungus *Beauveria bassiana* and completely inhibited the germination of another fungus *Cordyceps fumosorosea* (Wize) when added to agar media (Poprawski et al., 2000). Plants can also affect immune functions in lepidopteran larvae, through variation in encapsulation ability (Ojala et al., 2005), haemocyte numbers (Shikano et al., 2010), and phenoloxidase activity (Shikano et al., 2010; Smilanich et al., 2018), which are thought to influence the resistance of insects to pathogen challenge. Furthermore, in response to different types of diet or to counteract the ingestion of toxic plant defence compounds, herbivores are able to modify certain tissues, such as by thickening the peritrophic matrix (Chen et al., 2018), which lines the guts of most insects and has been shown to influence susceptibility to viral infection (Plymale et al., 2008; Shikano, 2017; Chen et al., 2018).

When different pathogen species (or strains) compete within the same host, they can interact directly and/or indirectly (Mideo, 2009), either for resources (exploitation competition; e.g. Perlman and Jaenike, 2001), by inhibiting each other (interference competition; e.g. Massey et al., 2004; Garbutt et al., 2011) or via the host immune system (inapparent competition; e.g. Raberg et al., 2006; Ulrich and Schmid-Hempel, 2012). The consequences of within-host pathogen competition are predicted to vary according to the type of interaction, the pathogen group and similarities between the competitors (Choisy and de Roode, 2010; Alizon et al., 2013; Clay and Rudolf, 2019). Pathogens infecting by the same route, sharing similar resource needs and activating a shared immune response are likely to have a different interaction compared to competing pathogens with separate resource niches or which trigger different immune responses (Ulrich and Schmid-Hempel, 2012). While pathogens sharing similar resources are likely to increase the host exploitation rate, thereby increasing damage to the host (virulence) (Brown et al., 2002),

inhibition of one pathogen by another might exclude one pathogen and result in a comparable outcome to when the dominant competitor infects alone (Massey et al., 2004). Within-host competition can also affect pathogen fitness by decreasing (or increasing) the duration of infection (Hodgson et al., 2004), and altering the production of transmission stages (Ben-Ami et al., 2011), which can have significant consequences for pathogen transmission and persistence in the host population (Mideo et al., 2008; Hesketh and Hails, 2015).

Theoretical models that have included priority effects (Cressler et al., 2014; Clay et al., 2019) and experimental studies on sequential co-infections have shown that the timing and the order of infection play an important role in the outcome, both in terms of host mortality and the successful production of pathogen transmission stages. It might be expected that the first pathogen to invade has an advantage, but results are highly variable and system dependent (Lohr et al., 2010; Doublet et al., 2015; Manzi et al., 2021). For example, Doublet *et al.* (2015) showed that the Deformed wing virus (DWV) was negatively affected when the microsporidian *Nosema ceranae* infected honey bees (*Apis mellifera*) first, whereas *N. ceranae* was not impacted by prior infection of the honey bees by DWV. Similarly, Lohr *et al.* (2010) found that the protozoan *Caullerya mesnili* benefited from prior infection of the waterflea *Daphnia galeata* by the fungus *Metschnikowia* sp. but the fungus was negatively impacted by prior infection of *D. galeata* with *C. mesnili*. Combining the effects of nutrition, co-infection and timing is rare and studies have primarily focused on a limited number of host-pathogen systems (Tritschler et al., 2017; Zilio and Koella, 2020). To our knowledge, there are no studies that have investigated the impact of host plant identity on co-infections in insect herbivores.

In this chapter, we investigate the impact of host plant identity on co-infection by two pathogens of the cabbage looper, *Trichoplusia ni*. We challenged larvae with the *T. ni* specific nucleopolyhedrovirus (TniSNPV) (a type of baculovirus) and the generalist entomopathogenic fungus, *Beauveria bassiana*, either alone or in combination, and fed challenged larvae with either broccoli, *Brassica oleracea* var. *italica*, or cucumber, *Cucumis sativus*. In addition, we examined the impact of pathogen priority on co-infection outcome, by challenging *T. ni* larvae with both pathogens either synchronously or by delaying the second pathogen infection by 48 hours. Both pathogens have very distinct life cycles (see Chapter 1, Fig. 1.2; Fig. 1.3); TniSNPV needs to be ingested in order to initiate infection and is likely to interact directly with host plant within the host midgut during

the initial infection period (Cory, 2010). Entomopathogenic fungi, such as *B. bassiana,* infect their host through the cuticle and thus plant identity is more likely to indirectly affect the fungus through changes in host development and condition (Ocampo-Hernández et al., 2019).

A previous study on *T. ni* larvae showed that broccoli was in general a more suitable host plant, in terms of developmental rate and survival, compared to cucumber (Shikano et al., 2010). In addition, *T. ni* larvae that were fed broccoli leaves prior to being challenged with TniSPNV had a greater number of haemocytes and higher chance of survival compared to those fed cucumber leaves (Shikano et al., 2010). Hence, we predicted that if we fed the *T. ni* larvae cucumber leaves after pathogen challenge, pathogen-induced mortality would also be greater compared to insects fed broccoli and this would be exacerbated when larvae were challenged with two pathogens. Based on earlier studies using artificial diet (Deschodt and Cory 2022, chapter 2), we also predicted that the two pathogens might respond differently to the two plant species, so that plant identity would alter the outcome of the mixed infection. Finally, the entomopathogenic fungus, *B. bassiana*, was the dominant pathogen when co-infected synchronously with the baculovirus on artificial diet (Deschodt and Cory 2022), thus, we expected that delaying fungal challenge would be advantageous to the virus, whereas infecting with the fungus first would essentially block TniSNPV infection entirely.

4.3. Materials and methods

4.3.1. Study system

Plants

Broccoli, *Brassica oleracea* (Centennial Broccoli, BR186), and cucumber, *Cucumis sativus* (Easy fortune organic, CU418), plants were grown in a greenhouse facility at SFU, Burnaby, British Columbia. Seeds (West Coast Seeds®, Ladner, BC) were planted in trays in Pro-Mix® seed starting mix and transplanted after the plants had reached the 4 to 6 true leaves stage. Each plant was grown in an individual pot containing a mixture of 50% mushroom manure and 50% peat moss. Plants were watered every other day and used in the assay when they were eight to 10 weeks old.

Insects

Trichoplusia ni larvae are generalist herbivores. They are important pests of cruciferous plants, as well as a diversity of greenhouse crops such as bell pepper, cucumber, and lettuce. *T. ni* larvae used in the experiment were reared individually from the neonate stage. Eggs were obtained from a long-term colony from our rearing facility at SFU. After hatch, *T. ni* larvae were maintained at 25°C with a 16L:8D photoperiod on a wheat germ-based artificial diet (Shikano and Cory, 2014) until they reached fourth instar.

Pathogens

The original isolate of TniSNPV was collected from a *T. ni* larva in the Fraser Valley, British Columbia (Janmaat and Myers, 2003) and amplified in *T. ni* before the experiment. Infected *T. ni* larvae were macerated in water and the suspension was semi-purified through multiple rounds of centrifugation. The number of occlusion bodies (OBs) was estimated using an improved Neubauer haemocytometer (Hausser Scientific, depth 0.1 mm) at 400x magnification. Four independent dilutions were counted, and the average was taken as the final concentration. BotaniGard® ES (initial concentration of $2.11x10^{10}$ spores/ml) was used as our original *B. bassiana* suspension (GHA strain). Fungal spore viability was verified before the assay by plating 150 µl of a 10^6 spores/ml suspension onto a Potato-Dextrose Agar (PDA) medium and left to germinate in a growth chamber at 24°C. After 24 h, germination was then assessed under a light microscope at 400x magnification (Inglis et al., 2012). Germination was over 95% on all 5 plates used to determine viability. Both viral and fungal suspensions used in the experiment were obtained by serial dilution of the original suspension immediately before pathogen challenge.

4.3.2. Experimental design

Pathogen challenge

Newly moulted fourth instar larvae were randomly selected and challenged with either a single pathogen, TniSNPV or *B. bassiana*, or both pathogens synchronously or sequentially. For each infection treatment, larvae were transferred individually into 12-well plates. As viral infection is initiated when OBs are ingested, larvae were exposed to 500 TniSNPV OBs (expected LD50 for 4^{th} instar *T. ni* larvae) by placing a 1 µl droplet of virus on a 3 x 2 mm plug of the standard rearing diet. Any larvae that did not eat the diet plug within the first 24 hrs were discarded from the experiment. Larvae were challenged with

$2.0x10^4$ of *B. bassiana* spore suspension (expected LD50 for 4th instar *T. ni* larvae) by administering a 1µl droplet to their dorsal abdomen to simulate natural fungal infection. Larvae challenged with both pathogens synchronously were exposed to *B. bassiana* immediately after being placed in the wells containing the diet plug with the virus. Larvae challenged with both pathogens sequentially, were challenged with either *TniSNPV* or *B. bassiana* first and then with the other pathogen 48 hours later as described above. To allow comparison between the delayed infection treatments and single pathogen challenge, we set up two additional single treatments where *T. ni* larvae were challenged with TniSNPV and *B. bassiana* alone on the second day of infection (see Table 4.1). The viral suspension used on the first infection day was stored at -20°C and used again on the second day of infection as the virus is very temperature stable. A fresh dilution of the fungal suspension was made prior to each infection date as previous experience had shown a reduction of spore viability after a few days. Unchallenged control larvae were treated with 1 µl of distilled water placed on their dorsal abdomen and on a 3 x 2 mm diet plug.

Table 4.1. Overview of the pathogen challenge treatments (pathogen dose and infection timing)

Pathogen	Infection	Timing	First infection (day 1)	Second infection (day 3)
Control	Control	Control	None	None
Virus	Single	Early	500 OBs of TniSNPV	None
Fungus	Single	Early	20,000 *B. bassiana* spores	None
Mix Synch	Mixed	Synchronous	500 OBs of TniSNPV + 20,000 *B. bassiana* spores	None
Mix virus first	Mixed	Delayed	500 OBs of TniSNPV	20,000 *B. bassiana* spores
Mix fungus first	Mixed	Delayed	20,000 *B. bassiana* spores	500 OBs of TniSNPV
Virus	Single	Delayed	None	500 OBs of TniSNPV
Fungus	Single	Delayed	None	500 OBs of TniSNPV

Host plant provision

Larvae from each of the eight pathogen exposures (unchallenged control, four single pathogen treatments, one mixed synchronous challenge and two mixed delayed infections; Table 4.1) were transferred to 2 oz SOLO® cups 24 hrs after the first pathogen challenge and assigned to one of the two host plants. Larvae in the delayed single and mixed infection treatments were transferred to a cup containing one of the host plants 24

hrs after the first pathogen challenge. The next day, they were removed from their cups and challenged with the assigned pathogen as described above and returned to the same cup 24 hrs later. Due to a shortage of leaves, control and single pathogen treatments on cucumber included 32 larvae; all the remaining treatments had a total of 64 larvae.

Insect Monitoring

After pathogen challenge, and for the remainder of the experiment, individual larvae were maintained at 24°C with a 16L:8D photoperiod. Cups were cleaned to limit the proliferation of external pathogens (mold) and fresh cut leaves were added daily. Larvae were fed *ad libitum*. Each larva was monitored twice a day (7am and 7pm) until death or pupation. Wherever possible, we ascribed the cause of death for each cadaver using visual symptoms. Larvae that succumb to TniSNPV or *B. bassiana* look very distinct and can be easily identified. Baculovirus infections usually cause the larvae to become pale and swollen compared to healthy larvae. At the end of baculovirus infection, the integuments of the larvae are fragile and easily ruptured, resulting in the release of millions of OBs. Conversely, larvae that succumb to fungal infection usually turn purple and become rigid when they die. After death, *B. bassiana* produces conidia that form a typical cloud of white spores around the surface of the cadavers. Fungal spore production is highly dependent on the temperature and humidity conditions at larval death, thus larvae that appeared to have died of fungal infection were first surface sterilized in 1% sodium hypochlorite solution, by dipping the whole cadavers into the solution for 1 min then rinsed in distilled water, to prevent any contamination of external pathogens. They were then placed in a humidity chamber (1oz SOLO® cup with damp cotton wool) at 24°C and checked daily for any sign of sporulation. Any unclear deaths were initially disinfected and treated as fungal death; if no sporulation occurred after 72 hrs the cadavers were smeared and examined under oil immersion on a light microscope (x1,000) for OBs (Giemsa stain) or fungal hyphae. Survivors were weighed 3 days after they formed pupae.

4.3.3. Statistical analysis

All analyses were conducted in R-4.0.3. Survival models presented below were run using the *survreg* function from the *survival* package (Therneau 2020) and the *pairwise_survdiff* function from the *survminer* package (Kassambara et al., 2021) was used to calculate pairwise comparison between group levels. All initial models included

interactions between all explanatory variables and non-significant terms were removed sequentially until the final minimal model was found.

Host survival

Larval survival was analyzed using a parametric survival model, with an exponential distribution (Weibull). Death or survival (pupation) was recorded up to 15 days after pathogen challenge (enough time for all pathogen deaths to occur and all surviving larvae to fully pupate). We first were interested in testing whether plant identity and timing of infection affected the outcome of single pathogen infections differently for our two pathogen species. Hence, we started by building a survival model only including the single pathogens treatments (virus or fungus), the timing of infection (early or late 4th instar) and the host plant (broccoli or cucumber). Then, we analysed larval survival in mixed pathogen infections, and we separated the analysis into two parts. First, we tested whether host plants altered larval survival in co-infection compared to single treatments. This was done by including the mixed synchronous infection treatment and the corresponding single pathogen treatments in the model, along with host plant, excluding potential effect of timing of infection. Then, we tested whether the pathogen infection time had an effect on the outcome of mixed pathogen infection and whether this was influenced by either of the host plants. We thus included in the model both host plants along with the timing of co-infection (synchronous, virus first or fungus first). Kaplan-Meier plots were used to visualize the survival curves.

To examine the effects that each pathogen had on the other in the co-infections, we then analysed two pathogen-specific survival models. First, we focused on death by viral infection (larvae swollen and pale with soft integuments), excluding any larvae that died of fungal infection. We ran a similar survival model as presented above looking at the effect of the host plant (broccoli or cucumber) as well as the pathogen treatment (virus single (early and delay), mixed synchronous, mixed virus first and mixed fungus first). We then ran a second model only including larvae that died of fungal infection (hard integument, purple coloured and then sporulated cadavers). Virtually all cadavers in the co-infection treatments showed visually identifiable symptoms of only one of the two pathogens, which was recorded as principal cause of death.

Within-host pathogen competition

For the mixed infection treatments, the nature of the pathogen interactions was determined using the formula described in Koppenhöffer and Kaya (1997). The expected additive proportional mortality (M_{exp}) in the mixed infections was estimated using the formula: $M_{exp}=M_{virus}+M_{fungus}*(1-M_{virus})$, where M_{virus} and M_{fungus} represent the respective observed proportional mortality in the single virus and fungus treatments, respectively. Then the expected mortality (M_{exp}) and observed (M_{obs}) mixed mortality (virus plus fungus combinations) were compared using a χ^2 test, $\chi^2= (M_{obs}- M_{exp})^2/ M_{exp}$ and compared to the χ^2 table value for 1 df (χ^2tab=3.841). If the χ^2 value was higher than the table value, the interaction between TniSNPV and *B. bassiana* was considered to be non-additive (or non-neutral). The interaction between TniSNPV and *B. bassiana* was considered synergistic if the difference between $M_{obs}- M_{exp}>0$ and antagonistic if $M_{obs}- M_{exp}<0$.

Sublethal effects

Finally, to determine if co-infection and host plant identity had any effect on larvae that survived infection, we analyzed pupal weight using an Analysis of Variance (ANOVA). Pupal weight in all our models was reflected using the following formula: for a given pupal weight i, reflected pupal $weight_i$=max(pupal weight +1)- pupal $weight_i$. Then reflected pupal weights were log_{10}-transformed to fit the assumptions of normality. First, we analyzed whether single or co-challenge influenced pupal weight by including pathogen treatment as three levels (control, single or mixed) along with host plants in the model. We then focused on single pathogen challenges and determined if larval age at pathogen challenge had an impact on subsequent pupal weight by including timing of infection and host plant. Lastly, we looked at potential differences between pupal weight in the mixed infection treatments by including both timing of infection (synchronous, fungus first or virus first) and host plants in the initial model. Tukey HSD comparison were performed when significant differences among treatments were detected.

4.4. Results

4.4.1. Overall host survival

Do host plant identity and host age (timing) differentially affect TniSNPV and B. bassiana *single infections?*

Host plant affected larval mortality, but only when the larvae were challenged later and fed cucumber, which resulted in a reduced survival rate (Table 4.2a; Fig. 4.1A). There was no difference in survival rate between broccoli and cucumber when larvae were challenged as newly moulted 4th instar or between early and late 4th instar larvae fed broccoli. Timing/age did not influence host survival rate when treated with the virus but delaying *B. bassiana* infection by 48 hours resulted in almost half as many larvae succumbing to fungal infection (Table 4.2a; Fig. 4.1B). The survival rate was significantly higher when larvae were challenged with TniSNPV compared to *B. bassiana* regardless of the host plant provided after pathogen challenge, such that larvae challenged with *B. bassiana* were 10 times more likely to die than those challenged with the virus (Hazard Ratio (HR)=13.39; 95% CI: 6.65-26.99).

Table 4.2. Effect of host plant and age at inoculation (timing) on *T. ni* larvae survival rate after (a) single pathogen challenge; (b) Single or mixed synchronous challenge, and (c) mixed pathogen challenge. Significant p values are highlighted in bold, and terms not included in the final model are italicized

Overall survival	Df	χ^2	p value
(a) Single			
Timing (early/ delayed)	1	5.12	**0.02**
Pathogen (virus/ fungus)	1	74.92	**<0.0001**
Plant (broccoli/ cucumber)	1	10.65	**0.001**
Timing x pathogen	1	16.90	**<0.0001**
Timing x plant	1	4.65	**0.03**
Pathogen x plant	1	0.80	0.38
Timing x pathogen x plant	1	0.68	0.41
(b) Single vs mixed			
Pathogen (virus/ fungus/ Mixed synchronous)	2	22.92	**<0.0001**
Plant (broccoli/ cucumber)	1	4.87	**0.03**
Pathogen x plant	2	2.51	0.28
(c) Mixed synchronous vs delayed			
Pathogen (Mixed synchronous/ fungus first/ virus first)	2	84.58	**<0.0001**
Plant (broccoli/ cucumber)	1	23.53	**<0.0001**
Pathogen x plant	2	20.75	**<0.0001**

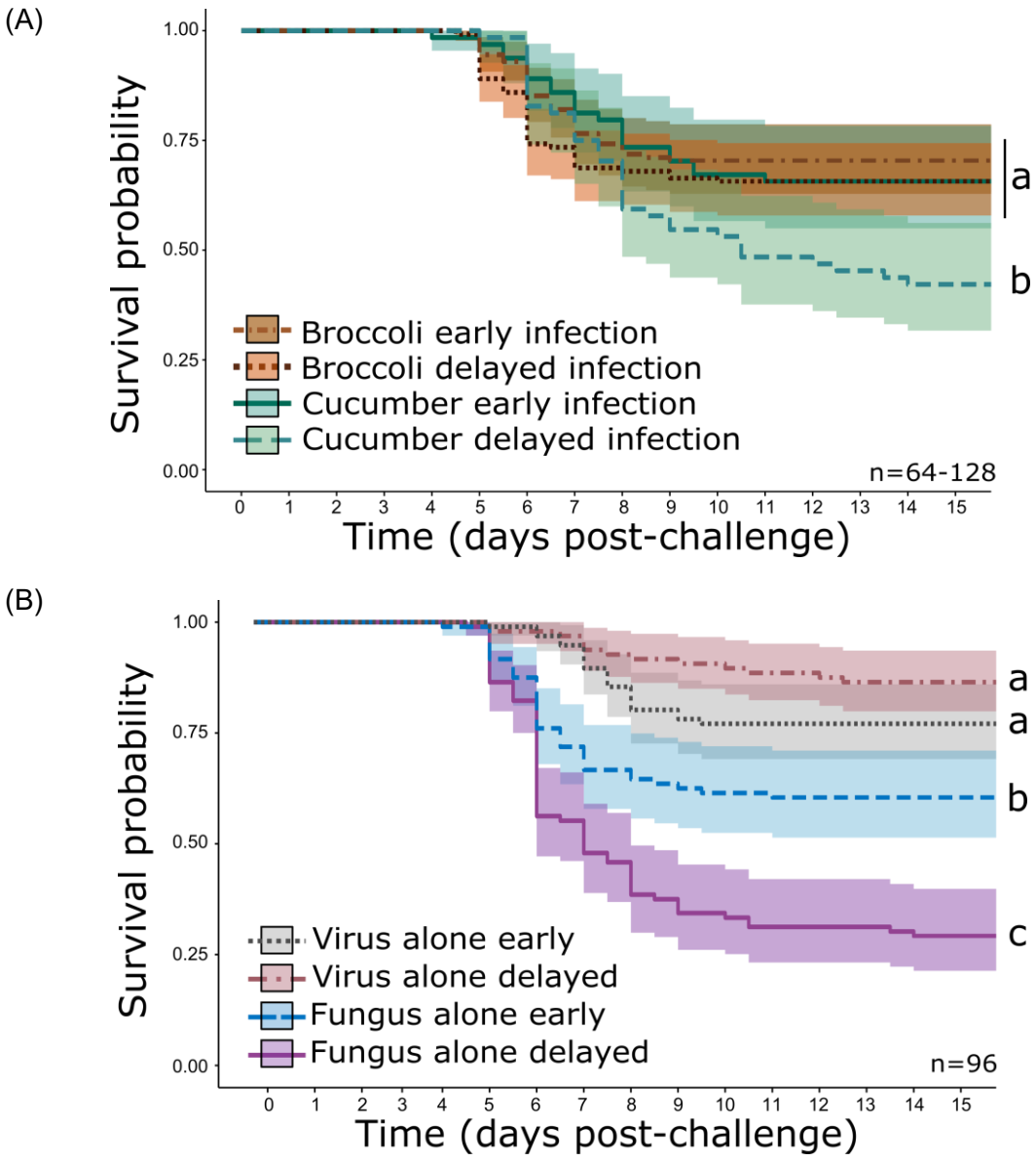

Figure 4.1. **Survival rate of *T. ni* larvae challenged with either a single dose of TniSNPV (500 OBs) or *B. bassiana* (20,000 spores) as newly moulted (early) or 48h old (delayed) 4th instars: (A) Fed either broccoli or cucumber leaves post-pathogen challenge and (B) challenged with either virus or fungus. Survival rates are visualised as Kaplan-Meier survival plots (with 95% confidence intervals). Letters show pairwise significant differences ($p<0.05$). Day 0 was set as the day of pathogen challenge for all treatments.**

Do host plant identity and co-infection alter larval survival (synchronous infection)?

Both pathogen treatment and host plant affected survival rates but there was no interaction between the two (Table 4.2b). Feeding on cucumber leaves post pathogen challenge increased the risk of larval death by around 50% (HR=1.49; 95% CI: 1.05-2.11), as well as decreasing survival time by about 40% and this was consistent across both single and mixed infections (Fig. 4.2A). Regardless of the host plant, mortality rate was the lowest when the insects were challenged with the virus alone, such that larvae in the mixed infection treatment were almost three times more likely to die of pathogen challenge than the larvae in the virus single treatment (HR=2.84; 95% CI: 1.75-4.62). However, there was no significant difference in *T. ni* survival rate between the single fungus and the mixed synchronous treatments (Fig. 4.2B).

Does host plant identity and the timing of pathogen challenge affect the outcome of mixed pathogen infections?

Host plant had a major impact on the outcome of the co-infections, and this was influenced by the timing of the mixed infections (Table 4.2c). On broccoli, survival rates were significantly different for all three mixed pathogen treatments. When *T. ni* larvae were challenged first with TniSNPV followed by *B. bassiana* 48 hours later the survival rate was more than 9 times lower than the reverse asynchronous treatment on the same host plant (HR=9.44, 95% CI: 5.18-17.20), with the synchronous co-infection treatment falling in between (Fig. 4.3A). Survival was poorer overall for larvae fed cucumber leaves, although again the asynchronous challenge with the virus preceding the fungus had significantly lower survival compared to the reverse situation (Fig. 4.3B). In addition, survival rates in both the mixed synchronous and fungus first treatments were significantly higher when larvae were fed broccoli leaves compared to any treatments where *T. ni* larvae were fed cucumber. However, larvae in the virus-first asynchronous treatment survival rates were significantly higher on cucumber, such that larvae fed broccoli tended to die quicker (between 6 and 8 days after the first pathogen challenge) compared to the larvae fed cucumber, which died between day 7 and 12 post pathogen challenge (Fig. 4.3, Appendix Fig. B1).

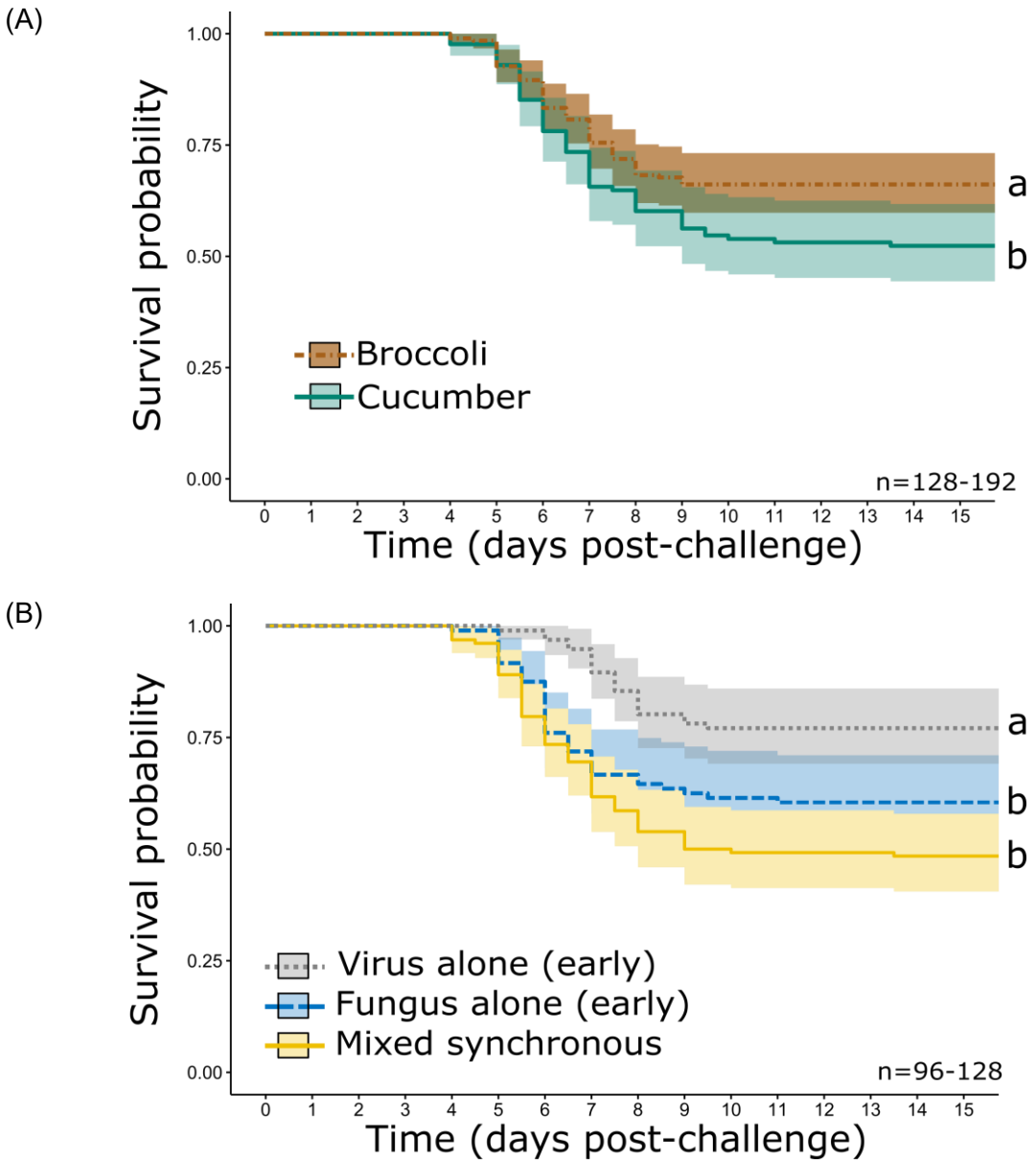

Figure 4.2. **Survival rate of 4th instar *T. ni* larvae (A) fed either broccoli or cucumber leaves post-pathogen challenge and (B) challenged with either a single dose of TniSNPV (500 OBs) or *B. bassiana* (20,000 spores) or both pathogens synchronously. Figure shows Kaplan-Meier survival plots (with 95% confidence intervals). Letters show pairwise significant differences ($p<0.05$).**

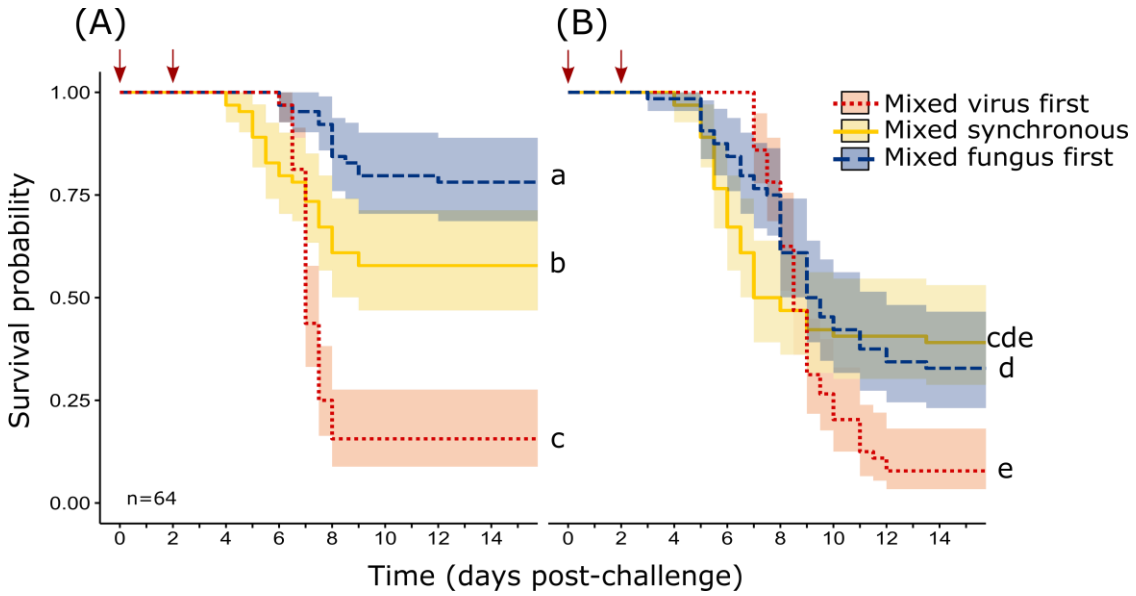

Figure 4.3. **Survival rate of 4th instar *T. ni* larvae co-challenged with TniSNPV (500 OBs) and *B. bassiana* (20,000 spores) synchronously or sequentially (virus first or fungus first) and fed: (A) broccoli or (B) cucumber leaves post-pathogen challenge. Figure shows Kaplan-Meier survival plots (with 95% confidence intervals). Letters show pairwise significant differences within the interaction term ($p<0.05$), red arrows indicate first and second pathogen challenge for the asynchronous mixed infections.**

4.4.2. Pathogen specific mortality

Virus infection

By only considering larvae that succumbed to viral infection in the mixed compared to virus single treatments, we were able to determine how host plant and co-infection timing affected TniSNPV mortality specifically (Pathogen x Plant: $\chi^2_4=9.73$, $p=0.04$; Pathogen: $\chi^2_4=27.50$, $p<0.0001$; Plant: $\chi^2_1=14.29$, $p<0.001$). Survival rate on broccoli was highest when larvae were challenged with the fungus first and that was significantly different from the reverse asynchronous mixed treatment as well as the early single virus challenge (Fig. 4.4A). The mixed synchronous treatment on broccoli was borderline in terms of difference ($p=0.057$). Feeding the insects cucumber had no significant effect on virus mortality across treatments (Fig. 4.4B). Larval survival rate due to viral infection was only significantly higher on broccoli in the mixed fungus-first treatment (Fig. 4.4, Appendix Fig. B2).

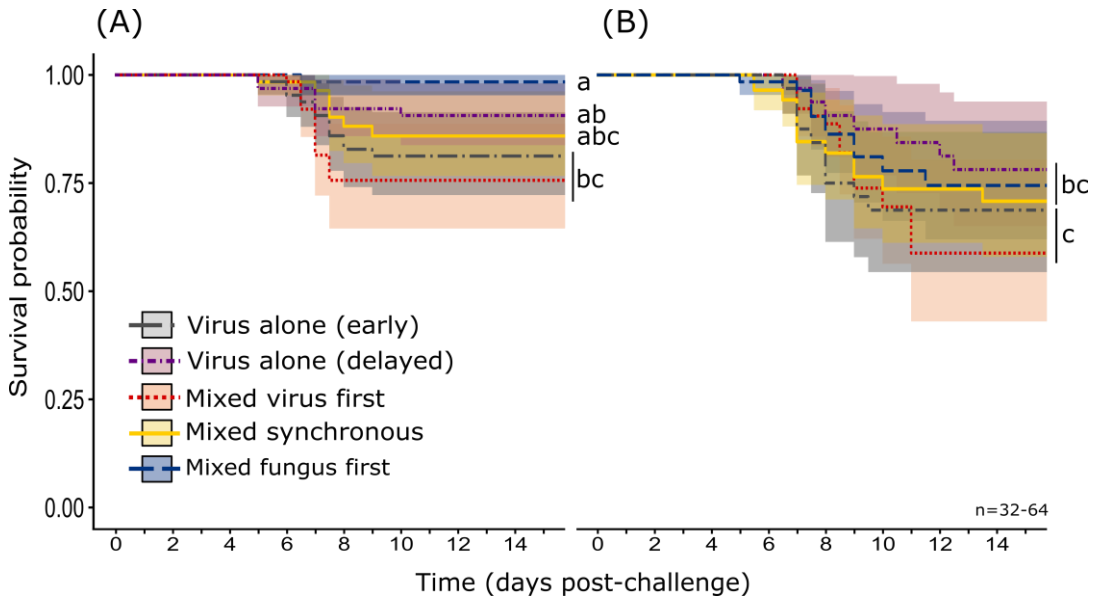

Figure 4.4. **Virus-specific survival rate of *T. ni* larvae challenged with a single dose of TniSNPV (500 OBs) either as newly moulted (early) or 48h old (delayed) 4th instars or co-challenged with *B. bassiana* (20,000 spores) synchronously or sequentially (virus first or fungus first) and fed: (A) broccoli or (B) cucumber leaves post-pathogen challenge. Figure shows Kaplan-Meier survival plots (with 95% confidence intervals). Letters show pairwise significant differences within the interaction term ($p<0.05$), day 0 set as the day for all viral challenges.**

Fungal infection

Host plant had a more pronounced effect on survival rate of fungally-infected insects (Pathogen x Plant: $\chi^2_4=19.89$, $p<0.001$; Pathogen: $\chi^2_4=83.66$, $p<0.0001$; Plant: $\chi^2_1=15.18$, $p<0.0001$). On both host plants, larval survival was the lowest in the delayed fungus only challenge and the asynchronous mixed infection treatment where *B. bassiana* was introduced 48 hours after TniSNPV (Fig. 4.5). However, on broccoli the highest level of survival was found in larvae challenged with *B. bassiana* first, such that the survival rate was more than 7 times higher than for the larvae in the reverse asynchronous treatment (HR=7.4, 95% CI: 3.9-14.0) and twice the survival in the corresponding fungus only (early) treatment (HR=2.3, 95% CI: 1.2-4.6; Fig. 4.5A). On cucumber leaves, the outcome was similar, but the response was more variable resulting in greater overlap. Unlike the insects fed on broccoli, larvae challenged with *B. bassiana* alone right after moulting to 4th instar had the highest survival rate, but that was not significantly different to larvae in the synchronous and fungus-first asynchronous treatment (Fig. 4.5B). Larvae in the mixed fungus-first treatment had a higher survival rate on broccoli compared to similarly

challenged insects fed cucumber leaves (Fig. 4.5, Appendix Fig. B3). However, larval in the reverse asynchronous treatment had a significantly lower survival rate as a result of fungal infection when were fed broccoli and succumbed to fungal infection faster (4 to 6 days post pathogen challenge) when fed broccoli compared to cucumber leaves (5 to 10 days after pathogen challenge).

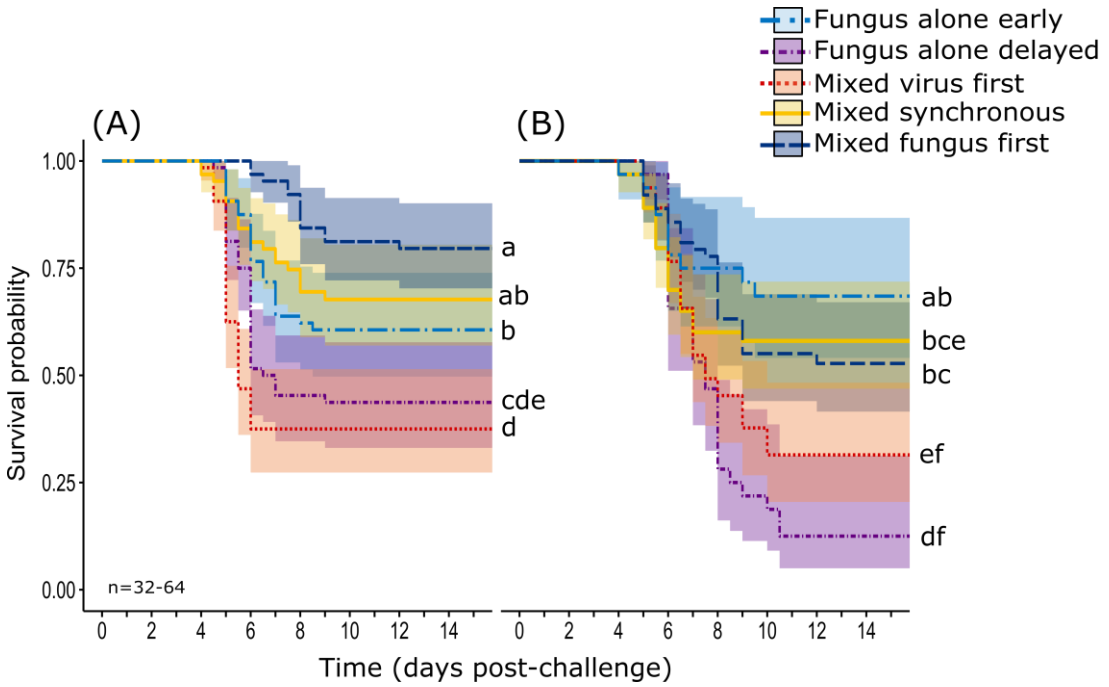

Figure 4.5. Fungus-specific survival rate of *T. ni* larvae challenged with a single dose of *B. bassiana* (20,000 spores) either as newly moulted (early) or 48h old (delayed) 4th instars or co-challenged with TniSNPV (500 OBs) synchronously or sequentially (virus first or fungus first) and fed: (A) broccoli or (B) cucumber leaves post-pathogen challenge. Figure shows Kaplan-Meier survival plots (with 95% confidence intervals). Letters show pairwise significant differences within the interaction term (p≤0.05), day 0 set as the day for all fungal challenges.

4.4.3. Pathogen within-host interaction

An alternative, simplified approach to summarizing the outcome of the mixed infections is to use the single pathogen treatments to estimate the expected mortality and determine whether the observed mortality in the mixed treatments is similar (neutral or additive), or significantly different (synergistic or antagonistic) from the prediction using the single treatments. This analysis indicates different outcomes for the asynchronous co-infections on the two plants, with exposure to the fungus first on broccoli being antagonistic

(lower observed mortality than expected), whereas the interaction was synergistic (higher observed mortality than expected) on cucumber (Table 4.3). When the virus was applied first, the outcome was synergistic on broccoli, but not on cucumber, although mortality was already high on this plant. Synchronous exposure to the two pathogens was either neutral on broccoli (observed mortality not different than the most virulent pathogen) or additive on cucumber (observed mortality equal to the sum of both single pathogen mortality)

Table 4.3. Observed larval mortality in 4th instar *T. ni*. larvae challenged with TniSNPV and *B. bassiana* alone, and observed and expercted mortality in 4th instar *T. ni*. larvae challenged with both pathogens synchronously or sequentially (delayed), on two of *T. ni* host plants (broccoli and cucumber). X^2 higher than the X^2tab (3.841) are highlighted in bold, (+) synergistic and (-) antagonistic interactions.

Pathogen treatment	Timing	Host plant	Observed mortality	Expected mortality	X^2	Interaction
Virus single	Early	Broccoli	0.19			
Fungus single	Early	Broccoli	0.40			
Virus single	Delayed	Broccoli	0.11			
Fungus single	Delayed	Broccoli	0.59			
Virus single	Early	Cucumber	0.33			
Fungus single	Early	Cucumber	0.33			
Virus single	Delayed	Cucumber	0.47			
Fungus single	Delayed	Cucumber	0.97			
Mix synch	Synchronous	Broccoli	0.43	0.52	1.25	neutral
Mix Virus first	Delayed	Broccoli	0.87	0.68	**5.31**	(+)
Mix fungus first	Delayed	Broccoli	0.22	0.47	**13.30**	(-)
Mix synch	Synchronous	Cucumber	0.63	0.55	1.16	additive
Mix Virus first	Delayed	Cucumber	0.93	0.98	0.26	neutral
Mix fungus first	Delayed	Cucumber	0.82	0.64	**5.60**	(+)

4.4.4. Pupal weight

Larvae fed with broccoli produced heavier pupae compared to those fed cucumber, 178.9 mg (± 3.3 SEM) and 124.2 mg (± 6.1 SEM) respectively, regardless of the pathogen treatment (host plant: $F_{(1, 414)}$=342.90, p<0.0001; host plant x infection: $F_{(2, 412)}$= 0.47, p=0.63). Larvae co-challenged with TniSNPV and *B. bassiana* formed pupae that were 20% lighter (135.6 mg ±3.8 SEM) than both the pupae from the control and single challenge treatments (infection: $F_{(2, 414)}$=33.52, p<0.0001; Fig. 4.6).

Focusing on single pathogen treatments, pupae were significantly (5.2%) heavier when larvae were fed broccoli and challenged as a new 4th instar (early) compared to larvae that were two days older when challenged, 163.78 mg ± 3.5 SEM and 155.23 mg ± 4.1 SEM respectively (host plant: $F_{(1, 203)}$=234.19, p<0.0001; timing: $F_{(1, 203)}$=5.44, p=0.02; host plant x timing: $F_{(1, 202)}$= 0.53, p=0.47). Aside from pupae being larger when fed broccoli leaves, pupal weight did not significantly differ among the mixed pathogen treatments (host plant: $F_{(1, 126)}$=100.5, p<0.0001; pathogen: $F_{(2, 124)}$=0.23, p=0.80; host plant x pathogen: $F_{(2, 122)}$= 1.94, p=0.15).

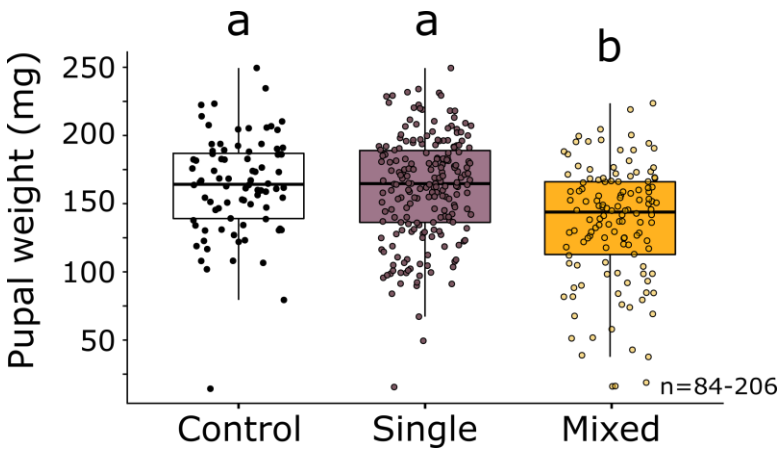

Figure 4.6. **Mean pupal weight from *T. ni* larvae in the control treatment or larvae that survived challenged with a single dose of TniSNPV (500 OBs) or *B. bassiana* (20,000 spores) or both together (Mixed). Boxes indicate 25th and 75th percentiles, lines within boxes indicate medians, and whiskers includes values within 1.5 times the interquartile range. Letters indicate significant differences between treatments at p<0.05 (Tukey's HSD).**

4.5. Discussion

We investigated whether host plant identity altered the outcome of mixed pathogen infection and how this was influenced by the order of infection using a virus and a fungus. We compared larval survival after single and mixed pathogen infection on two *T. ni* host plants, broccoli and cucumber. The results show that host plant identity is an important driver of co-infection outcome, as its impact varied with the type of pathogen and which pathogen was introduced first. While broccoli was the better host plant for *T. ni* larvae, mixed infection outcomes varied from neutral, or similar to the most virulent pathogen in the single treatments (*B. bassiana*) when both pathogens were introduced at the same time to synergistic (more virulent than the sum of the mortality of both pathogen in the

single treatments) and antagonistic (less virulent than the most virulent pathogen in the single treatment) depending on the timing of pathogen introduction and host plant. Both pathogens responded differently to the two host plants and *B. bassiana* was the most affected. Although the results discussed in Chapters 2 & 3 showed a clear dominance of *B. bassiana* over TniSNPV in synchronous mixed infections on artificial diet, this was not observed on either of the host plants.

Host plant and pathogen infections

Our results support our prediction that TniSNPV and *B. bassiana* generally caused greater overall mortality when larvae are fed cucumber leaves in single as well as in mixed infections. While *T. ni* is a pest on both plants (Tallamy et al., 1997; Sarfraz et al., 2011), the brassica broccoli has been shown to be a more suitable host plant, in terms of larval weight gain (Dussourd and Denno, 1994) and pupal size (Shikano et al., 2010). This was confirmed in our study, with a higher pupal weight for insects fed on broccoli. Although there is considerable evidence that host plant species, or even variety, can have major impacts on the outcome of single pathogen infections in insects (e.g. Shikano et al., 2010; Shaurub et al., 2016; Ocampo-Hernández et al., 2019), this study is the first to draw attention to the effect of host plant on the outcome of mixed infections post-pathogen challenge. Indeed, while the difference between the asynchronous treatments on broccoli was relatively large, the higher overall mortality on cucumber resulted in fewer differences between the mixed treatments. Broccoli and cucumber belong to two different families, Brassicaceae and Cucurbitaceae respectively, with distinct physical characteristics and secondary chemical profiles (Dussourd and Denno, 1994; Walz et al., 2004). As their main defence, broccoli and other Brassicaceae contain glucosinolates, which are detoxified in cabbage looper larvae (Winde and Wittstock, 2011). Cucumber, on the other hand, contains cucurbitacins, highly bitter terpenoids which act as feeding deterrents (Dussourd, 1997; Tallamy et al., 1997), and which have been shown to be sequestered in the hemolymph of beetle species (Chrysomelidae) (Ferguson et al., 1985; Opitz and Müller, 2009). Although cabbage looper larvae have been shown not to be deterred by cucurbitacin-treated food (Tallamy et al., 1997), the study did not look at the effect of cucurbitacin on other life-history traits, such as growth or development. By rearing the larvae on the same artificial diet before and during challenge, differences in larval weight or condition prior to pathogen exposure were eliminated (Cory and Hoover, 2006; Shikano, 2017). In addition, dosing the insects on diet plugs rules out any direct effect of the plants

on the baculovirus in the midgut. Hence any differences in susceptibility to single or mixed pathogen infection are the consequences of the host ingesting the two different host plants after pathogen exposure. Different host plants can elicit the activation of different immune functions in insects (Karlsson Green, 2021); however, their link to pathogen susceptibility is poorly understood. Entomopathogenic fungus interaction with host plants has been mainly focused on spore acquisition or the inhibition of the germination process (Ugine et al., 2007; Müller and Riederer, 2005; Lacey and Mercadier, 1998). Nevertheless, there is some evidence that plant defensive compounds can affect fungal development within the host. For example, corn earworm, *Heliothis zea*, larvae feeding on artificial diet containing the tomato defensive compound, α-tomatine, limited the development of the fungus *Nomuraea rileyi* (Gallardo et al., 1990). In addition, the surface of cucumber leaves is often covered with many non-glandular trichomes, creating a physical barrier to herbivory (Coapio et al., 2017; Kariyat et al., 2019), limiting contact with plant surface and obstructing larval locomotion (Pete and Shanower, 2001). Broccoli leaves, on the other hand, do not contain trichomes; however, the wax coating on the leaf surface can limit the insects' grip on the leaf, potentially impeding larval movement and feeding behaviour (Pete and Shanower 2001). Although unchallenged *T. ni* larvae were able to complete their development and reach pupation on both host plants, the increase in host susceptibility on cucumber, particularly in the mixed infection treatments, could be the consequence of limited access to resources to maintain an immune response (Cotter and Al Shareefi, 2022; Valtonen et al., 2010) and/or a slower developmental rate weakening overall larval condition and increasing susceptibility to infection. The history of the hosts with regard to diet and pathogen exposure should also be considered; the insects in our study came from a long-term colony that had been reared pathogen-free on artificial diet for many generations, which might have affected their response to the plants, although the larvae did respond well to the shift to natural plant food.

Priority effects and mixed infections

The time of arrival of each pathogen was particularly important to the outcome of mixed infection, but it was also highly influenced by the host plant the larvae fed on post-pathogen challenge. On broccoli, the different outcomes of the mixed infection treatments in term of larval mortality suggests the presence of a potential trade-off between host plant quality and resistance to multiple pathogens associated with timing of pathogen arrival. The interaction between TniSNPV and *B. bassiana* on broccoli was found to be neutral in

synchronous infection, whereas challenging larvae with *B. bassiana* first resulted in lower mortality than expected from the single treatments alone and introducing the virus first caused higher mortality. Similar to our findings, synchronous infection of the fall armyworm, *Spodoptera frugiperda,* and the velvetbean caterpillar, *Anticarsia gemmatalis* larvae, with NPVs and *Metarhizium rileyi* strains showed additive interaction (similar observed mortality than expected from single treatments), whereas asynchronous infection led to synergistic or antagonistic interactions (higher and lower observed mortality than expected, respectively), depending on the host species (Souza et al., 2019). Moreover, when the sugarcane borer, *D. saccharalis* larvae, were challenged with their granulovirus (*Diatraea saccharalis* GV) 36 hours prior to fungal challenge, the outcome of the interaction was shown to be antagonistic (lower LC50 in the mixed infections) with *B. bassiana* but not with *Metarhizium anisopliae* (Pauli et al., 2018). Although baculoviruses and entomopathogenic fungi initiate infection at different locations (midgut versus dorsal cuticle), direct or indirect competition to access resources by each pathogen could lead to an increase in the rate of host exploitation (Choisy and de Roode, 2010; Day, 2002). When TniSNPV is introduced 48 hours prior to *B. bassiana*, both pathogens were expected to kill the host at about the same time based on the single infections. However, since only one pathogen was able to produce transmission stages at the end of successful infections, this suggests that the competition between two pathogens was more likely to have happened earlier in the infection process and that one pathogen quickly dominated the host overall. Interestingly, when the virus was introduced first, *T. ni* larvae died faster when fed broccoli compared to cucumber (Appendix Fig. B1). This was mainly the result of a faster speed of kill for *B. bassiana* on broccoli (Appendix Fig. B3). It is possible that the slower larval development on cucumber, the poorer host plant, slowed down pathogen exploitation, as the production of transmission stages for baculoviruses and fungi is associated with larval age or size (Luz and Fargues, 1998; Cory and Myers, 2003). Although *B. bassiana* appears to be particularly efficient in utilizing host resources on artificial diet (see Chapters 2 & 3), there was no evidence of this on these two host plants. The antagonistic interaction observed between *B. bassiana* and TniSNPV on broccoli suggests that on the better host plant, competition between the two pathogens might be greater, potentially as a result of an increase in resources available and/or the better host condition on broccoli, enabling the larvae to limit fungal and later viral infection. Mounting and maintaining an immune response against pathogen invasion is energetically costly, potentially even more so in mixed infections (Zuk and Stoehr, 2002; Ulrich and Schmid-

Hempel, 2012), and under variable food regimes (Jokela et al., 2005; Fellous and Koella, 2010; Vale et al., 2013). However, more experimental data are needed to determine whether these outcomes are driven by pathogen competition for host resources or the effect of the different plants on the host immunocompetence (Ulrich and Schmid-Hempel, 2012).

Pathogen-specific mortality and competition

Looking at each pathogen separately, both species were affected by the host plant and the order of introduction. We expected *B. bassiana* to be the dominant pathogen due to its shorter incubation time, and its previously demonstrated negative effect on virus-specific mortality on artificial diet (Chapters 2 & 3). By inoculating TniSNPV 48 hours prior to *B. bassiana*, the aim was to provide TniSNPV with more time to invade the host and potentially outcompete the faster-acting fungus. However, virus infection (larval survival) did not differ between any of the mixed infection treatments and their respective single treatments. This suggests that studies on artificial diet might poorly predict the outcome of mixed pathogen interactions compared to more natural food resources. Indeed, the spotted stem borer, *Chilo partellus*, infected with either *B. bassiana* or *Metarhizium anisopliae* showed an increase in fungal mortality when fed maize leaves after pathogen challenge compared to the larvae fed artificial diet throughout their entire development (Tefera and Pringle, 2003). The absence of a negative effect of *B. bassiana* on virus-induced mortality could be the result of direct interaction between the pathogen and plant secondary chemicals or the indirect effect of plant chemical compounds through changes in immunity, such as *T. ni* larvae being better at limiting fungal infection leading to the establishment of TniSNPV. Similarly, challenging the larvae with *B. bassiana* first and then TniSNPV, which would have been expected to be even more advantageous for *B. bassiana*, showed a reduction in fungal induced mortality compared to the respective single fungus treatment, but only on broccoli. This could be the result of a better immune response to fungal infection by *T. ni* larvae due to host plant or an increase in immune activation after the virus enters the host (Scholefield et al., 2019; Pan et al., 2021), affecting the development of *B. bassiana*. Indeed, infection of the corn earworm, *Helicoverpa zea,* with its NPV elicited a higher response, including an increase in hemocyte counts and then later a decrease in encapsulation ability, whereas *B. bassiana* infection in the same host did not activate any of the measured immune response (Black

et al., 2022). Yet, more data are needed to disentangle the influence of the host plant on pathogen competition and how they can influence priority effects.

Susceptibility to fungal challenge increased considerably between newly moulted 4th instar larvae and two-day-old 4th instars, whereas there was no difference for the viral challenge. Intrastadial variation has been shown to make a difference in larval susceptibility to baculovirus infection in the spongy moth, *Lymantria dispar* larvae but has not been studied in other species (Hoover et al., 2002; Grove and Hoover, 2007). Little is known about changes in larval susceptibility to fungal challenge within a single instar. Successful fungal infection is related to successful germination of fungal spores on the host cuticle. However, it would seem more likely that larvae would be more susceptible to fungal infection around ecdysis, as moulting involves substantial larval transformations, including the formation of a thin new cuticle, the shedding of the previous cuticle, and finally the thickening of the newly formed cuticle (Way, 1950; Csikós et al., 1999; Lee and Wilson, 2006). Younger tobacco hornworm larvae, *Manduca sexta*, have been shown to quickly recognise and limit the propagation of the entomopathogenic bacterium *Photorhabdus luminescens* (Eleftherianos et al 2008), while silk moth larvae, *Bombyx mori*, showed that moulting fluid excreted during ecdysis can protect against bacterial and fungal pathogens (Zhang et al., 2014). The larvae in the asynchronous infection did spend 24 hrs on the host plants prior to the second pathogen challenge. Thus, it cannot be excluded that this could have affected the host response to pathogen infection. However, no effect was apparent in the viral infection treatments, but host plant identity could have had an impact on the host, increasing susceptibility to only the fungus. Further studies are needed to determine mechanisms in the variation in intrastadial susceptibility to pathogen infections and the relationship between host plant, host age and timing in mixed pathogen infections.

Sublethal effects

Finally, we also examined the influence of co-infection and host plants on the pupal weight of larvae that survived pathogen challenge, as an indicator of potential sublethal effects and a proxy for female fecundity (Greenberg et al., 2001). We predicted that a double challenge, particularly of two very different pathogens, would be more costly to repel than a single infection of either (Ulrich and Schmid-Hempel, 2012). This was supported by our results as the survivors of mixed pathogen infections were always lighter

than the single infections and untreated controls. Earlier studies on artificial diet showed a selection for larger pupae after fungal infection but did not show an increased cost of surviving multiple infections when *T. ni* larvae were provided with a diluted diet (reduced quantity) or different quality diets (varying P:C ratios) (see Chapters 2 & 3). Costs of surviving pathogen infection are highly dependent on the quality and availability of the resource (Jokela et al., 2005; Valtonen et al., 2010; Boots, 2011), as described earlier, host plant contains many chemical compounds that can alter insect host development and condition. Thus, a less complex artificial diet, which contains antimicrobials could have represented a better resource for larval development and thus decreased the potential cost of surviving infection (Tefera and Pringle, 2003), in addition to the larvae coming from a long-term colony reared on artificial diet. Indeed, increased resource quantity has been shown to limit the negative effect of within-host pathogen competition on host fitness in the yellow fever mosquito, *Aedes aegypti,* infected with the microsporidium, *Vavraia culicis,* and the protozoan, *Ascogregarina culicis* (Fellous and Koella, 2009).

In summary, we show that host plant quality had a major impact on the outcome of mixed pathogen infections. On a poor-quality host plant, the overall survival was poor and the outcome of mixed infections tended to be similar, while on a better quality host plant within-host pathogen interactions were governed by pathogen time of arrival. These results highlight the importance of plant identity in host's survival to pathogen infection and mixed pathogen infection dynamics.

Chapter contributions

Pauline Deschodt and Dr. Jenny Cory conceived the idea and designed the experiment; Pauline, Jessi Li and Soobin Yim collected the data; Dr. Jenny Cory provided funding and materials, Pauline and Dr. Jenny Cory analysed the data; Pauline drafted the chapter; Pauline and Dr. Jenny Cory edited the chapter.

4.6. References

Alizon, S., de Roode, J. C., and Michalakis, Y. (2013). Multiple infections and the evolution of virulence. *Ecol. Lett.* 16, 556–567. doi:10.1111/ele.12076.

Aljbory, Z., and Chen, M.-S. (2018). Indirect plant defence against insect herbivores: a review. Insect Sci. 25, 2–23. doi:10.1111/1744-7917.12436.

Awmack, C. S., and Leather, S. R. (2002). Host plant quality and fecundity in herbivorous insects. *Annu. Rev. Entomol.* 47, 817–844. doi:10.1146/annurev.ento.47.091201.145300.

Black, J. L., Lorenz, G. M., Cato, A. J., Bateman, N. R., and Seiter, N. J. (2022). Efficacy of *Helicoverpa armigera* nucleopolyhedrovirus on soybean for control of *Helicoverpa zea* (boddie) (Lepidoptera: Noctuidae) in Arkansas agriculture. *Insects* 13, 1–12. doi:10.3390/insects13010091.

Bartolomé, C., Buendía-Abad, M., Benito, M., Sobrino, B., Amigo, J., Carracedo, A., Martín-Hernández, R., Higes, M., and Maside, X. (2020). Longitudinal analysis on parasite diversity in honeybee colonies: new taxa, high frequency of mixed infections and seasonal patterns of variation. *Sci. Rep*. 10, 10454. doi:10.1038/s41598-020-67183-3.

Boots, M. (2011). The evolution of resistance to a parasite is determined by resources. *Am. Nat.* 178, 214–220. doi:10.1086/660833.

Brown, S. P., Hochberg, M. E., and Grenfell, B. T. (2002). Does multiple infection select for raised virulence? *Trends Microbiol.* 10, 401–405. doi:10.1016/s0966-842x(02)02413-7.

Chen, E., Kolosov, D., O'Donnell, M. J., Erlandson, M. A., McNeil, J. N., and Donly, C. (2018). The effect of diet on midgut and resulting changes in infectiousness of AcMNPV baculovirus in the cabbage looper, *Trichoplusia ni*. *Front. Physiol.* 9, 1348. doi:10.3389/fphys.2018.01348.

Chen, M.-S. (2008). Inducible direct plant defense against insect herbivores: A review. *Insect Science* 15, 101–114. doi:10.1111/j.1744-7917.2008.00190.x.

Choisy, M., and de Roode, J. C. (2010). Mixed infections and the evolution of virulence: effects of resource competition, parasite plasticity, and impaired host immunity. *Am. Nat.* 175, E105-18. doi:10.1086/651587.

Clay, P. A., Cortez, M. H., Duffy, M. A., and Rudolf, V. H. W. (2019). Priority effects within coinfected hosts can drive unexpected population-scale patterns of parasite prevalence. *Oikos* 128, 571–583. doi:10.1111/oik.05937.

Clay, P. A., and Rudolf, V. H. W. (2019). How parasite interaction strategies alter virulence evolution in multi-parasite communities. *Evolution* 73, 2189–2203. doi:10.1111/evo.13843.

Coapio, G. G., Cruz-López, L., Guerenstein, P., Malo, E. A., and Rojas, J. C. (2017). Oviposition preference and larval performance and behavior of *Trichoplusia ni* (Lepidoptera: Noctuidae) on host and nonhost plants. *Arthropod Plant Interact.* 12, 1–10. doi:10.1007/s11829-017-9566-9.

Cory, J. S., and Ericsson, J. D. (2010). Fungal entomopathogens in a tritrophic context. *Biocontrol* 55, 75–88. doi:10.1007/s10526-009-9247-4.

Cory, J. S., and Hoover, K. (2006). Plant-mediated effects in insect-pathogen interactions. *Trends Ecol. Evol.* 21, 278–286. doi:10.1016/j.tree.2006.02.005.

Cory, J. S., and Myers, J. H. (2003). The ecology and evolution of insect baculoviruses. *Annu. Rev. Ecol. Evol. Syst.* 34, 239–272. doi:10.1146/annurev.ecolsys.34.011802.132402.

Cory, J. S. (2010). "The Ecology of baculoviruses," in *Insect Virology*, eds. S. Asgari and K. Johnson (Caister Academic Press, Norfolk), 411–427.

Cotter, S. C., and Al Shareefi, E. (2022). Nutritional ecology, infection and immune defence - exploring the mechanisms. *Curr. Opin. Insect Sci.* 49, xx-yy. doi:10.1016/j.cois.2021.12.002.

Cressler, C. E., Nelson, W. A., Day, T., and McCauley, E. (2014). Disentangling the interaction among host resources, the immune system and pathogens. *Ecol. Lett.* 17, 284–293. doi:10.1111/ele.12229.

Csikós, G., Molnár, K., Borhegyi, N. H., Talián, G. C., and Sass, M. (1999). Insect cuticle, an in vivo model of protein trafficking. *J. Cell Sci.* 112, 2113–2124. doi:10.1242/jcs.112.13.2113.

Day, T. (2002). On the evolution of virulence and the relationship between various measures of mortality. *Proc. Biol. Sci.* 269, 1317–1323. doi:10.1098/rspb.2002.2021.

Doublet, V., Natsopoulou, M. E., Zschiesche, L., and Paxton, R. J. (2015). Within-host competition among the honey bees pathogens *Nosema ceranae* and Deformed wing virus is asymmetric and to the disadvantage of the virus. *J. Invertebr. Pathol.* 124, 31–34. doi:10.1016/j.jip.2014.10.007.

Duffey, S. S., Hoover, K., Bonning, B., and Hammock, B. D. (1995). "The impact of host-plant on the efficacy of baculoviruses," in *Reviews in pesticide toxicology*, eds. M. Roe and R. Kuhr (CTI Toxicology Communications, Raleigh, NC.), 137–275.

Dussourd, D. E., and Denno, R. F. (1994). Host Range of Generalist Caterpillars: Trenching Permits Feeding on Plants with Secretory Canals. *Ecology* 75, 69–78. doi:10.2307/1939383.

Dussourd, D. E. (1997). Plant exudates trigger leaf-trenching by cabbage loopers, Trichoplusia ni (Noctuidae). *Oecologia* 112, 362–369. doi:10.1007/s004420050321.

Fellous, S., and Koella, J. C. (2010). Cost of co-infection controlled by infectious dose combinations and food availability. *Oecologia* 162, 935–940. doi:10.1007/s00442-009-1535-2.

Fellous, S., and Koella, J. C. (2009). Infectious dose affects the outcome of the within-host competition between parasites. *Am. Nat.* 173, E177-84. doi:10.1086/598490.

Ferguson, J. E., Metcalf, R. L., and Fischer, D. C. (1985). Disposition and fate of cucurbitacin B in five species of diabroticites. *J. Chem. Ecol.* 11, 1307–1321. doi:10.1007/BF01024118.

Gallardo, F., Boethel, D. J., Fuxa, J. R., and Richter, A. (1990). Susceptibility of *Heliothis zea* (Boddie) larvae to *Nomuraea rileyi* (Farlow) Samson: effects of α-tomatine at the third trophic level. *J. Chem. Ecol.* 16, 1751–1759. doi:10.1007/BF01020492.

Greenberg, S. M., Sappington, T. W., Legaspi, B. C., Liu, T. X., and Sétamou, M. (2001). Feeding and life history of *Spodoptera exigua* (Lepidoptera: Noctuidae) on different host plants. *AN* 94, 566–575. doi:10.1603/0013-8746(2001)094[0566:FALHOS]2.0.CO;2.

Grove, M. J., and Hoover, K. (2007). Intrastadial developmental resistance of third instar gypsy moths (*Lymantria dispar L.*) to *L. dispar* nucleopolyhedrovirus. *Biological Control* 40, 355–361. doi:10.1016/j.biocontrol.2006.12.003.

Hoover, K., Alaniz, S. A., Yee, J. L., Rocke, D. M., Hammock, B. D., and Duffey, S. S. (1998a). Dietary protein and chlorogenic acid effect on baculoviral disease of noctuid (Lepidoptera: Noctuidae) larvae. *Environ. Entomol.* 27, 1264–1272. doi:10.1093/ee/27.5.1264.

Hoover, K., Grove, M. J., and Su, S. (2002). Systemic component to intrastadial developmental resistance in Lymantria dispar to its baculovirus. *Biological Control* 25, 92–98. doi:10.1016/S1049-9644(02)00041-5.

Hoover, K., Stout, M. J., Alaniz, S. A., Hammock, B. D., and Duffey, S. S. (1998b). Influence of induced plant defenses in cotton and tomato on the efficacy of baculoviruses on noctuid larvae. *Journal of Chemical Ecology*.

Inglis, G. D., Enkerli, J., and Goettel, M. S. (2012). “Laboratory techniques used for entomopathogenic fungi,” in *Manual of techniques in invertebrate pathology*, ed. L. A. Lacey (Elsevier), 189–253. doi:10.1016/B978-0-12-386899-2.00007-5.

Jafary, M., Karimzadeh, J., Farazmand, H., and Rezapanah, M. (2016). Plant-mediated vulnerability of an insect herbivore to *Bacillus thuringiensis* in a plant-herbivore-pathogen system. *Biocontrol Sci. Technol.* 26, 104–115. doi:10.1080/09583157.2015.1078872.

Janmaat, A. F., Bergmann, L., and Ericsson, J. (2014). Effect of low levels of *Bacillus thuringiensis* exposure on the growth, food consumption and digestion efficiencies of *Trichoplusia ni* resistant and susceptible to Bt. *J. Invertebr. Pathol.* 119, 32–39. doi:10.1016/j.jip.2014.04.001.

Janmaat, A. F., and Myers, J. (2003). Rapid evolution and the cost of resistance to *Bacillus thuringiensis* in greenhouse populations of cabbage loopers, *Trichoplusia ni. Proc. Biol. Sci.* 270, 2263–2270. doi:10.1098/rspb.2003.2497.

Jansen, M. P. T., and Stamp, N. E. (1997). Effects of light availability on host plant chemistry and the consequences for behavior and growth of an insect herbivore. *Entomol. Exp. Appl.* 82, 319–333. doi:10.1046/j.1570-7458.1997.00146.x.

Ji, X. Y., Wan, N. F., Liu, J., and Jiang, J. X. (2016). Nucleopolyhedrovirus infection and/or parasitism by *Microplitis pallidipes* affected haemolymph titre of 20-hydroxyecdysone in *Spodoptera exigua* larvae. *J. Appl. Entomology* 140, 142–149. doi:10.1111/jen.12230.

Johnson, P. T. J., and Buller, I. D. (2011). Parasite competition hidden by correlated coinfection: using surveys and experiments to understand parasite interactions. *Ecology* 92, 535–541. doi:10.1890/10-0570.1.

Jokela, J., Taskinen, J., Mutikainen, P., and Kopp, K. (2005). Virulence of parasites in hosts under environmental stress: experiments with anoxia and starvation. *Oikos* 108, 156–164. doi:10.1111/j.0030-1299.2005.13185.x.

Kariyat, R. R., Raya, C. E., Chavana, J., Cantu, J., Guzman, G., and Sasidharan, L. (2019). Feeding on glandular and non-glandular leaf trichomes negatively affect growth and development in tobacco hornworm (*Manduca sexta*) caterpillars. *Arthropod Plant Interact.* 13, 321–333. doi:10.1007/s11829-019-09678-z.

Karlsson Green, K. (2021). The effects of host plant species and larval density on immune function in the polyphagous moth *Spodoptera littoralis*. *Ecol. Evol.* 11, 10090–10097. doi:10.1002/ece3.7802.

Kassambara, A., Kosinsk, M., and Biecek, P. (2021). survminer: Drawing survival curves using 'ggplot2'. R package version 0.4.9. https://CRAN.R-project.org/package=survminer

Koppenhöfer, A. M., and Kaya, H. K. (1997). Additive and synergistic interaction between entomopathogenic nematodes and *Bacillus thuringiensis* for scarab grub control. *Biological Control* 8, 131–137. doi:10.1006/bcon.1996.0498.

Lacey, L. A., and Mercadier, G. (1998). The effect of selected allelochemicals on germination of conidia and blastospores and mycelial growth of the entomopathogenic fungus, *Paecilomyces fumosoroseus* (Deuteromycotina: Hyphomycetes). *Mycopathologia* 142, 17–25. doi:10.1023/A:1006963016316.

Lee, K. P., Simpson, S. J., and Wilson, K. (2008). Dietary protein-quality influences melanization and immune function in an insect. *Funct. Ecol.* 22, 1052–1061. doi:10.1111/j.1365-2435.2008.01459.x.

Lee, K. P., and Wilson, K. (2006). Melanism in a larval Lepidoptera: repeatability and heritability of a dynamic trait. *Ecol. Entomol.* 31, 196–205. doi:10.1111/j.0307-6946.2006.00780.x.

Lochmiller, R. L., and Deerenberg, C. (2000). Trade-offs in evolutionary immunology: just what is the cost of immunity? *Oikos* 88, 87–98. doi:10.1034/j.1600-0706.2000.880110.x.

Lohr, J. N., Yin, M., and Wolinska, J. (2010). Prior residency does not always pay off-co-infections in *Daphnia*. *Parasitology* 137, 1493–1500. doi:10.1017/S0031182010000296.

Luz, C., and Fargues, J. (1998). Factors affecting conidial production of *Beauveria bassiana* from fungus-killed cadavers of *Rhodnius prolixus*. *J. Invertebr. Pathol.* 72, 97–103. doi:10.1006/jipa.1998.4774.

Manzi, F., Halle, S., Seemann, L., Ben-Ami, F., and Wolinska, J. (2021). Sequential infection of *Daphnia magna* by a gut microsporidium followed by a haemolymph yeast decreases transmission of both parasites. *Parasitology*, 1–12. doi:10.1017/S0031182021001384.

Massey, R. C., Buckling, A., and ffrench-Constant, R. (2004). Interference competition and parasite virulence. *Proc. Biol. Sci.* 271, 785–788. doi:10.1098/rspb.2004.2676.

Müller, C., and Riederer, M. (2005). Plant surface properties in chemical ecology. *J. Chem. Ecol.* 31, 2621–2651. doi:10.1007/s10886-005-7617-7.

Ocampo-Hernández, J. A., Tamayo-Mejía, F., Tamez-Guerra, P., Gao, Y., and Guzmán-Franco, A. W. (2019). Different host plant species modifies the susceptibility of *Bactericera cockerelli* to the entomopathogenic fungus *Beauveria bassiana*. *J. Appl. Entomology* 143, 984–991. doi:10.1111/jen.12680.

Ojala, K., Julkunen-Tiitto, R., Lindström, L., and Mappes, J. (2005). Diet affects the immune defence and life-history traits of an Arctiid moth *Parasemia plantaginis*. *Evol Ecol Res* 7, 1153–1170.

Opitz, S. E. W., and Müller, C. (2009). Plant chemistry and insect sequestration. *Chemoecology* 19, 117–154. doi:10.1007/s00049-009-0018-6.

Pan, Q., Shikano, I., Felton, G. W., Liu, T.-X., and Hoover, K. (2021). Host permissiveness to baculovirus influences time-dependent immune responses and fitness costs. *Insect Sci.* 28, 103–114. doi:10.1111/1744-7917.12755.

Pauli, G., Moura Mascarin, G., Eilenberg, J., and Delalibera Júnior, I. (2018). Within-host competition between two entomopathogenic fungi and a granulovirus in *Diatraea saccharalis* (Lepidoptera: Crambidae). *Insects* 9. doi:10.3390/insects9020064.

Pike, V. L., Lythgoe, K. A., and King, K. C. (2019). On the diverse and opposing effects of nutrition on pathogen virulence. *Proc. Biol. Sci.* 286, 20191220. doi:10.1098/rspb.2019.1220.

Plymale, R., Grove, M. J., Cox-Foster, D., Ostiguy, N., and Hoover, K. (2008). Plant-mediated alteration of the peritrophic matrix and baculovirus infection in lepidopteran larvae. *J. Insect Physiol.* 54, 737–749. doi:10.1016/j.jinsphys.2008.02.005.

Poprawski, T. J., Greenberg, S. M., and Ciomperlik, M. A. (2000). Effect of host plant on *Beauveria bassiana* - and *Paecilomyces fumosoroseus* -induced mortality of *Trialeurodes vaporariorum* (Homoptera: Aleyrodidae). *Environ. Entomol.* 29, 1048–1053. doi:10.1603/0046-225X-29.5.1048.

Raymond, B., Vanbergen, A., Pearce, I., Hartley, S., Cory, J., and Hails, R. (2002). Host plant species can influence the fitness of herbivore pathogens: the winter moth and its nucleopolyhedrovirus. *Oecologia* 131, 533–541. doi:10.1007/s00442-002-0926-4.

Saeed, S., Sayyed, A. H., and Ahmad, I. (2010). Effect of host plants on life-history traits of *Spodoptera exigua* (Lepidoptera: Noctuidae). *J Pest Sci (2004)* 83, 165–172. doi:10.1007/s10340-009-0283-8.

Sarfraz, R. M., Cervantes, V., and Myers, J. H. (2011). The effect of host plant species on performance and movement behaviour of the cabbage looper *Trichoplusia ni* and their potential influences on infection by *Autographa californica* multiple nucleopolyhedrovirus. *Agric. For. Entomol.* 13, 157–164. doi:10.1111/j.1461-9563.2010.00504.x.

Schmid-Hempel, P. (2005). Evolutionary ecology of insect immune defenses. *Annu. Rev. Entomol.* 50, 529–551. doi:10.1146/annurev.ento.50.071803.130420.

Scholefield, J. A., Shikano, I., Lowenberger, C. A., and Cory, J. S. (2019). The impact of baculovirus challenge on immunity: The effect of dose and time after infection. *J. Invertebr. Pathol.* 167, 107232. doi:10.1016/j.jip.2019.107232.

Shaurub, E.-S. H., Reyad, N. F., Abdel-Wahab, H. A., and Ahmed, S. H. (2016). Mortality and nematode production in *Spodoptera littoralis* larvae in relation to dual infection with *Steinernema riobrave*, *Heterorhabditis bacteriophora*, and *Beauveria bassiana*, and the host plant. *Biological Control* 103, 86–94. doi:10.1016/j.biocontrol.2016.08.007.

Shikano, I., and Cory, J. S. (2014). Genetic resistance to *Bacillus thuringiensis* alters feeding behaviour in the cabbage looper, *Trichoplusia ni*. *PLoS ONE* 9, e85709. doi:10.1371/journal.pone.0085709.

Shikano, I., Ericsson, J. D., Cory, J. S., and Myers, J. H. (2010). Indirect plant-mediated effects on insect immunity and disease resistance in a tritrophic system. *Basic and Applied Ecology* 11, 15–22. doi:10.1016/j.baae.2009.06.008.

Shikano, I. (2017). Evolutionary ecology of multitrophic interactions between plants, insect herbivores and entomopathogens. *J. Chem. Ecol.* 43, 586–598. doi:10.1007/s10886-017-0850-z.

Simpson, S. J., Clissold, F. J., Lihoreau, M., Ponton, F., Wilder, S. M., and Raubenheimer, D. (2015). Recent advances in the integrative nutrition of arthropods. *Annu. Rev. Entomol.* 60, 293–311. doi:10.1146/annurev-ento-010814-020917.

Smilanich, A. M., Langus, T. C., Doan, L., Dyer, L. A., Harrison, J. G., Hsueh, J., and Teglas, M. B. (2018). Host plant associated enhancement of immunity and survival in virus infected caterpillars. *J. Invertebr. Pathol.* 151, 102–112. doi:10.1016/j.jip.2017.11.006.

Smith, V. H., Ii, T. P. J., Smith, M. S., Smith, V. H., Jones, T. P. I., and Smith, M. S. (2005). Host nutrition and infectious disease: an ecological view. *Front Ecol Environ* 3, 268–274.

Souza, M. L., Sanches, M. M., Souza, D. A. de, Faria, M., Espinel-Correal, C., Sihler, W., and Lopes, R. B. (2019). Within-host interactions of *Metarhizium rileyi* strains and nucleopolyhedroviruses in *Spodoptera frugiperda* and *Anticarsia gemmatalis* (Lepidoptera: Noctuidae). *J. Invertebr. Pathol.* 162, 10–18. doi:10.1016/j.jip.2019.01.006.

Stirnadel, H. A., and Ebert, D. (1997). Prevalence, host specificity and impact on host fecundity of microparasites and epibionts in three sympatric *Daphnia* species. *The Journal of animal ecology* 66, 212. doi:10.2307/6023.

Tallamy, D. W., Stull, J., Ehresman, N. P., Gorski, P. M., and Mason, C. E. (1997). Cucurbitacins as feeding and oviposition deterrents to insects. *Environ. Entomol.* 26, 678–683. doi:10.1093/ee/26.3.678.

Tefera, T., and Pringle, K. L. (2003). Food consumption by *Chilo partellus* (Lepidoptera: Pyralidae) larvae infected with *Beauveria bassiana* and *Metarhizium anisopliae* and effects of feeding natural versus artificial diets on mortality and mycosis. *J. Invertebr. Pathol.* 84, 220–225. doi:10.1016/j.jip.2003.11.001.

Therneau T (2020). A package for survival analysis in R. R package version 3.2-7, URL: https://CRAN.R-project.org/package=survival.

Tikkanen, O. P., Niemelä, P., and Keränen, J. (2000). Growth and development of a generalist insect herbivore, *Operophtera brumata*, on original and alternative host plants. *Oecologia* 122, 529–536. doi:10.1007/s004420050976.

Tritschler, M., Vollmann, J. J., Yañez, O., Chejanovsky, N., Crailsheim, K., and Neumann, P. (2017). Protein nutrition governs within-host race of honey bee pathogens. *Sci. Rep.* 7, 1–11. doi:10.1038/s41598-017-15358-w.

Ugine, T. A., Wraight, S. P., and Sanderson, J. P. (2007). A tritrophic effect of host plant on susceptibility of western flower thrips to the entomopathogenic fungus *Beauveria bassiana*. *J. Invertebr. Pathol.* 96, 162–172. doi:10.1016/j.jip.2007.05.004.

Ulrich, Y., and Schmid-Hempel, P. (2012). Host modulation of parasite competition in multiple infections. *Proc. Biol. Sci.* 279, 2982–2989. doi:10.1098/rspb.2012.0474.

Vale, P. F., Choisy, M., and Little, T. J. (2013). Host nutrition alters the variance in parasite transmission potential. *Biol. Lett.* 9, 20121145. doi:10.1098/rsbl.2012.1145.

Valtonen, T. M., Kleino, A., Rämet, M., and Rantala, M. J. (2010). Starvation reveals maintenance cost of humoral immunity. *Evol. Biol.* 37, 49–57. doi:10.1007/s11692-009-9078-3.

Walz, C., Giavalisco, P., Schad, M., Juenger, M., Klose, J., and Kehr, J. (2004). Proteomics of curcurbit phloem exudate reveals a network of defence proteins. *Phytochemistry* 65, 1795–1804. doi:10.1016/j.phytochem.2004.04.006.

Way, M. J. (1950). The structure and development of the larval cuticle of *Diataraxia oleracea* (Lepidoptera). *Q. J. Microsc. Sci.* 91, 145–182.

Wetzel, W. C., Kharouba, H. M., Robinson, M., Holyoak, M., and Karban, R. (2016). Variability in plant nutrients reduces insect herbivore performance. *Nature* 539, 425–427. doi:10.1038/nature20140.

Winde, I., and Wittstock, U. (2011). Insect herbivore counteradaptations to the plant glucosinolate-myrosinase system. *Phytochemistry* 72, 1566–1575. doi:10.1016/j.phytochem.2011.01.016.

Zhang, J., Lu, A., Kong, L., Zhang, Q., and Ling, E. (2014). Functional analysis of insect molting fluid proteins on the protection and regulation of ecdysis. *J. Biol. Chem.* 289, 35891–35906. doi:10.1074/jbc.M114.599597.

Zilio, G., and Koella, J. C. (2020). Sequential co-infections drive parasite competition and the outcome of infection. *J. Anim. Ecol.* 89, 2367–2377. doi:10.1111/1365-2656.13302.

Zuk, M., and Stoehr, A. M. (2002). Immune defense and host life history. *Am. Nat.* 160 Suppl 4, S9–S22. doi:10.1086/342131.

Chapter 5.

Host plant is a key determinant of the outcome of combined field application of TniSNPV and *Beauveria bassiana* against the cabbage looper

5.1. Abstract

Microbial insecticides are an important weapon in the pest management armoury but their use is still relatively limited. One approach to broadening their use could be to combine different pathogens in mixed applications to increase pest mortality or treat a complex of pests. However, little is known about whether increasing insect pathogen diversity in the field will improve pest management. In this chapter, we investigated the compatibility of applying two pathogens of the cabbage looper, *Trichoplusia ni*, the *T. ni* specific nucleopolyhedrovirus (TniSNPV) and the generalist entomopathogenic fungus *Beauveria bassiana* on two different host plants, tomato and broccoli. In a field trial, both pathogens were sprayed alone or in combination, either synchronously or with TniSNPV being introduced a day before *B. bassiana*. Healthy 3rd instar *T. ni* larvae were introduced to the plants before application and then collected by destructive sampling a day after the last pathogen application and kept in laboratory conditions on artificial diet to determine the time and cause of death. Larval survival was generally higher on broccoli, especially when *B. bassiana* was applied. Mixed pathogen applications did not result in an increase in larval mortality compared to TniSNPV applied alone. However, virus-induced mortality was negatively affected by the presence of *B. bassiana* regardless of whether TniSNPV was applied at the same time or 24 hrs before the fungus. Fungus-induced mortality did not differ between single and mixed pathogen applications on either of the host plants. Interestingly, on tomato, the cadaver weight of larvae that succumbed to fungal infection was significantly lower when both pathogens were applied synchronously than when *B. bassiana* was sprayed alone. Our results validate our previous findings in laboratory conditions showing the negative effects of *B. bassiana* on TniSNPV, suggesting that applying this combination of entomopathogens would not be beneficial for pest management. The study also demonstrates that host plant identity is an important driver of the efficacy of single or multiple pathogen applications and needs to be considered when designing pest management programs.

5.2. Introduction

Predators, parasitoids, and pathogens are of considerable importance as biological control agents in insects (Lacey et al., 2015; Brodeur and Boivin, 2006). While research has generally focused on a single natural enemy species at a time, there is increasing interest in understanding whether increasing species richness and diversity could improve pest population management in the field (Denoth et al., 2002; Cardinale et al., 2006). Studies that have manipulated the number of natural enemies have shown that increasing diversity can lead to an increase in pest consumption, either due to resource partitioning, when species complement each other in terms of resource needs and mode of action (Finke and Snyder, 2008, 2010), or interspecific facilitation, when the presence of one natural enemy species increases host or prey susceptibility to another natural enemy species (Cardinale et al., 2003; Finke and Snyder, 2010; Jabbour et al., 2011). Nevertheless, competition and interference between introduced natural enemy species can severely impact the benefits of increasing diversity (Rosenheim, 1995; Denoth et al., 2002). More experimental studies are needed to determine whether increasing the number of natural enemies introduced has a beneficial effect on pest suppression compared to single species introductions.

Entomopathogen combination has led to mixed results regarding the efficacy and complementarity of the different pathogen groups commonly used for pest management (Denoth et al., 2002; Lacey et al., 2015; Cory and Deschodt, 2017). Moreover, since 1970, about 85% of experimental studies that have investigated entomopathogen interactions have taken place in laboratory, controlled conditions (Deschodt unpubl. data). These studies have primarily focused on a few pathogen groups, mainly the interaction between the entomopathogenic bacteria, *Bacillus thuringiensis* (Bt), and the fungus, *Beauveria bassiana* (e.g. Costa et al., 2001; Lewis, 1996; Mantzoukas et al., 2013; Sayed and Behle, 2017a); between the nematodes *Heterorhabditis* sp and *B. bassiana* or *Metarhizium* sp (e.g. Ansari et al., 2006; Acevedo et al., 2007; Williams et al., 2013; Ibrahim et al., 2019) or baculovirus interactions, particularly between nucleopolyhedroviruses and granuloviruses (e.g. Shapiro, 2000; Jehle et al., 2003; Bonsall and Benmayor, 2005; Han et al., 2015). Only a few studies have determined pathogen compatibility in the field (Bt-virus: Pingel and Lewis, 1997; Bt-nematode: Bauer et al., 1998; nematode-fungus: Ansari et al., 2006; Williams et al., 2013). Hence, more experimental studies are needed to better

understand interactions between entomopathogens and to determine species compatibility against insect pests.

Pathogen efficacy in the field not only depends on the pathogen species and mode of action but can also vary considerably between crop plants (Cory and Hoover, 2006; Agrawal, 2000; Shikano, 2017). Indeed, host plants can affect entomopathogens in multiple ways including, the persistence of pathogen on the plant (Sukirno et al., 2018; Mwanza et al., 2021; Sutanto et al., 2022), insect behaviour and pathogen acquisition (Sarfraz et al., 2011; Coapio et al., 2017), as well as host susceptibility to pathogen infection (Foster et al., 1992; Hoover et al., 1998; Cory and Ericsson, 2010; Jafary et al., 2016). Moreover, variation in plant quality, both in terms of nutrient composition and the presence of defensive compounds, can also alter overall host condition and immunocompetence, indirectly affecting the susceptibility of herbivores to pathogen infection (Lampert, 2012; Jafary et al., 2016; Kumar, 2017; Shikano et al., 2018; Ocampo-Hernández et al., 2019). Variation in the efficacy of microbial control agents in the field reduces their reliability and can be costly for growers. Polyphagous pests can feed and develop on a broad range of crop plants; however, their development and overall condition are likely to vary with plant species (Awmack and Leather, 2002; Cameron et al., 2007). Insect feeding behaviour on different host plants, as well as their movement and overall development, can have a major impact on the likelihood of being infected by pathogens. Yet, little is known about the effect of host plants on mixed pathogen infections and the factors influencing the efficacy of pathogen combinations.

In this study, we examine the efficacy and compatibility of the field application of two pathogens of the cabbage looper larvae, *Trichoplusa ni* Hübner (Lepidoptera: Noctuidae), *T. ni* single nucleopolyhedrovirus (TniSNPV) and the generalist entomopathogenic fungus, *B. bassiana* on two host plants, tomato, *Solanum lycopersicum*, and broccoli, *Brassica oleracea*. Based on early laboratory studies on diet, we predicted (i) a negative interaction when both pathogens were applied together, thus increased mortality when applied asynchronously, (ii) mortality would be higher on the lower quality plant. In addition, we predicted that (iii) pathogen concentrations could be reduced in mixed applications and still maintain an effective level of control which could save the growers' expense. Finally, we hypothesized that larvae in the mixed application treatment would die earlier, especially on the low-quality plant tomato, affecting pathogen replication and recycling within-host population, which could have long-term

consequences on pathogen presence within the host population and pathogen transmission potential.

5.3. Materials and Methods

5.3.1. Insects

In Fall (September) 2019, *T. ni* eggs were obtained from Insect Production Services (Natural Resources Canada, Sault Ste Marie, ON). After hatch larvae were maintained at 25°C with a 16L:8D photoperiod and reared on a standard wheat germ-based artificial diet (modified from Shikano and Cory 2014). Newly moulted 3rd instar larvae used in the experiments were reared individually from the neonate stage.

5.3.2. Pathogens

The *Trichoplusia ni* single nucleopolyhedrovirus (TniSNPV) isolate was initially collected from an infected *T. ni* larva collected from a greenhouse in the Fraser Valley, British Columbia in 2000 (Janmaat & Myers 2003). TniSNPV was then amplified in *T. ni* larvae and semi-purified using several rounds of centrifugation to remove debris. The entomopathogenic fungus, *Beauveria bassiana*, was obtained by diluting the original commercial product, BotaniGardES®, *B. bassiana* GHA strain (initial concentration of viable spores: $8.6*10^6$ spores/ml). Fungal spore viability was tested before pathogen dilution by plating 150 µl of a 10^6 spores/ml suspension onto five plates containing Potato-Dextrose Agar (PDA) medium, spores were left to germinate in a growth chamber at 24°C for 24 h. Germination was then assessed under a light microscope at 400x magnification (Inglis et al., 2012). Spore germination in all five plates was over 95%. Both viral and fungal final suspensions used in the experiment were obtained through serial dilution of the original suspension.

5.3.3. Experimental set up

Host plant

A total of 45 Centennial broccoli and 45 Moneymaker tomato plants were grown from seeds (West Coast Seeds ®, BC, Canada) in a greenhouse at SFU in a 1:1 ratio of mushroom manure and peat moss mixture. When seedlings reached the 4-true leaves

stage, each plant was transferred to an individual 10 L pot with the same soil mixture and grown in the same greenhouse conditions for six weeks. Each 6-week-old individual plant was enclosed in ½ inch chicken wire and white mesh nylon fabric. The protective structure was large enough to enable the plants to continue growing without being restrained by the mesh fabric. As the evening temperatures in September 2019 started to drop, the plants were kept in greenhouse conditions (apart from the spray application) for the duration of the experiment. Plants were 8-weeks-old at the start of the assay.

Pathogen dilution

Pathogen dilutions were prepared on the day of the application to avoid viability problems due to storage. The pathogens were applied outside at ultra-low volumes using an Ulva+ sprayer (Micron ©, Herefordshire, UK), using the yellow nozzle (flow rate at 1m/s walking speed: 25ml/ min). Two sprayers were used, one for each pathogen, to avoid contamination between pathogen treatments. Pathogen applications were chosen according to the recommended field rate for *B. bassiana* commercial products (Behle 2006; Garcia-Guttierrez et al 2010) and both field application studies using NPVs on *T. ni* larvae and other lepidopteran hosts (Bianchi et al 2000; Ashour et al 2007; Landwehr 2021) along with the data collected in Chapter 2, 3 and 4 in laboratory conditions for TniSNPV application. The TniSNPV suspension was diluted and applied at the recommended rate (Full), $8*10^7$ OBs/m^2, or half the recommended rate (Half). Similarly, *B. bassiana* was applied at either 2 or $4*10^8$ conidia/m^2, the latter corresponding to the recommended rates (Table 5.1). Pathogens were sprayed in the early evening (~8 pm) to reduce the effects of UV on the activity of the pathogens.

Spray trial

Larvae were introduced to the host plant before pathogen application to give them time to settle on the plant. Twenty third instar *T. ni* larvae were placed onto every plant through an opening at the top of the mesh cages. Pathogens were then applied the next day (24h later) at both rates (Half/ Full), either singly, together synchronously or asynchronously, with *Tni*SNPV being applied 24 h before *B. bassiana* (Table 5.1). For each pathogen application treatment, 10 individual plants (5 broccoli and 5 tomato plants) were randomly selected and arranged in a row with a 50 cm interval between each plant to avoid *T. ni* larvae moving between plants. The mesh cages were carefully removed before each application, pathogens were applied to the row of plants and each plant was

then placed back in the greenhouse in a random manner. For the asynchronous treatment, plants that were sprayed with virus on day one were taken back outside 24 h later to be sprayed with the assigned fungus suspension and placed back in the greenhouse. An additional 5 broccoli and 5 tomato plants were not sprayed but moved in a similar matter as the other plants to mimic any stress of plant handling on larval behaviour.

Table 5.1. Summary of the pathogen treatments and timing of application.

Pathogen	Infection	Application rate	First infection (day 1)	Second infection (day 2)
Control	Control	Control	None	None
Virus	Single	Half	$4*10^7$ OBs/m^2	None
Virus	Single	Full	$8*10^7$ OBs/m^2	None
Fungus	Single	Half	$2*10^8$ conidia/m^2	None
Fungus	Single	Full	$4*10^8$ conidia/m^2	None
Mix Synchronous	Mixed	Half	$4*10^7$ OBs/m^2 + $2*10^8$ conidia/m^2	None
Mix Synchronous	Mixed	Full	$8*10^7$ OBs/m^2 + $4*10^8$ conidia/m^2	None
Mixed delayed	Mixed	Half	$4*10^7$ OBs/m^2	$2*10^8$ conidia/m^2
Mixed delayed	Mixed	Full	$8*10^7$ OBs/m^2	$4*10^8$ conidia/m^2

Sampling and monitoring

All the larvae were collected from each plant 24h after the last spray application and kept individually in 1 oz SOLO® cups. Larvae were maintained at 25°C and reared on an artificial wheat germ-based diet (Shikano and Cory 2014). Larvae in each treatment were monitored daily until death or pupation. Larvae that die of baculovirus infection are usually pale with a soft tegument which easily ruptures to release millions of OBs, whereas larvae that succumb to the fungal challenge are purple-coloured and rigid, which made the cause of death easily identifiable. Each cadaver was weighed at the time of death; because of their fragile condition, viral cadavers were placed in a 1.5 ml tube and then weighed. Larvae killed by *B. bassiana* were surface sterilized by dipping the cadavers in 1% sodium hypochlorite solution for 1 min followed by two rinses with distilled water. These cadavers were then placed in humidity chambers (1 oz SOLO® cup with 3 cm^2 cotton humidified with 500 µl distilled water) and kept at 24°C to allow the fungus to sporulate and confirm a fungal infection. Any unclear deaths were initially treated as fungal

cadavers to check for sporulation; if no sporulation occurred after 72 hrs the cadavers were smeared and checked under a light microscope at 400x for OBs or fungal hyphae. Sporulation dates were recorded. Pupae were collected and weighed 3 days after pupation.

5.3.4. Statistical analysis

All analysis were carried out using R version-4.0.3. Survival models were run using the *survreg* function from the *survival* package (Therneau, 2020) and the *pairwise survdiff* function from the *survminer* package (Kassambara et al., 2021) was used to calculate pairwise comparison between group levels. Contrasts between groups in generalized linear models were determine using the *emmeans* function from the *emmeans* package (Length 2020). For each model, interactions between all explanatory variables were first included in the initial model and then removed if not significant, then any non-significant single explanatory variable not included in an interaction term were removed until the minimum adequate model was found.

First, overall pathogen-induced larval mortality was analyzed using a generalized linear model (GLM) with a binomial distribution. Initially all explanatory variables (host plant (2) and application treatment (8)) and their interactions were included in initial the model. Due to a number of unknown deaths in the control treatment, especially on tomato plants, overall pathogen mortality was corrected using the following formula:

$$Corrected_{dead} = Treat_{dead} - Treat_{total} \times (Control_{dead} / Control_{total})$$

$$Corrected_{total\ larvae} = Treat_{total} \times (1 - (Control_{dead} / Control_{total}))$$

$Treat_{total}$ and $Treat_{dead}$ are the total number and the number of dead larvae in the treatment respectively and $Control_{total}$ and $Control_{dead}$ the total number and the number of dead larvae in the control treatment.

To determine the effect of pathogen combination and host plant on larval survival we ran a series of parametric survival models, with an exponential distribution (Weibull). First, we investigated the differences between the single pathogen applications, including the two application rates (Half/ Full) and the two host plants. We then used a similar model to determine potential differences in the mixed infection treatments (synchronous vs

delayed application). Finally, we analyzed the survival rate of larvae sprayed with a similar formulation by comparing the mixed treatment with both pathogens applied at medium rates and the single treatments applied at the high rate on each host plant.

To investigate how pathogens affected each other after mixed pathogen application, we compared difference in virus-specific mortality between treatments using a GLM model with binomial distribution. Included in the initial model were the pathogen applications (virus alone, synchronous vs delayed application), the two application rates (Half/ Full) and the two host plants, excluding the single fungus application treatments and all the larvae that died of fungal infection from the model. Then we ran a similar model but this time focusing on fungus-specific survival, excluding virus single application treatments. As overdispersion was detected for the virus-specific mortality, a quasi-likelihood approach was used.

We then analyzed cadaver weight for both viral and fungal cadavers as the size can be related to the potential inoculum for secondary transmission. Cadaver weights were square root transformed to fit the assumption of normality and analyzed using linear models. Initial models included host plant, pathogen treatment, application rates, and time to death (linear and quadratic term).

Finally, we analyzed pupal weight to determine the effects of host plant on larval development and potential sublethal effects of pathogen application for *T.ni* larvae that survived infection. We first analyzed pupal weight from the control treatment to examine the effect of host plant alone. Then we looked at differences in pupal weight between the control and pathogen treatments. Due to the lack of successful pupation on tomato, only pupae from the broccoli treatments were analyzed in this section. Non-parametric Kruskal Wallis tests were used to compare pupal weights, as the data could not be normalized.

5.4. Results

5.4.1. Overall mortality

The overall mortality of *T. ni* larvae collected after pathogen application was significantly higher on tomato compared to insects collected from broccoli, 97% (± 2.1 SEM) and 73% (± 5.6 SEM) respectively, regardless of the pathogen treatment (host plant: χ^2_1=67.76, p<0.0001; host plant x pathogen treatment: χ^2_7=0.78, p=1.00). Larval mortality was on average 41% lower when *B. bassiana* was applied alone (55% ± 9.3 SEM) compared to the single virus and mixed application treatments (synchronous or delayed). There was no significant difference in overall mortality between the single virus and the mixed pathogen treatments (Pathogen treatment: χ^2_7=175.81, p<0.0001; Fig. 5.1).

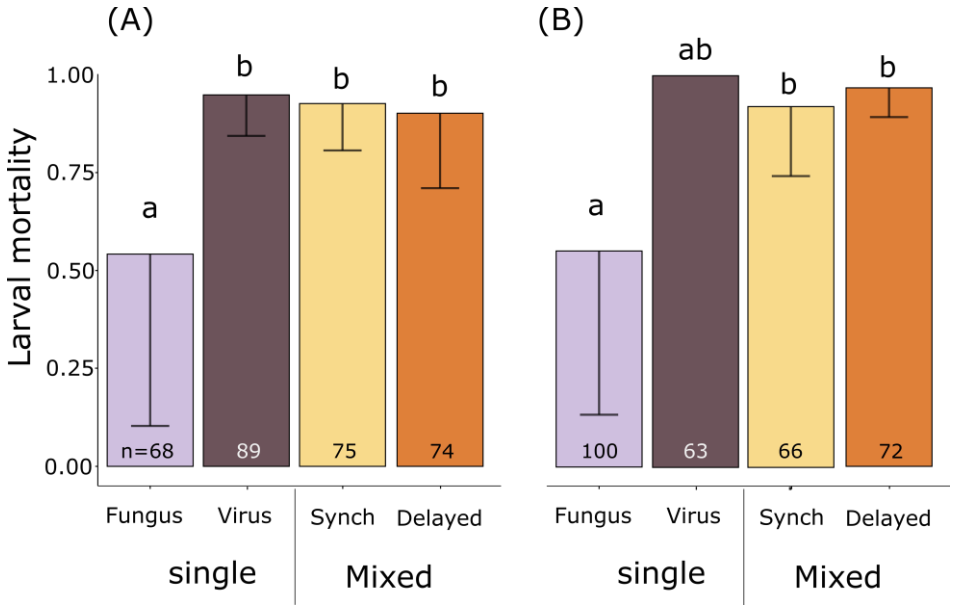

Figure 5.1. Corrected mean (± s.e.) overall mortality of *T. ni* larvae after field application of TniSNPV and *B. bassiana* at (A) half (4*10^7 OBs/m^2 and 2*10^8 conidia/m^2) and (B) full rate (8*10^7 OBs/m^2 and 4*10^8 conidia/m^2), either alone (single) or together (Mixed) synchronously (Synch) or application of TniSNPV 24 h prior to *B. bassiana* application (Delayed). Error bars show the standard deviation. Letters indicate significant differences between all pathogen treatments (p<0.05).

5.4.2. Larval survival

Single pathogen application

Larval survival after single pathogen application was affected by the pathogen species (virus or fungus), the application rate (Half/ Full) and the host plant identity (Table 5.2a). On broccoli plants, the larval survival rate was 93% higher when *B. bassiana* was applied compared to TniSNPV (HR=0.07; 95% CI=0.04-11.27) and no significant difference was found between application rates (Fig. 5.2). On tomato plants, the larval survival rate was 56% higher in the half rate fungus treatment compared to fungus applied at the full rate (HR=0.44; 95% CI=0.31-0.65). No difference was found in the single virus treatments on tomato. Survival rate in the single virus treatment did not differ between host plant, but survival rate in the single fungus treatment decreased when larvae fed on tomato compared to broccoli (Fig. 5.2).

Table 5.2. Effect of host plant and pathogen application rate (Half/ Full) on *T. ni* survival rate after (a) single application of TniSNPV or *B. bassiana* or (b) both pathogens together, synchronously or TniSNPV applied 24h prior to *B. bassiana* (delayed). Significant p values are highlighted in bold.

	(a) Single applications		(b) Mixed applications	
	χ^2_1	p	χ^2_1	p
Host plant	62.99	**<0.0001**	40.62	**<0.0001**
pathogen	114.66	**<0.0001**	6.10	**0.01**
Application rate	19.91	**<0.0001**	5.57	**0.02**
Host plant x pathogen	87.26	**<0.0001**	0.87	0.35
Host plant x application rate	0.69	0.41	1.72	0.19
Pathogen x application rate	1.99	0.16	3.67	0.06
Host plant x pathogen x Application rate	5.80	**0.02**	3.10	0.08

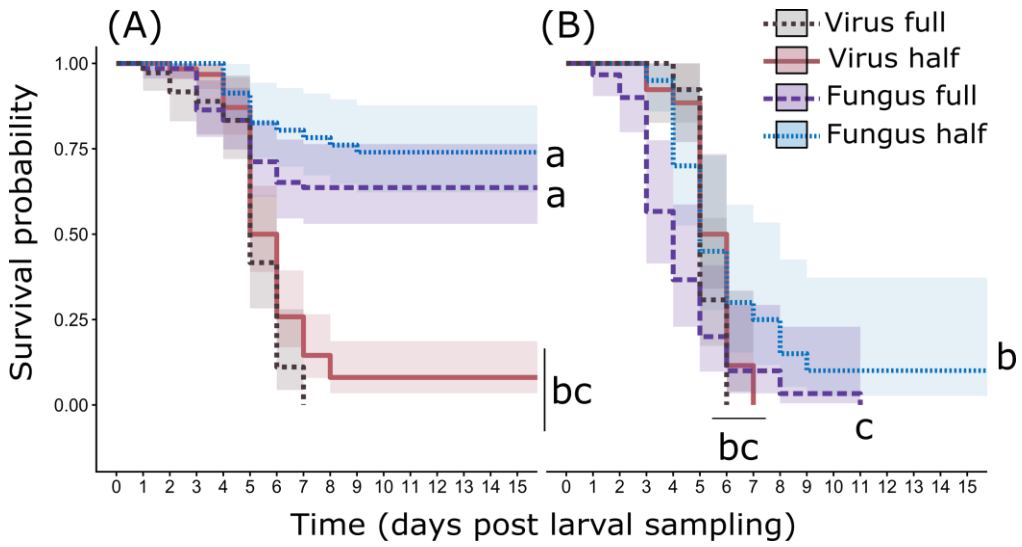

Figure 5.2. **Survival of *T. ni* larvae on (A) broccoli or (B) tomato plants after single application of TniSPNV or *B. bassiana* at half ($4*10^7$ OBs/m^2 and $2*10^8$ conidia/m^2) or full rate ($8*10^7$ OBs/m^2 and $1*10^8$ conidia/m^2). Figure shows Kaplan-Meier survival plots (with 95% confidence intervals). Letters show significant differences between treatments in a full pairwise comparison between host plant, pathogen treatment and application rate ($p<0.05$), N=62-96.**

Mixed infection treatments

Larval survival in the mixed pathogen applications was significantly higher on broccoli compared to larvae sprayed on tomato plants (Table 5.2b), such that larvae on tomato plants were more than twice as likely to die than larvae on broccoli plants (HR=2.5, 95% CI: 1.90-3.32; Fig. 5.3A). Larvae sprayed with the two pathogens synchronously were about 40% more likely to die compared to larvae sprayed with virus 24h before fungal application (HR=1.36, 95% CI: 1.07-0.74; Fig. 5.3B). Finally, regardless of the host plant or the application combination (synchronous or delayed), larvae in the treatment where both pathogens were applied at half the recommended dosage had a 26% higher survival rate, than when both pathogens were applied at the recommended rate (HR=0.74, 95% CI: 0.58-0.95; Fig. 5.3C).

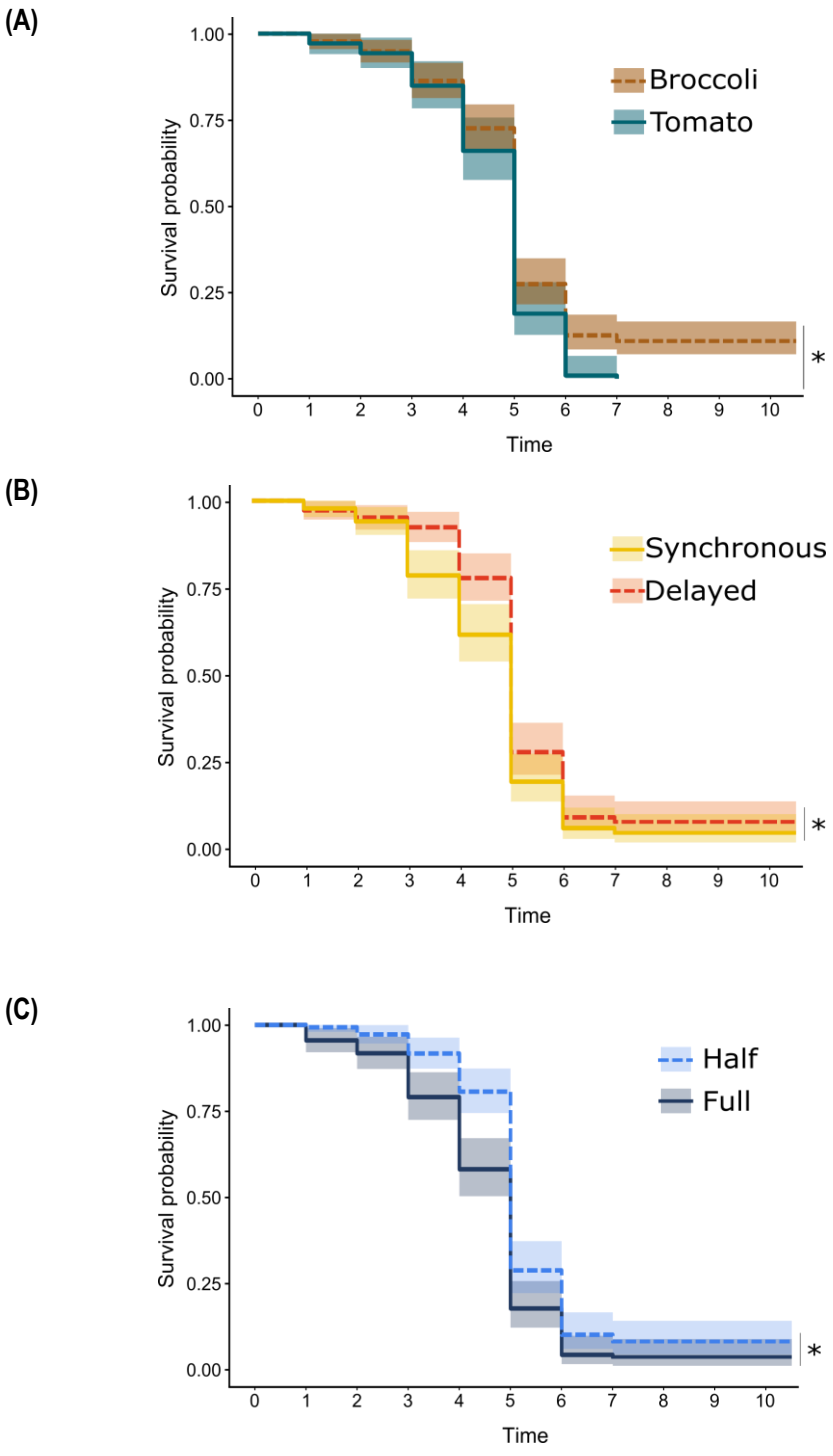

Figure 5.3: **Survival of *T. ni* larvae after field application of both TniSNPV and B. bassiana (A) either synchronously or TniSNPV applied 24 h prior to B. bassiana (delayed), N=136-145, (B) on broccoli and tomato plant, N=106-175, and (C) at half ($4*10^7$ OBs/m^2 and $2*10^8$ conidia/m^2) or full rate ($8*10^7$ OBs/m^2 and $1*10^8$ conidia/m^2), N=135-146. Figure shows Kaplan-Meier survival plots (with 95% confidence intervals). Stars show significant differences bewteewn treatments ($p<0.05$), N=20-68.**

Can dose rate be halved in mixed applications?

Applying both pathogens together at half the recommended rate did not lower larval survival more than TniSNPV applied alone at the full recommended rate. When pathogens were sprayed on broccoli plants, the survival rate was significantly higher when *B. bassiana* was applied at the highest rate ($4*10^8$ conidia/m^2) alone and no difference were observed for the other treatments (Host plant x pathogen: χ^2_3= 80.56, p<0.0001; host plant: χ^2_1= 112.99, p<0.0001; pathogen: χ^2_3= 166.85, p<0.0001; Fig.5.4). On tomato plants, no significant difference was observed between any of the single and mixed treatments.

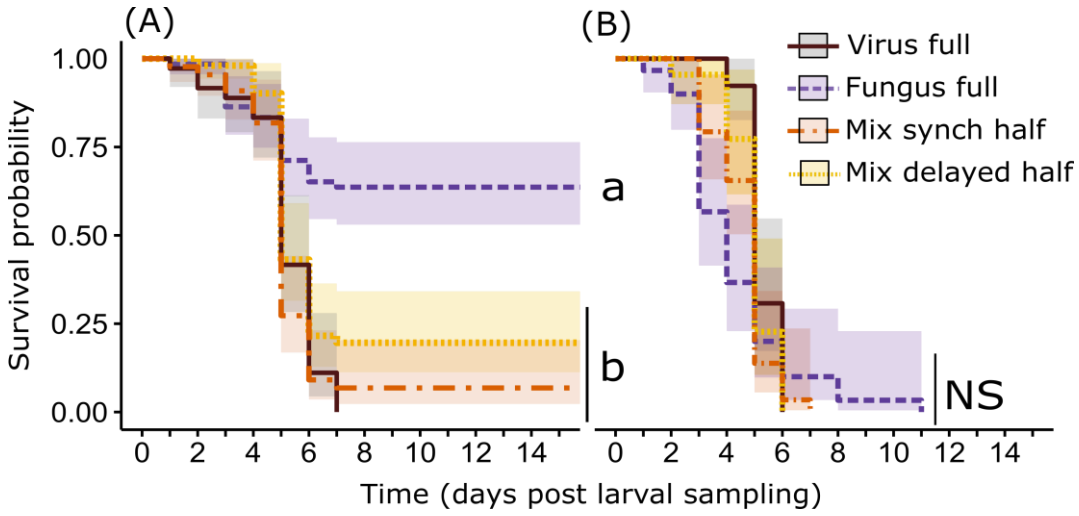

Figure 5.4. **Survival of *T. ni* larvae on (A) broccoli or (B) tomato, after single application of TniSPNV or *B. bassiana* at full recommended rate ($3.85x10^7$ OBs/m^2 and $2x10^8$ conidia/m^2respectively) or mixed application of TniSNPV and *B. bassiana*, both at half the recommended rate, synchronously (Mix synch) or delayed (Mix delayed) with TniSNPV applied on the plants 24 h before *B. Bassiana*. Figure shows Kaplan-Meier survival plots (with 95% confidence intervals). Letters show pairwise differences between pathogen treatments within each host plant (p<0.05), N=62-96.**

5.4.3. Pathogen-specific mortality

Virus-induced mortality

To evaluate how the pathogens affected each other in the different mixed applications, we looked at the mortality from each pathogen specifically. Virus-induced mortality decreased by 50% in any treatment where *B. bassiana* was applied with TniSNPV, regardless of the host plant and the dose applied (Table 5.3a; Fig. 5.5). There was no difference in virus-induced mortality between the two host plants or application rates.

Table 5.3. Effect of host plant, application rate (Half/ Full) and pathogen treatment (Single, mixed synchronous or delayed) on (a) virus-specific and (b) fungus-specific *T. ni* larvae mortality. Significant p values are highlighted in bold.

	(a) Virus-specific			(b) Fungus-specific		
	DF	F	p	DF	F	p
Host plant	1	0.14	0.71	1	24.51	**<0.0001**
pathogen	2	28.34	**<0.0001**	2	1.97	0.15
Application rate	1	1.75	0.19	1	10.13	**0.003**
Host plant x pathogen	2	2.23	0.12	2	4.18	**0.02**
Host plant x Application rate	1	1.37	0.25	1	3.01	0.09
Pathogen x Application rate	2	2.16	0.13	2	0.20	0.82
Host plant x pathogen x Application rate	2	1.32	0.28	2	2.73	0.08

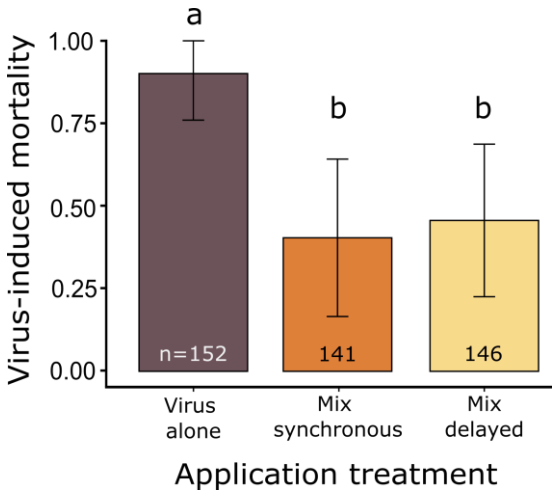

Figure 5.5. Virus-induced mortality (mean ± s.e.) of *T. ni* larvae after application of TniSNPV either alone or with *B. bassiana* synchronously or with 24h delay (Mix delayed). Letters show significant differences betweeb treatments (pairwise posthoc, p<0.05).

Fungus-induced mortality

Beauveria bassiana induced mortality was significantly affected by the host plant but this was also influenced by the pathogen treatment (Table 5.3b), such that larval mortality due to fungus was only significantly higher on tomato compared to broccoli when *B. bassiana* was applied alone (Fig. 5.6). Within each host plant, there was no significant difference in fungal-induced mortality between the single and the mixed pathogen treatments. Regardless of the host plant or pathogen treatment, fungus-induced mortality at the recommended rate was about 30% higher than when applied at half the recommended rate (Table 5.3b).

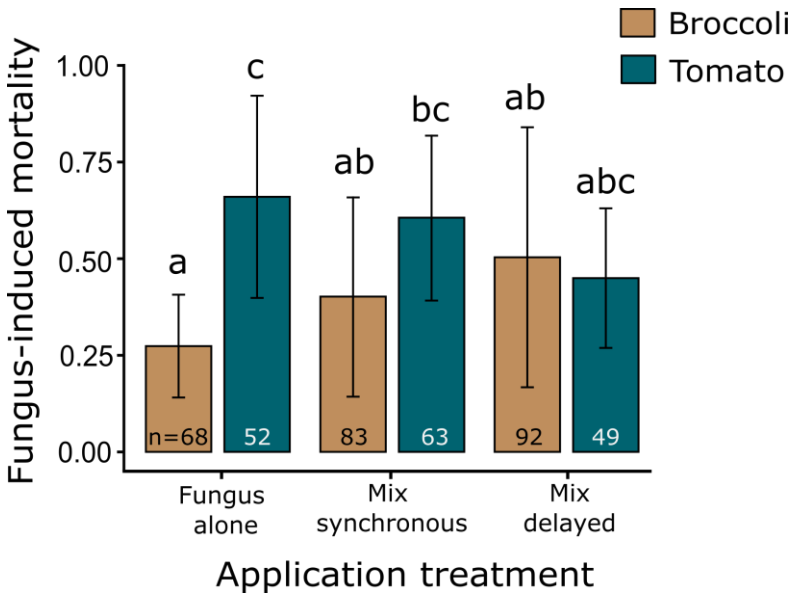

Figure 5.6. Fungus-induced mortality (mean ± s.e.) of *T. ni* larvae on broccoli and tomato after field application of *B. bassiana* alone or with TniSNPV, either synchronously (Mix synch) or with TniSNPV applied 24h prior *B. bassiana* (Mix delayed). Letters show significant differences between treatments (pairwise posthoc, p<0.05).

5.4.4. Cadaver weights

Cadavers from viral deaths

Larvae that succumbed to viral infection were more than twice as heavy when they fed on broccoli compared to the larvae from tomato, 191.8 mg (± 8.0 SEM) and 91.6mg (± 6.7 SEM) respectively, regardless of the treatment or the application rate (Table 5.4a). Cadaver weight increased with increasing time to death (Table 5.4a). Moreover, cadavers

from the delayed mixed application treatment tended to be heavier (170.1 mg) than the single virus (154.9 mg) and mixed synchronous (143.5 mg) application treatments; however, this was only marginally significant (p=0.06).

Table 5.4. Effect of host plant, application rate (Half/ Full) and treatment (single, mixed synchronous or delayed) on cadaver weight of *T. ni* larvae that died of (a) viral and (b) fungal infection. Significant p values are highlighted in bold.

	(a) Viral cadavers			(b) Fungal cadavers		
	Df	Fvalue	p	Df	Fvalue	p
Host plant	1, 188	89.74	**<0.0001**	1, 157	51.67	**<0.0001**
pathogen	2, 186	2.85	0.06	2, 157	0.51	0.60
Application rate	1, 184	0.02	0.90	1, 155	1.58	0.21
Time to death	1, 188	46.51	**<0.0001**	1, 157	44.92	**<0.0001**
Time to death ^2	1, 184	2.32	0.13	1, 156	2.92	0.09
Host plant x pathogen	2, 179	0.49	0.61	2, 157	3.85	**0.02**
Host plant x Application rate	1, 179	0.06	0.80	1, 152	1.33	0.25
Pathogen x Application rate	2, 179	0.43	0.65	2, 152	1.51	0.22
Host plant x pathogen x Application rate	2, 177	0.63	0.53	2, 150	0.30	0.74

Cadavers killed by fungus

Cadaver weight of *T. ni* larvae that succumbed to fungal infection was significantly higher on broccoli compared to tomato (Table 5.4b). While there was no difference in weight between pathogen treatments for larvae on broccoli (~168.7 mg ±7.3 SEM), cadaver weights on tomato plant were quite low, with the lowest weight found in the mixed synchronous treatment (46.3 mg ± 4.7 SEM) and the highest weight found for the single fungus application treatment (76.0 mg ± 4.9SEM) with the delayed application treatment falling between the two (Fig. 5.7).

5.4.5. Pupal weight

Larvae that pupated in the control treatments were significantly heavier on broccoli compared to larvae collected from tomato plants, 190.9 mg (± 5.5 SEM) and 165.0 mg (± 9.8 SEM) respectively (Host plant: χ^2_1=5.41, p=0.02). There was, however, no significant differences between pupal weight in the control and larvae that survived pathogen

application (single and mixed) when considering larvae that pupated within the broccoli treatment only (Pathogen treatment: χ^2_4=7.67, p=0.10).

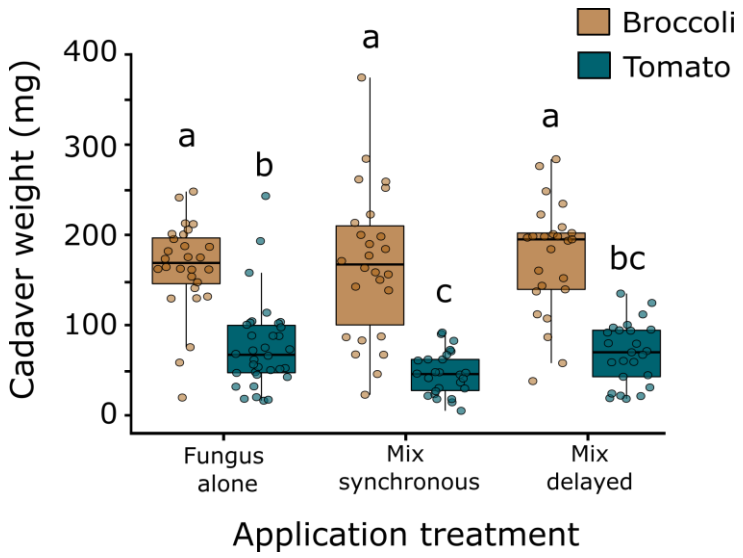

Figure 5.7. Cadaver weight of *T. ni* larvae that died of fungal infection after single field application of *B. bassiana* or mixed application of *B. bassiana* and TniSNPV either synchronously or TniSNPV sprayed 24 h before *B. bassiana* (Mix delayed). Boxes indicate 25th and 75th percentiles, lines within boxes indicate medians, and whiskers includes values within 1.5 times the interquartile range. Letters indicate significant differences between treatments at p<0.05, N=25-34.

5.5. Discussion

Understanding mixed pathogen interactions is crucial for assessing the potential compatibility of using multiple control agents in the field. Our results show that overall larval mortality in the mixed pathogen applications did not differ from single virus applications and that TniSNPV was negatively affected by the presence of *B. bassiana*. Host plant identity had a major impact on pathogen infection and is thus an important factor to consider when designing pest control programs.

Single applications

Host plant identity was a key factor in fungal infection, such that *B. bassiana* generally caused higher larval mortality when applied on tomatoes, whereas plant species did not affect TniSNPV mortality in single applications. The higher pupal weight in the unchallenged group on broccoli compared to the larvae on tomato support the results of

other studies showing that broccoli is generally a better host plant for *T. ni* (Hoover et al., 1998; Janmaat and Myers, 2005; Shikano et al., 2010). Broccoli and tomato plants are very different in terms of their architecture and leaf characteristics, as well as their defensive compounds. While broccoli leaves are waxy and mainly smooth, tomato leaves have glandular trichomes that contain toxic chemicals to repel insect herbivores (Peter and Shanower 2001; Kennedy 2003; Tian et al. 2012). In addition, broccoli contains glucosinolates (Fenwick et al., 1983; Possenti et al., 2016) which need to be detoxified by the larvae (Jeschke et al., 2016), whereas tomato plants contain glycoalkaloids such as tomatine that serve as feeding deterrents (Sarfraz et al., 2011; Hauri et al., 2021). Cabbage looper larvae are *Brassica* specialists, thus *T. ni* larvae are more likely to show higher cost of detoxifying tomato chemical compounds, such as longer developmental time, lower larval weight, or survival, compared to larvae fed broccoli (Meneses-Arias et al., 2000; Shikano et al., 2010). In addition, as viral OBs need to be ingested to initiate infection, the high larval susceptibility to infection could have been the result of proteinase inhibitors present in tomatoes which are known to impact nutrient intake and larval survival through degradation of essential amino acids in the gut of *T. ni* larvae (Gonzales-Vigil et al., 2011). The high viral mortality of *T. ni* larvae on broccoli could be the result of increased ingestion of OBs through increased foliage consumption, although this was not measured. Indeed, the vulnerability of *T. ni* and other lepidopteran larvae to NPV has been shown to increase with the amount of foliage eaten (Dwyer et al., 2005; Bianchi et al., 2000; Sarfraz et al., 2011). Alternatively, the lower susceptibility of *T. ni* larvae to *B. bassiana* applied on broccoli could have been an indirect result of the host plant effect on larval growth or resistance (Shikano et al., 2010), or direct effect of the host plant and plant volatiles on the fungus, affecting fungal spore acquisition, persistence, or germination (Brown et al., 1995; Cory and Ericsson 2010). In addition, direct contact between the fungal spores and the insect host at the time of application can be critical for efficacy of fungal pathogen application (Brown et al., 1995; Behle, 2006). Thus, on broccoli where *T. ni* larvae are generally positioned on the underside of the leaves and hidden in the most central parts of the plants, direct contact between fungal spores and *T. ni* larvae could have been limited, reducing the overall efficacy of *B. bassiana* application (Behle, 2006; Jaronski, 2010).

Mixed application and host plant

The high pathogen mortality on broccoli and tomato in single pathogen applications, particularly after viral application, resulted in high overall larval mortality on both host plants in the mixed application treatments, unfortunately limiting the analysis of the interaction between the pathogens. Nevertheless, field application of *Spodoptera frugiperda* nucleopolyhedrovirus (SfMNPV) and *Metarhizium rileyi* against the fall armyworm, *Spodoptera frugiperda* larvae, also showed no beneficial effect of combined application of this generalist fungus and baculovirus, such that larval mortality in the mixed treatment six days after pathogen application did not significantly differ from any of the single pathogen application. However, field studies examining the interaction between entomopathogens have primarily focused on a few systems, mainly including commercial products or a limited number of pathogen species, (e.g. *Bacillus thuringiensis* - *fungus* (Sayed and Behle, 2017b; Fite et al., 2020; Wraight and Ramos, 2005); Bt-NPV (Pingel and Lewis, 1997), *Heterorhabditis bacteriophora* - *Metarhizium* sp. (Ansari et al., 2006; Rauch et al., 2017)). Souza *et al*., (2019), also showed that in laboratory, controlled conditions, the outcome of the combination of AgMNPV and *Metarhizium rileyi,* as well as SfMNPV and *Metarhizium rileyi* in the velvetbean caterpillar, *Anticarsia gemmatalis*, and the fall armyworm, *Spodoptera frugiperda* larvae respectively, resulted in similar levels of mortality compared to at least one of the single pathogen infections. Likewise, co-infection of 3rd instar *Diatraea saccharalis* larvae with its granulovirus (DsGV) and either *B. bassiana* or *B. brongniartii* resulted in a similar mortality level to a single DsGV infection (Lecuona and Alves, 1988). However, more field data are needed to better understand the factors affecting virus-fungus interactions in a more realistic environment.

We expected that reducing pathogen concentrations in mixed applications could still result in high larval mortality and potentially save the growers' expense. Unfortunately, the high level of mortality obtained in the single application treatments limits our comparison between single and mixed applications. Although, variation in TniSNPV dose in our previous studies either did not result in different larval mortality between single virus and mixed treatments (See chapter 3) or showed higher mortality overall in mixed infections regardless of the virus dose (Deschodt and Cory 2022). Interestingly here, even when mortality was quite high in all treatments, TniSNPV was always negatively affected by the presence of *B. bassiana,* even when the virus application occurred 24 h before application of the fungus, while fungus-induced mortality did not differ between single and

mixed pathogen application on either of the host plants. Additionally, fungus-induced mortality, which was generally higher on tomato when applied alone, did not vary between host plants when both pathogens were applied. Although in laboratory conditions, *B. bassiana* had a limited effect on TniSNPV on host plants (see chapter 4), these results generally agree with the overall dominance of *B. bassiana* in the system. In similar virus-fungus studies, pathogen speed of kill has been shown to be particularly important in determining dominance. Indeed, spongy moth larvae, *Lymantria dispar*, infected by both LdMNPV and *Entomophaga maimaiga* synchronously, generally died of fungal infection due to its more rapid speed of kill (Malakar et al., 1999). Whereas in another system, when *Anticarsia gemmatalis* larvae were simultaneously infected with the fungus *Metarhizium rileyi* and its NPV, AgMNPV, larval mortality was mainly caused by AgMNPV due to rapid colonization and high virulence of viral strain used (Moscardi and Soza 2007; Souza et al 2019;). In mixed application treatments, we might have expected to see an increase in fungal mortality as larval activity increases in NPV-infected larvae, potentially increasing the contact between the host and fungal infective spores (Evans and Allaway, 1983). However, mortality due to fungus was highly variable within each of the treatments which might have obscured any differences. Fungus development is highly dependent on environmental conditions (temperature, humidity) (Wraight et al., 2000). Thus it is possible that small changes in local environmental factors more than pathogen interactions affected *B. bassiana* efficacy in the field.

Cadaver weight/ recycling

The impact of mixed pathogen application in the field can also be relevant in terms of the pathogen inoculum recycling throughout the growing season, particularly in the case of multivoltine pests and pest complexes or longer living crops. After the initial round of infection, host cadavers on the plant can become a new source of inoculum that can lead to secondary cycles of transmission within the host population (Thomas et al., 1995; Hesketh and Hails, 2015). Our results showed that pathogen speed of kill and host plant were key factors in the final cadaver weight, with larger cadavers found on broccoli plants. Our results also showed that in the synchronous application of TniSNPV and *B. bassiana*, larvae which died of fungus weighed less than cadavers from any other treatment on tomato. Although the time of infection cannot be specifically determined, this suggests that on the less suitable host plant, tomato, there might be a higher cost of fighting infections when both pathogens are present (Choisy and de Roode, 2010). Alternatively, *B. bassiana*

could increase host exploitation due to pathogen competition and/or limited host resources (Cressler et al., 2014) thus killing the host more rapidly. Although there are other factors that will likely affect the production of fungal spores in the field, particularly humidity and temperature (Glare and Milner, 1991; Hajek and St. Leger, 1994), larger cadavers are likely to produce higher viral (Raymond and Hails, 2007; Tseng and Myers, 2014) or fungal (Glare and Milner, 1991; Sun et al., 2002) yield. Similarly, yield is often reduced for pathogens that kill their host at an earlier stage (Hodgson et al., 2004; Redman et al., 2016). Although mixed pathogen applications did not affect the weight of the larvae that died of viral infection compared to single TniSNPV application. When a larval cadaver liquefies, OB dispersal is limited, at least initially, and thus changes in OB yield do not necessarily directly link strongly to changes in virus transmission if a new host does not come in contact with the discrete patch of infective transmission stages (Evans and Allaway, 1983; Hesketh and Hails, 2015). It would be interesting to analyze further the impact of host plants and multiple pathogen applications in this system (yield, pathogen transmission) to better understand the impact of pathogen competition on host population dynamics and longer-term host-pathogen cycles.

Sublethal effects

Pupal weight in female Lepidoptera is strongly linked to fecundity (Milks et al., 1998), with smaller pupae more likely developing into smaller, less fecund adults (Greenberg et al., 2001). While, costs of surviving pathogen infection, including reduced female pupal mass and fecundity, have been recorded in Lepidoptera as a result of baculovirus challenge (e.g. Milks et al., 1998; Myers et al., 2000), we found no evidence of sublethal effects after being challenged by one or two pathogens, or selection for larger larvae, at least on broccoli. This suggests that there are limited trade-offs against disease resistance on the better host plant, broccoli. To determine the influence of host plants on survivors and transgenerational impacts, it would be interesting to also examine other traits, such as egg number and quality or offspring development and survival.

In conclusion, combined field application of TniSNPV and *B. bassiana* did not show an increase in efficacy against the cabbage looper larvae, particularly compared to single TniSNPV applications. A negative effect of the presence of *B. bassiana* on TniSNPV induced mortality was seen in all mixed pathogen application treatments which confirms the negative interaction observed in laboratory for this system. Although manipulating

pathogen combinations and concentrations to maximize host mortality in the field could be advantageous, the data presented here does not indicate that there is likely to be a benefit in combining TniSNPV and *B. bassiana* in term of overall larval mortality. Nevertheless, this study highlights the importance of considering crop identity in studying pathogen interaction and testing their compatibility in the field.

Chapter contributions

Pauline Deschodt and Dr. Jenny Cory conceived the idea and designed the experiment; Pauline and Brandon Dang collected the data; Dr. Jenny Cory provided funding and materials, Pauline and Dr. Jenny Cory analysed the data; Pauline drafted the chapter; Pauline and Dr. Jenny Cory edited the chapter.

5.6. References

Acevedo, J. P. M., Samuels, R. I., Machado, I. R., and Dolinski, C. (2007). Interactions between isolates of the entomopathogenic fungus Metarhizium anisopliae and the entomopathogenic nematode Heterorhabditis bacteriophora JPM4 during infection of the sugar cane borer Diatraea saccharalis (Lepidoptera: Pyralidae). *J. Invertebr. Pathol.* 96, 187–192. doi:10.1016/j.jip.2007.04.003.

Agrawal, A. A. (2000). Mechanisms, ecological consequences and agricultural implications of tri-trophic interactions. *Curr. Opin. Plant Biol.* 3, 329–335. doi:10.1016/S1369-5266(00)00089-3.

Ansari, M. A., Shah, F. A., Tirry, L., and Moens, M. (2006). Field trials against *Hoplia philanthus* (Coleoptera: Scarabaeidae) with a combination of an entomopathogenic nematode and the fungus *Metarhizium anisopliae* CLO 53. *Biological Control* 39, 453–459. doi:10.1016/j.biocontrol.2006.07.004.

Awmack, C. S., and Leather, S. R. (2002). Host plant quality and fecundity in herbivorous insects. *Annu. Rev. Entomol.* 47, 817–844. doi:10.1146/annurev.ento.47.091201.145300.

Bauer, L. S., Miller, D. L., Maddox, J. V., and McManus, M. L. (1998). Interactions between a Nosema sp. (Microspora: nosematidae) and nuclear polyhedrosis virus infecting the gypsy moth, Lymantria dispar (Lepidoptera: Lymantriidae). *J. Invertebr. Pathol.* 72, 147–153. doi:10.1006/jipa.1998.4773.

Behle, R. W. (2006). Importance of direct spray and spray residue contact for infection of *Trichoplusia ni* larvae by field applications of *Beauveria bassiana*. *J. Econ. Entomol.* 99, 1120–1128. doi:10.1603/0022-0493-99.4.1120.

Bianchi, F. J. J. A., Joosten, N. N., Vlak, J. M., and van der Werf, W. (2000). Greenhouse evaluation of dose– and time–mortality relationships of two nucleopolyhedroviruses for the control of beet armyworm, *Spodoptera exigua*, on chrysanthemum. *Biological Control* 19, 252–258. doi:10.1006/bcon.2000.0869.

Bonsall, M. B., and Benmayor, R. (2005). Multiple infections alter density dependence in host-pathogen interactions. *J. Anim. Ecology* 74, 937–945. doi:10.1111/j.1365-2656.2005.00991.x.

Brodeur, J., and Boivin, G. eds. (2006). *Trophic and guild in biological interactions control*. Dordrecht: Springer Netherlands doi:10.1007/1-4020-4767-3.

Brown GC, Prochaska GL, Hildebrand DF, Nordin GL, Jackson DM (1995) Green leaf volatiles inhibit conidial germination of the entomopathogen *Pandora neoaphidis* (Entomophthorales: Entomophthoraceae). *Environ Entomol* 24:1637–1643

Cameron, J. H., Isman, M. B., and Upadhyaya, M. K. (2007). *Trichoplusia ni* growth and preference on broccoli and eight common agricultural weeds. *Can. J. Plant Sci.* 87, 413–421. doi:10.4141/P06-032.

Cardinale, B. J., Harvey, C. T., Gross, K., and Ives, A. R. (2003). Biodiversity and biocontrol: emergent impacts of a multi-enemy assemblage on pest suppression and crop yield in an agroecosystem. *Ecol. Lett.* 6, 857–865. doi:10.1046/j.1461-0248.2003.00508.x.

Cardinale, B. J., Srivastava, D. S., Duffy, J. E., Wright, J. P., Downing, A. L., Sankaran, M., and Jouseau, C. (2006). Effects of biodiversity on the functioning of trophic groups and ecosystems. *Nature* 443, 989–992. doi:10.1038/nature05202.

Choisy, M., and de Roode, J. C. (2010). Mixed infections and the evolution of virulence: effects of resource competition, parasite plasticity, and impaired host immunity. *Am. Nat.* 175, E105-18. doi:10.1086/651587.

Coapio, G. G., Cruz-López, L., Guerenstein, P., Malo, E. A., and Rojas, J. C. (2017). Oviposition preference and larval performance and behavior of *Trichoplusia ni* (Lepidoptera: Noctuidae) on host and nonhost plants. *Arthropod Plant Interact.* 12, 1–10. doi:10.1007/s11829-017-9566-9.

Cory, J. S., and Deschodt, P. S. (2017). “The Biotic Environment,” in *Ecology of invertebrate diseases*, eds. A. E. Hajek and D. I. Shapiro-Ilan (Chichester, UK: John Wiley & Sons, Ltd), 187–212. doi:10.1002/9781119256106.ch6.

Cory, J. S., and Ericsson, J. D. (2010). Fungal entomopathogens in a tritrophic context. *Biocontrol* 55, 75–88. doi:10.1007/s10526-009-9247-4.

Cory, J. S., and Hoover, K. (2006). Plant-mediated effects in insect-pathogen interactions. *Trends Ecol. Evol.* 21, 278–286. doi:10.1016/j.tree.2006.02.005.

Costa, S. D., Barbercheck, M. E., and Kennedy, G. G. (2001). Mortality of Colorado potato beetle (*Leptinotarsa decemlineata*) after sublethal stress with the CryIIIA delta-endotoxin of *Bacillus thuringiensis* and subsequent exposure to *Beauveria bassiana. J. Invertebr. Pathol.* 77, 173–179. doi:10.1006/jipa.2001.5017.

Cressler, C. E., Nelson, W. A., Day, T., and McCauley, E. (2014). Disentangling the interaction among host resources, the immune system and pathogens. *Ecol. Lett.* 17, 284–293. doi:10.1111/ele.12229.

Denoth, M., Frid, L., and Myers, J. H. (2002). Multiple agents in biological control: improving the odds? *Biological Control* 24, 20–30. doi:10.1016/S1049-9644(02)00002-6.

Dwyer, G., Firestone, J., and Stevens, T. E. (2005). Should models of disease dynamics in herbivorous insects include the effects of variability in host-plant foliage quality? *Am. Nat.* 165, 16–31. doi:10.1086/426603.

Evans, H. F., and Allaway, G. P. (1983). Dynamics of baculovirus growth and dispersal in *Mamestra brassicae* L. (Lepidoptera: Noctuidae) larval populations introduced into small cabbage plots. *Appl. Environ. Microbiol.* 45, 493–501. doi:10.1128/aem.45.2.493-501.1983.

Fenwick, G. R., Heaney, R. K., Mullin, W. J., & VanEtten, C. H. (1983). Glucosinolates and their breakdown products in food and food plants. *CRC Critical Reviews in Food Science and Nutrition*, 18, 123-201.

Finke, D. L., and Snyder, W. E. (2010). Conserving the benefits of predator biodiversity. *Biological Conservation* 143, 2260–2269. doi:10.1016/j.biocon.2010.03.022.

Finke, D. L., and Snyder, W. E. (2008). Niche partitioning increases resource exploitation by diverse communities. *Science* 321, 1488–1490. doi:10.1126/science.1160854.

Fite, T., Tefera, T., Negeri, M., Damte, T., and Sori, W. (2020). Evaluation of *Beauveria bassiana* , *Metarhizium anisopliae* , and *Bacillus thuringiensis* for the management of *Helicoverpa armigera* (Hubner) (Lepidoptera: Noctuidae) under laboratory and field conditions. *Biocontrol Sci. Technol.* 30, 278–295. doi:10.1080/09583157.2019.1707481.

Foster, M. A., Schultz, J. C., and Hunter, M. D. (1992). Modelling Gypsy moth-virus-leaf chemistry interactions: Implications of plant quality for pest and pathogen dynamics. *The Journal of animal ecology* 61, 509. doi:10.2307/5606.

Glare, T. R., and Milner, R. J. (1991). “Ecology of entomopathogenic fungi,” in *Handbook of Applied Mycology. Volume 2: Humans, Animals, and Insects*, eds. D. K. Arora, L. Ajello, and K. G. Mukerji, 547–612.

Gonzales-Vigil, E., Bianchetti, C. M., Phillips, G. N., & Howe, G. A. (2011). Adaptive evolution of threonine deaminase in plant defense against insect herbivores. *Proceedings of the National Academy of Sciences*, 108, 5897-5902.

Greenberg, S. M., Sappington, T. W., Legaspi, B. C., Liu, T. X., and Sétamou, M. (2001). Feeding and life history of *Spodoptera exigua* (Lepidoptera: Noctuidae) on different host plants. *AN* 94, 566–575. doi:10.1603/0013-8746(2001)094[0566:FALHOS]2.0.CO;2.

Hajek, A. E., and St. Leger, R. J. (1994). Interactions between fungal pathogens and insect hosts. *Annu. Rev. Entomol.* 39, 293–322. doi:10.1146/annurev.en.39.010194.001453.

Han, G., Li, C., Liu, Q., and Xu, J. (2015). Synergistic Effect of Combining Plutella xylostella Granulovirus and Bacillus thuringiensis at Sublethal Dosages on Controlling of Diamondback Moth (Lepidoptera: Plutellidae). *J. Econ. Entomol.* 108, 2184–2191. doi:10.1093/jee/tov182.

Hauri, K. C., Glassmire, A. E., and Wetzel, W. C. (2021). Chemical diversity rather than cultivar diversity predicts natural enemy control of herbivore pests. *Ecol. Appl.*, e02289. doi:10.1002/eap.2289.

Hesketh, H., and Hails, R. S. (2015). *Bacillus thuringiensis* impacts on primary and secondary baculovirus transmission dynamics in Lepidoptera. *J. Invertebr. Pathol.* 132, 171–181. doi:10.1016/j.jip.2015.09.008.

Hodgson, D. J., Hitchman, R. B., Vanbergen, A. J., Hails, R. S., Possee, R. D., and Cory, J. S. (2004). Host ecology determines the relative fitness of virus genotypes in mixed-genotype nucleopolyhedrovirus infections. *J. Evol. Biol.* 17, 1018–1025. doi:10.1111/j.1420-9101.2004.00750.x.

Hoover, K., Stout, M. J., Alaniz, S. A., Hammock, B. D., and Duffey, S. S. (1998). Influence of induced plant defenses in cotton and tomato on the efficacy of baculoviruses on noctuid larvae. *Journal of Chemical Ecology*.

Ibrahim, S. A. M., Salem, H. H. A., and Taha, M. A. (2019). Dual application of entomopathogenic nematodes and fungi on immune and antioxidant enzymes of the greater wax moth, Galleria mellonella L. *Egypt. J. Biol. Pest Control* 29, 20. doi:10.1186/s41938-019-0125-9.

Inglis, G. D., Enkerli, J., and Goettel, M. S. (2012). “Laboratory techniques used for entomopathogenic fungi,” in *Manual of techniques in invertebrate pathology*, ed. L. A. Lacey (Elsevier), 189–253. doi:10.1016/B978-0-12-386899-2.00007-5.

Jabbour, R., Crowder, D. W., Aultman, E. A., and Snyder, W. E. (2011). Entomopathogen biodiversity increases host mortality. *Biological Control* 59, 277–283. doi:10.1016/j.biocontrol.2011.07.016.

Jafary, M., Karimzadeh, J., Farazmand, H., and Rezapanah, M. (2016). Plant-mediated vulnerability of an insect herbivore to *Bacillus thuringiensis* in a plant-herbivore-pathogen system. *Biocontrol Sci. Technol.* 26, 104–115. doi:10.1080/09583157.2015.1078872.

Janmaat, A. F., and Myers, J. H. (2005). The cost of resistance to *Bacillus thuringiensis* varies with the host plant of *Trichoplusia ni*. *Proc. Biol. Sci.* 272, 1031–1038. doi:10.1098/rspb.2004.3040.

Jaronski, S. T. (2010). Ecological factors in the inundative use of fungal entomopathogens. *Biocontrol* 55, 159–185. doi:10.1007/s10526-009-9248-3.

Jehle, J. A., Fritsch, E., Huber, J., and Backhaus, H. (2003). Intra-specific and inter-specific recombination of tortricid-specific granuloviruses during co-infection in insect larvae. *Arch. Virol.* 148, 1317–1333. doi:10.1007/s00705-003-0104-y.

Jeschke, V., Gershenzon, J., & Vassão, D. G. (2016b). Insect detoxification of glucosinolates and their hydrolysis products. *Advances in Botanical Research*, 80, 199-245.

Kassambara, A., Kosinsk, M., and Biecek, P. (2021). survminer: Drawing survival curves using 'ggplot2'. R package version 0.4.9. https://CRAN.R-project.org/package=survminer

Kennedy, G. G. (2003). Tomato, pests, parasitoids, and predators: tritrophic interactions involving the genus *Lycopersicon*. *Annual Review of Entomology*, 48, 51-72.

Kumar, S. (2017). Plant secondary metabolites (PSMs) of Brassicaceae and their role in plant defense against insect herbivores – A review. *JANS* 9, 508–519. doi:10.31018/jans.v9i1.1222.

Lacey, L. A., Grzywacz, D., Shapiro-Ilan, D. I., Frutos, R., Brownbridge, M., and Goettel, M. S. (2015). Insect pathogens as biological control agents: Back to the future. *J. Invertebr. Pathol.* 132, 1–41. doi:10.1016/j.jip.2015.07.009.

Lampert, E. (2012). Influences of plant traits on immune responses of specialist and generalist herbivores. *Insects* 3, 573–592. doi:10.3390/insects3020573.

Lecuona, R. E., and Alves, S. B. (1988). Efficiency of *Beauveria bassiana* (Bals.) Vuill., *B. brongniartii* (Sacc.) Petch. and granulose virus on *Diatraea saccharalis* (F., 1794) at different temperatures. *J. Appl. Entomology* 105, 223–228. doi:10.1111/j.1439-0418.1988.tb00181.x.

Length, R. V. (2020). Emmeans: Estimated marginal means, aka least-squares means. R package version 1.5.3. https://CRAN.R-project.org/package=emmeans

Lewis, L. (1996). Aptness of insecticides (*Bacillus thuringiensis* and carbofuran) with endophytic *Beauveria bassiana*, in suppressing larval populations of the European corn borer. *Agriculture, Ecosystems & Environment* 57, 27–34. doi:10.1016/0167-8809(95)01011-4.

Malakar, R., Elkinton, J. S., Carroll, S. D., and Amico, V. D. (1999). Interactions between two gypsy moth (Lepidoptera: Lymantriidae) pathogens : Nucleopolyhedrovirus and *Entomophaga maimaiga* (Zygomycetes: Entomophthorales): Field studies and a simulation model. *Biol Control* 16, 189–198.

Mantzoukas, S., Milonas, P., Kontodimas, D., and Angelopoulos, K. (2013). Interaction between the entomopathogenic bacterium *Bacillus thuringiensis* subsp. kurstaki and two entomopathogenic fungi in bio-control of *Sesamia nonagrioides* (Lefebvre) (Lepidoptera: Noctuidae). *Ann. Microbiol.* 63, 1083–1091. doi:10.1007/s13213-012-0565-x.

Meneses-Arias, M. G., Solis-Montero, L., Malo, E. A., & Rojas, J. C. (2020). Tomato variety affects larval survival but not female preference of the generalist moth *Trichoplusia ni*. *Entomologia Experimentalis et Applicata*, 168, 105-112.

Mwanza, P., Jukes, M., Dealtry, G., Lee, M., and Moore, S. (2021). Selection for and analysis of UV-resistant *Cryptophlebia Leucotreta* granulovirus-SA as a biopesticide for *Thaumatotibia leucotreta*. *Viruses* 14, 1–13. doi:10.3390/v14010028.

Ocampo-Hernández, J. A., Tamayo-Mejía, F., Tamez-Guerra, P., Gao, Y., and Guzmán-Franco, A. W. (2019). Different host plant species modifies the susceptibility of *Bactericera cockerelli* to the entomopathogenic fungus *Beauveria bassiana*. *J. Appl. Entomology* 143, 984–991. doi:10.1111/jen.12680.

Peter, A. J., and Shanower, T. G. (2001). Role of plant surface in resistance to insect herbivores, in insects and plant defence dynamics, ed. T. N. Ananthakrishnan (Enfield, NH, USA.: Science Publishers, Inc.), 107–132.

Pingel, R. L., and Lewis, L. C. (1997). Field application of *Bacillus thuringiensis* and *Anagrapha falcifera* multiple nucleopolyhedrovirus against the corn earworm, (Lepidoptera: Noctuidae). *J. Econ. Entomol.* 90, 1195–1199. doi:10.1093/jee/90.5.1195.

Possenti, M., Baima, S., Raffo, A., Durazzo, A., Giusti, A. M., & Natella, F. (2017). Glucosinolates in food. In Mérillon, J. -M., & Ramawat, K. G. (eds), *Glucosinolates References Series in Phytochemistry*: 87-132. New York (NY): Springer International.

Rauch, H., Steinwender, B. M., Mayerhofer, J., Sigsgaard, L., Eilenberg, J., Enkerli, J., Zelger, R., and Strasser, H. (2017). Field efficacy of *Heterorhabditis bacteriophora* (Nematoda: Heterorhabditidae), *Metarhizium brunneum* (Hypocreales: Clavicipitaceae), and chemical insecticide combinations for *Diabrotica virgifera virgifera* larval management. *Biological Control* 107, 1–10. doi:10.1016/j.biocontrol.2017.01.007.

Raymond, B., and Hails, R. S. (2007). Variation in plant resource quality and the transmission and fitness of the winter moth, *Operophtera brumata* nucleopolyhedrovirus. *Biological Control* 41, 237–245. doi:10.1016/j.biocontrol.2007.02.005.

Redman, E. M., Wilson, K., and Cory, J. S. (2016). Trade-offs and mixed infections in an obligate-killing insect pathogen. *J. Anim. Ecol.* 85, 1200–1209. doi:10.1111/1365-2656.12547.

Rosenheim, J. A. (1995). Intraguild predation amoing biological- control agents: Theory and evidence. *Biol Control* 5, 303–335.

Roy, H. E., Steinkraus, D. C., Eilenberg, J., Hajek, A. E., and Pell, J. K. (2006). Bizarre interactions and endgames: entomopathogenic fungi and their arthropod hosts. *Annu. Rev. Entomol.* 51, 331–357. doi:10.1146/annurev.ento.51.110104.150941.

Sarfraz, R. M., Cervantes, V., and Myers, J. H. (2011). The effect of host plant species on performance and movement behaviour of the cabbage looper *Trichoplusia ni* and their potential influences on infection by *Autographa californica* multiple nucleopolyhedrovirus. *Agric. For. Entomol.* 13, 157–164. doi:10.1111/j.1461-9563.2010.00504.x.

Sayed, A. M. M., and Behle, R. W. (2017a). Comparing formulations for a mixed-microbial biopesticide with *Bacillus thuringiensis* var.*kurstaki* and *Beauveria bassiana* blastospores. *Archives Of Phytopathology And Plant Protection* 50, 745–760. doi:10.1080/03235408.2017.1372942.

Sayed, A. M. M., and Behle, R. W. (2017b). Evaluating a dual microbial agent biopesticide with *Bacillus thuringiensis* var. *kurstaki* and *Beauveria bassiana* blastospores . *Biocontrol Sci. Technol.* 27, 461–474. doi:10.1080/09583157.2017.1303662.

Shapiro, M. (2000). Effect of two granulosis viruses on the activity of the gypsy moth (Lepidoptera: Lymantriidae) nuclear polyhedrosis virus. *J. Econ. Entomol.* 93, 1633–1637. doi:10.1603/0022-0493-93.6.1633.

Shikano, I., Akhtar, Y., and Isman, M. B. (2010). Relationship between adult and larval host plant selection and larval performance in the generalist moth, *Trichoplusia ni*. *Arthropod Plant Interact.* 4, 197–205. doi:10.1007/s11829-010-9100-9.

Shikano, I., McCarthy, E., Hayes-Plazolles, N., Slavicek, J. M., and Hoover, K. (2018). Jasmonic acid-induced plant defenses delay caterpillar developmental resistance to a baculovirus: Slow-growth, high-mortality hypothesis in plant-insect-pathogen interactions. *J. Invertebr. Pathol.* 158, 16–23. doi:10.1016/j.jip.2018.09.001.

Shikano, I. (2017). Evolutionary ecology of multitrophic interactions between plants, insect herbivores and entomopathogens. *J. Chem. Ecol.* 43, 586–598. doi:10.1007/s10886-017-0850-z.

Souza, M. L., Sanches, M. M., Souza, D. A. de, Faria, M., Espinel-Correal, C., Sihler, W., and Lopes, R. B. (2019). Within-host interactions of *Metarhizium rileyi* strains and nucleopolyhedroviruses in *Spodoptera frugiperda* and *Anticarsia gemmatalis* (Lepidoptera: Noctuidae). *J. Invertebr. Pathol.* 162, 10–18. doi:10.1016/j.jip.2019.01.006.

Sukirno, S., Tufail, M., Rasool, K. G., Salamouny, S. E., Sutanto, K. D., and Aldawood, bdulrahman S. (2018). The efficacy and persistence of *Spodoptera littoralis* nucleopolyhedrovirus (SpliMNPV) applied in UV protectants against the beet armyworm, *Spodoptera exigua* (Hubner) (Lepidoptera: Noctuidae) under Saudi field conditions. *Pak. J. Zool.* 50. doi:10.17582/journal.pjz/2018.50.5.1895.1902.

Sun, J., Fuxa, J. R., and Henderson, G. (2002). Sporulation of *Metarhizium anisopliae* and *Beauveria bassiana* on *Coptotermes formosanus* and in vitro. *Journal of invertebrate pathology* 81, 78–85. doi:10.1016/S0022-2011(02)00152-0.

Sutanto, K. D., Husain, M., Rasool, K. G., Malik, A. F., Al-Qahtani, W. H., and Aldawood, A. S. (2022). Persistency of indigenous and exotic entomopathogenic fungi isolates under Ultraviolet B (UV-B) irradiation to enhance field application efficacy and obtain sustainable control of the red palm weevil. *Insects* 13, 1–13. doi:10.3390/insects13010103.

Therneau, T. (2020). A Package for survival analysis in R. R package version 3.2-7, URL:https://CRAN.R-project.org/package=survival.

Thomas, M. B., Wood, S. N., and Lomer, C. J. (1995). Biological control of locusts and grasshoppers using a fungal pathogen: the importance of secondary cycling. *Proc. R. Soc. Lond. B* 259, 265–270. doi:10.1098/rspb.1995.0039.

Tian, D., Tooker, J., Peiffer, M., Chung, S. H., and Felton, G. W. (2012). Role of trichomes in defense against herbivores: comparison of herbivore response to woolly and hairless trichome mutants in tomato (Solanum lycopersicum). *Planta* 236, 1053–1066. doi:10.1007/s00425-012-1651-9.

Tseng, M., and Myers, J. H. (2014). The relationship between parasite fitness and host condition in an insect--virus system. *PLoS ONE* 9, e106401. doi:10.1371/journal.pone.0106401.

Williams, C. D., Dillon, A. B., Harvey, C. D., Hennessy, R., Namara, L. M., and Griffin, C. T. (2013). Control of a major pest of forestry, *Hylobius abietis*, with entomopathogenic nematodes and fungi using eradicant and prophylactic strategies. *Forest Ecology and Management* 305, 212–222. doi:10.1016/j.foreco.2013.05.055.

Wraight, S. P., and Ramos, M. E. (2005). Synergistic interaction between *Beauveria bassiana*- and *Bacillus thuringiensis tenebrionis*-based biopesticides applied against field populations of Colorado potato beetle larvae. *J. Invertebr. Pathol.* 90, 139–150. doi:10.1016/j.jip.2005.09.005.

Chapter 6.

Conclusion

Pathogens are rarely found in isolation in a host. However, invertebrate pathology has traditionally focused on single host-single pathogen interactions on a limited number of pathogen and/or host species and under controlled environmental conditions. In this thesis, I investigated the effect of host nutrition on the interaction between two pathogens of the cabbage looper, *Trichoplusia ni*, the baculovirus, *T. ni* single nucleopolyhedrovirus (TniSNPV) and the entomopathogenic fungus, *Beauveria bassiana*. I started by examining the effect of dietary dilution and diet quality (Protein-to-Carbohydrate ratio) on the outcome of mixed pathogen infection on both host fitness, in terms of mortality and pupal weight, as well as pathogen fitness, analysing the speed of kill, pathogen production of transmission stages (yield) and virus virulence. I then assessed the effect of natural resources (host plant) and priority effects on the outcome of mixed infection. Lastly, I investigated the potential of combining both pathogens in the field to manage *T. ni* populations. Due to its rapid speed of kill, *B. bassiana* was overall the dominant pathogen in the system and this was shown by the negative effect of the presence of the fungus on virus-specific mortality in the mixed infection treatments. As the host represents a limited resource environment for co-infecting pathogens, we expected to observe higher or faster mortality in the mixed infection treatments. However, the outcome of mixed pathogen infections, both in terms of host mortality and within-host pathogen competition, was difficult to predict and was dependent on the timing of pathogen arrival, host resources and pathogen infection dose. It is clear from the results that more experimental data, including a broader range of pathogen species and host conditions, are needed to be able to better understand disease severity and build a predictive framework that would be necessary to improve the use of multiple biological control agents in the field.

6.1. Summary

Host resources play an important role in host-pathogen interactions (Smith, 2007; Cressler et al., 2014; Lange et al., 2014; Cotter and Al Shareefi, 2022). Resource quality and quantity can be highly variable in the environment and both the overall access or availability of resources (quantity) along with the actual ratios of macronutrients (quality)

have been identified to be important in insect growth, development, or survival (Krittika et al., 2020; Lee et al., 2008; Vanni and Lampert 1992). When provided with the opportunity to make choices insects have been shown to choose specific protein-to-carbohydrate ratios or combinations to balance their intakes and maximise their development or survival (Lee et al., 2006; Povey et al., 2014). When insects are not able to balance their intake of macronutrients, their response to pathogen infection can be affected (Lee et al., 2006; Gómez-Moracho et al 2021; Ponton et al., 2019). However, little is understood about whether resources matter in the context of mixed pathogen infections. In both chapters 2 and 3, varying the quantity or quality of artificial diet after pathogen challenge did not affect the overall mortality. Although several studies have shown the negative effect of resource dilution or the effect of varying protein-to-carbohydrate ratios on survival to pathogen challenge, many studies have focused on more extreme scenarios. One important aspect to consider is the timing of dietary change provided to the host. Here, the larvae were reared under similar conditions until pathogen challenge. Hence the overall nutritional status of each larva was comparable until infection and the experiment focused on the direct effects of resources on both host survival and pathogen replication. In the wild, changes in food availability or quality may happen at any time, which could change host condition prior to infection or increase the likelihood of infection by increasing developmental time.

Resources can affect the strength and rapidity of the deployment of the host immune response to infection (Pedersen and Fenton, 2007; Cotter and Al Shareefi, 2022) but this is also shared between competing pathogens. The outcome of mixed pathogen infection will be linked to how both the host and pathogen utilize shared resources (Cressler et al., 2014). Understanding what resources are needed by competing pathogens inside the host will help elucidate pathogen interactions and enable us to better predict the outcome of infections. On diluted diet, in chapter 2, the yield of both pathogens was not affected by either the competition with the other pathogen or the dilution of the diet. However, both pathogens responded differently to changes in diet quality. Indeed, when using unbalanced diets (P:C ratios) in Chapter 3, *B. bassiana* spore production was not affected by the diet, but fungus yield increased when co-infected with TniSNPV at low dose. On the other hand, virus yield decreased with increasing protein in the diet but did not change in co-infections. Interestingly, only one of the two pathogens was able to release significant numbers of infective propagules at the end of each successful infection.

This suggests that there is competition between the pathogens, but it happens early in the infection cycle and that the pathogen that produced transmission stages was able to exclude the other. TniSNPV and *B. bassiana* have very different life cycles, including the initiation of infection, transmission stages production as well as transmission strategies (Volkman, 1997; Butt et al., 2016). Hence, it is not surprising that the speed of kill and production of transmission stages responded differently to changes in host nutritional status.

Another important factor driving the outcome of pathogen competition is the timing of infection and which pathogen arrives before the other (Clay et al 2020). A pathogen infecting the host first might be expected to have a competitive advantage over pathogens introduced later (e.g. Pilarska et al., 2006; Zilio and Koella, 2020), although this is not always the case (e.g. Lohr et al., 2010; Manzi et al., 2021). Moreover, prior infection can weaken or exhaust the host immune response, facilitating the introduction of a second pathogen or alternatively, prior infection can trigger host defences that will prevent subsequent infections (Clay et al., 2019). In both chapters 4 and 5, pathogens were introduced either synchronously or one pathogen before the other. The effect of the timing of infection on the outcome of mixed infection was dependent on the pathogen species, but also the host plant provided after pathogen challenge. In chapter 4, the timing of pathogen arrival seemed to be more important on the better host plant (broccoli). Indeed, differences between co-infection treatments varied greatly when the larvae were fed broccoli compared to those fed cucumber leaves. As host susceptibility to pathogen infection varies with the host plant identity and timing of pathogen arrival, these results are particularly important as both pathogens can be used as microbial control agents. Hence their use and potential combination in the field need to be evaluated taking into account host plant, as well as timing of application to increase pest control and limit pathogen competition. Unfortunately, in chapter 5, TniSNPV field application induced a high level of larval mortality which did not enable us to fully evaluate the interaction between *B. bassiana* and TniSNPV in spray applications. However, these results showed that the likelihood of a pest being infected is highly dependent on the crop identity and the pathogen infection pathway, giving further insight into the complex interactions that can modulate pathogen efficacy in the field.

Finally, it is essential to better understand the long-term effect of pathogen competition on both host and pathogen evolution. Different pathogen species cause

different degrees of harm to their hosts, defined as virulence. The prevailing theory is that the evolution of pathogen virulence is driven by a trade-off between the replication within the host and transmission potential (Alizon et al., 2009), although the trade-off hypothesis has been challenged (Brown et al., 2006). Mixed infections are predicted to increase virulence (de Roode et al., 2005; Bell et al., 2006), thus, understanding how multiple species interact within a host and how this affects population dynamics and host-pathogen evolution are crucial issues in disease ecology and evolution. Yet, little is understood about the process underlying the evolution of genetic diversity in pathogen populations and there are few empirical data on the long-term evolution of pathogen virulence in the context of pathogen co-infection. Both pathogens are obligate killers, meaning that they need to kill their host to release (virus) or even produce (fungus) infectious propagules. In chapter 3, I focused on viral occlusion body production and virus virulence after a single passage in 4th instar *T. ni* larvae. I did observe an overall increase in virulence between the initial infection and the passaged virus experiment, but this was not clearly related to the diet or co-infection treatment. Co-infection with the fungus, balance of macronutrients in the diet and initial virus dose altered both the overall OB size and virus virulence. Interestingly, differences in OB size and virulence were mainly found on high protein diet and for larvae inoculated with a high virus dose (LD75 for 4th instar *T. ni* larvae). This suggests that at the highest dose competition for resources between the virus and the host might be greater. Moreover, we can assume that TniSNPV was a stronger competitor when inoculated at the highest dose and thus within-host competition between TniSNPV and *B. bassiana* can be expected to have a stronger influence on OB morphology and virus virulence. However, the interactions between co-infection, virus dose and diet quality were complex and alteration in OB volume was not shown to be directly linked to variation in virulence.

6.2. Future directions

The complexity of host nutritional ecology combined with variation in species identity, the size of the pathogen challenge and timing and order of infection, makes studying co-infections challenging. To improve our understanding of within-host pathogen interactions, studies have used ecological theory (Graham, 2008; Mideo, 2009; Seabloom et al., 2015). After conducting a meta-analysis on more than 50 experiments on mice and helminth co-infection, Graham (2008) observed that it is possible to explain different

outcomes in parasite interactions using bottom-up or top-down ecological processes. Similarly, in a review, Mideo (2009) divided within-host competitions into three categories based on ecological principles: exploitation competition (competition to access shared host resources), immune-mediated competition (competition to escape the host immune response) and interference competition (competition between species competitors within the host). Our ability to better predict the outcome of mixed pathogen infections is dependent on our understanding of context-dependent host- and pathogen-pathogen interactions. Studies addressing mixed-pathogen infections in insects have generated mixed results regarding the general outcome of the interaction, mainly focusing on overall host mortality and speed of kill. Moreover, from the pathogen's perspective, the main challenge is to be able to successfully invade the host, produce and release transmission stages. However, the effects of within-host pathogen competition on pathogen fitness are rarely evaluated.

A number of experimental studies have evaluated the connection between host resources and host response to single pathogen infection, mainly in terms of host mortality and pathogen speed of kill (Huber et al., 2012; Budischak and Cressler, 2018). The host requires resources to reproduce and respond to pathogen infection as mounting an immune response can be costly (Schmid-Hempel, 2005). On the other hand, infecting pathogens also requires resources to reproduce. Hence, how both host and pathogen interact with the resource supply is likely to determine whether an increase in host resources is likely to increase, decrease or not affect pathogen within-host replication (Cressler et al., 2014). Interestingly, when considering a broad range of different taxa, changes in host nutrition both in terms of quality and quantity appear to have limited effects on host mortality in single pathogen infections (Pike et al., 2019). Although as the authors suggest, it is unlikely that changes in resources affect both vertebrate and invertebrate hosts in a similar way as the vertebrate immune response is more complex and likely to require higher energy resources. Indeed, six out of the seven studies on invertebrates included in the meta-analysis suggest that better host nutrition will be favourable for pathogen fitness in contrast to studies analysing vertebrate-pathogen interactions. However, there is still a large discrepancy between studies analysing the effect of host resources on mixed pathogen infection outcome, including the study system (4 out of 6 used in Pike et al., 2019 involved *Daphnia sp.*), the level of the study (individual host vs populations), along with the type of resource provided (e.g. quality vs quantity, artificial

diet vs natural resources) which reduced our ability to draw general conclusions on the relationship between host resources and the outcome of pathogen infection. Moreover, within the same seven studies on invertebrate in the review, the hosts were introduced to the different food treatments before pathogen challenge in three of the studies, whereas the different food treatments were provided after pathogen challenge in three other studies, making it difficult to compare the outcome between the studies. Indeed, specific effects of host resources are harder to unravel as host exposure to diet treatments prior to pathogen challenge is likely to affect the host's overall conditions and response to pathogen infection. A follow-up analysis should take into account timing of infection and details on the diet treatments when comparing the effects of host resources on host and pathogen fitness in single pathogen infections. Since studies analysing the effect of host resources on the outcome of mixed infection in invertebrates are still limited, more experimental data are needed to evaluate the extent of the role of host nutrition in mixed pathogen infection and the effect on pathogen fitness in different systems. Nevertheless, in the future, both the effect of mixed infections on the host as well as pathogen fitness should be evaluated whenever it is possible to fully comprehend the host-pathogen dynamics and facilitate the design of integrated pest management strategies.

Pathogen survival in the wild depends mainly on its ability to persist in the environment, to be transmitted to a new host, and the host availability in the community (Begon et al., 1992). In addition, the species traits that might make a pathogen a good competitor within the host might not be the same as those that are needed for successful transmission within the host population (Alizon et al., 2013). Both TniSNPV and *B. bassiana* are primarily horizontally transmitted via OBs and spores respectively. To be able to predict the effect of species interactions on disease transmission, it is necessary to define the structure and dynamics of the community (Hajek et al., 2015; Hicks et al., 2015) as well as the role of host plant identity and host nutrition. Given the current use of pathogen in insect pest management strategies, it is also necessary to assess the factors affecting the outcome of pathogen interactions at both within and between-host levels and establish frameworks to help us better predict biological control success. An interesting follow-up study would be to determine how mixed infection affects both pathogen transmission in the field and whether host plant identity impacts the transmission of competing pathogen within the host population.

6.3. Concluding remarks

This thesis highlights the key role that host nutrition can play in pathogen-pathogen interactions. I found that resource quality, in particular, is important in driving the outcome of mixed infections. In addition, host plant identity is a key factor that needs to be taken into account when examining the efficacy of microbial biological control agents, even more so, when trying to combine multiple different species of microbial control agents to increase the efficacy of pest management strategies.

6.4. References

Alizon, S., Hurford, A., Mideo, N., and Van Baalen, M. (2009). Virulence evolution and the trade-off hypothesis: history, current state of affairs and the future. *J. Evol. Biol*. 22, 245–259. doi:10.1111/j.1420-9101.2008.01658.x.

Alizon, S., de Roode, J. C., and Michalakis, Y. (2013). Multiple infections and the evolution of virulence. *Ecol. Lett.* 16, 556–567. doi:10.1111/ele.12076.

Begon, M., Bowers, R.G., Kadianakis, N., and Hodgkinson, D.E. (1992). Disease and community structure: the importance of host self-regulation in a host-host-pathogen model. *Am. Nat.* 1131–1150.

Bell, A. S., De Roode, J. C., Sim, D., and Read, A. F. (2006). Within-host competition in genetically diverse malaria infections: parasite virulence and competitive success. *Evolution*, 60(7), 1358-1371.

Brown, N. F., Wickham, M. E., Coombes, B. K., and Finlay, B. B. (2006). Crossing the line: selection and evolution of virulence traits. *PLoS Pathog*, 2(5), e42.

Budischak, S. A., and Cressler, C. E. (2018). Fueling defense: effects of resources on the ecology and evolution of tolerance to parasite infection. *Front. Immunol*. 9, 2453. doi:10.3389/fimmu.2018.02453.

Clay, P. A., Duffy, M. A., and Rudolf, V. H. W. (2020). Within-host priority effects and epidemic timing determine outbreak severity in co-infected populations. *Proc. Biol. Sci*. 287, 20200046. doi:10.1098/rspb.2020.0046.

Clay, P. A., and Rudolf, V. H. W. (2019). How parasite interaction strategies alter virulence evolution in multi-parasite communities. *Evolution* 73, 2189–2203. doi:10.1111/evo.13843.

Cressler, C. E., Nelson, W. A., Day, T., and McCauley, E. (2014). Disentangling the interaction among host resources, the immune system and pathogens. *Ecol. Lett.* 17, 284–293. doi:10.1111/ele.12229.

Cotter, S. C., and Al Shareefi, E. (2022). Nutritional ecology, infection and immune defence - exploring the mechanisms. *Curr. Opin. Insect Sci.* 49, xx-yy. doi:10.1016/j.cois.2021.12.002.

de Roode, J. C., Pansini, R., Cheesman, S. J., Helinski, M. E., Huijben, S., Wargo, A. R., and Read, A. F. (2005). Virulence and competitive ability in genetically diverse malaria infections. *Proceedings of the National Academy of Sciences of the United States of America*, 102(21), 7624-7628.

Gómez-Moracho, T., Durand, T., Pasquaretta, C., Heeb, P., and Lihoreau, M. (2021). Artificial diets modulate infection rates by *Nosema ceranae* in bumblebees. *Microorganisms* 9. doi:10.3390/microorganisms9010158.

Graham, A.L., (2008). Ecological rules governing helminth-¬microparasite coinfection. *PNAS* 105, 566-570.

Hajek, A.E., Tobin, P.C., and Haynes, K.J. (2015). Replacement of a dominant viral pathogen by a fungal pathogen does not alter the collapse of a regional forest insect outbreak. *Oecologia* 177, 785--797.

Hicks, J. P., Hails, R. S., and Sait, S. M. (2015). Scale-dependent, contrasting effects of habitat fragmentation on host-natural enemy trophic interactions. *Landscape ecology*, 30(8), 1371-1385.

Huber, D., Römheld, V., and Weinmann, M. (2012). and Relationship between Nutrition, Plant Diseases and Pests," in Marschner's mineral nutrition of higher plants (*Elsevier*), 283–298. doi:10.1016/B978-0-12-384905-2.00010-8.

Krittika, S., and Yadav, P. (2020). Dietary protein restriction deciphers new relationships between lifespan, fecundity and activity levels in fruit flies *Drosophila melanogaster*. *Sci. Rep.* 10, 1–8. doi:10.1038/s41598-020-66372-4.

Lange, B., Reuter, M., Ebert, D., Muylaert, K., and Decaestecker, E. (2014). Diet quality determines interspecific parasite interactions in host populations. *Ecol. Evol*. 4, 3093–3102. doi:10.1002/ece3.1167.

Lee, K. P., Simpson, S. J., and Wilson, K. (2008). Dietary protein-quality influences melanization and immune function in an insect. *Funct. Ecol.* 22, 1052–1061. doi:10.1111/j.1365-2435.2008.01459.x.

Lee, K., Cory, J., Wilson, K., Raubenheimer, D., and Simpson, S. (2006). Flexible diet choice offsets protein costs of pathogen resistance in a caterpillar. *Proc. R. Soc. Lond. Biol*. 273, 823-829.

Lohr, J. N., Yin, M., and Wolinska, J. (2010). Prior residency does not always pay off-co-infections in *Daphnia*. *Parasitology* 137, 1493–1500. doi:10.1017/S0031182010000296.

Manzi, F., Halle, S., Seemann, L., Ben-Ami, F., and Wolinska, J. (2021). Sequential infection of *Daphnia magna* by a gut microsporidium followed by a haemolymph yeast decreases transmission of both parasites. *Parasitology*, 1–12. doi:10.1017/S0031182021001384.

Mideo, N., 2009. Parasite adaptations to within host competition. *Trends Parasitol*. 25, 261-268.

Pedersen, A. B., and Fenton, A. (2007). Emphasizing the ecology in parasite community ecology. *Trends Ecol. Evol.* 22, 133–139. doi:10.1016/j.tree.2006.11.005.

Pike, V. L., Lythgoe, K. A., and King, K. C. (2019). On the diverse and opposing effects of nutrition on pathogen virulence. *Proc. Biol. Sci.* 286, 20191220. doi:10.1098/rspb.2019.1220.

Pilarska, D. K., Solter, L. F., Kereselidze, M., Linde, A., and Hoch, G. (2006). Microsporidian infections in *Lymantria dispar* larvae: interactions and effects of multiple species infections on pathogen horizontal transmission. *J. Invertebr. Pathol.* 93, 105–113. doi:10.1016/j.jip.2006.05.003.

Ponton, F., Morimoto, J., Robinson, K., Kumar, S. S., Cotter, S. C., Wilson, K., and Simpson, S. J. (2019). Macronutrients modulate survival to infection and immunity in *Drosophila*. *J. Anim. Ecol*. 00, 1–11. doi:10.1111/1365-2656.13126.

Povey, S., Cotter, S. C., Simpson, S. J., and Wilson, K. (2014). Dynamics of macronutrient self-medication and illness-induced anorexia in virally infected insects. *Journal of Animal Ecology*, 83(1), 245-255.

Schmid-Hempel, P. (2005). Evolutionary ecology of insect immune defenses. *Annu. Rev. Entomol.* 50, 529–551. doi:10.1146/annurev.ento.50.071803.130420

Seabloom, E. W., Borer, E. T., Gross, K., Kendig, A. E., Lacroix, C., Mitchell, C. E., Mordecai, E. A., and Power, A. G. (2015). The community ecology of pathogens: coinfection, coexistence and community composition. Ecol. Lett. 18, 401–415. doi:10.1111/ele.12418.

Smith, V. (2007). Host resource supplies influence the dynamics and outcome of infectious disease. *Integr. Comp. Biol.* 47, 310–316. doi:10.1093/icb/icm006.

Vanni, M. J., and Lampert, W. (1992). Food quality effects on life history traits and fitness in the generalist herbivore *Daphnia*. *Oecologia* 92, 48–57. doi:10.1007/BF00317261.

Zilio, G., and Koella, J. C. (2020). Sequential co-infections drive parasite competition and the outcome of infection. *J. Anim. Ecol.* 89, 2367–2377. doi:10.1111/1365-2656.13302.

Appendix A.

Effect of increase protein on virus-induced mortality

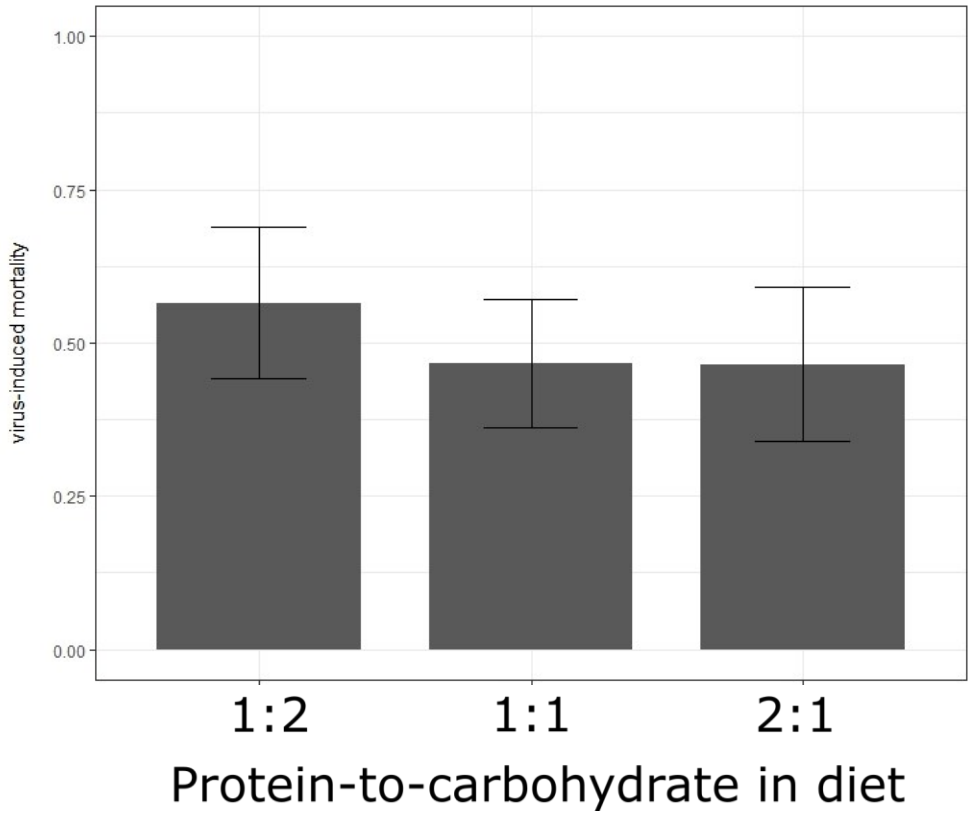

Figure A1: **Virus-induced mortality of fourth instar *T. ni* larvae (mean ± s.e.) fed on different quality diets (P:C ratios).**

Appendix B

Larval survival and host plant identity

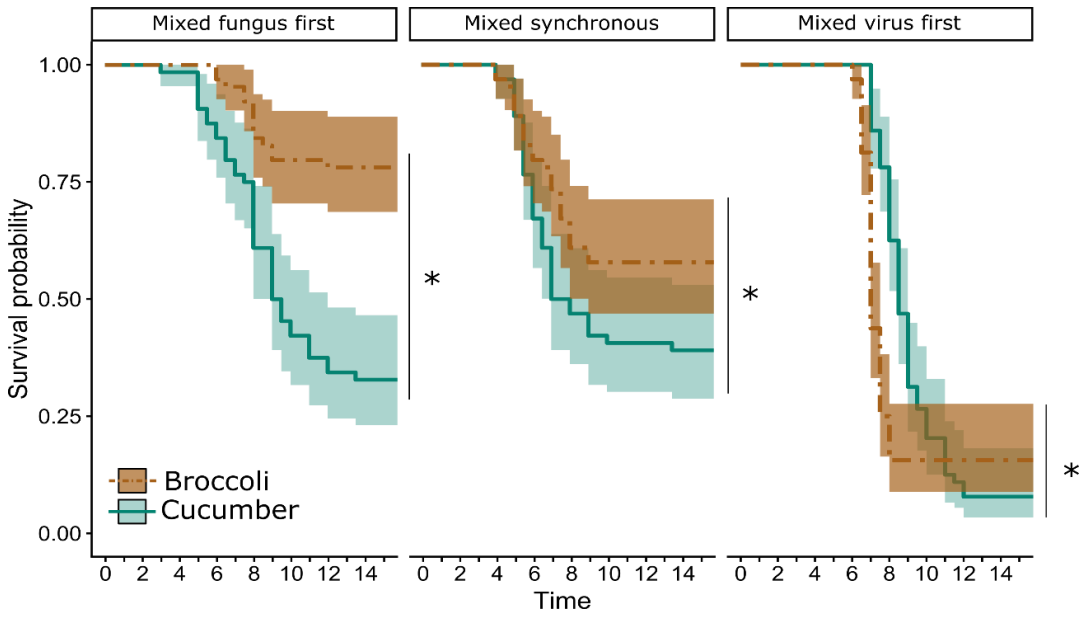

Figure B1: **Kaplan-Meier survival plot (with 95% confidence intervals) for 4th instar *T. ni* larvae challenged with TniSNPV (500 OBs) and *B. bassiana* (20,000 spores) either synchronously or sequentially and fed broccoli or cucumber leaves post-pathogen challenge. Stars show significant differences between each host plant ($p<0.05$).**

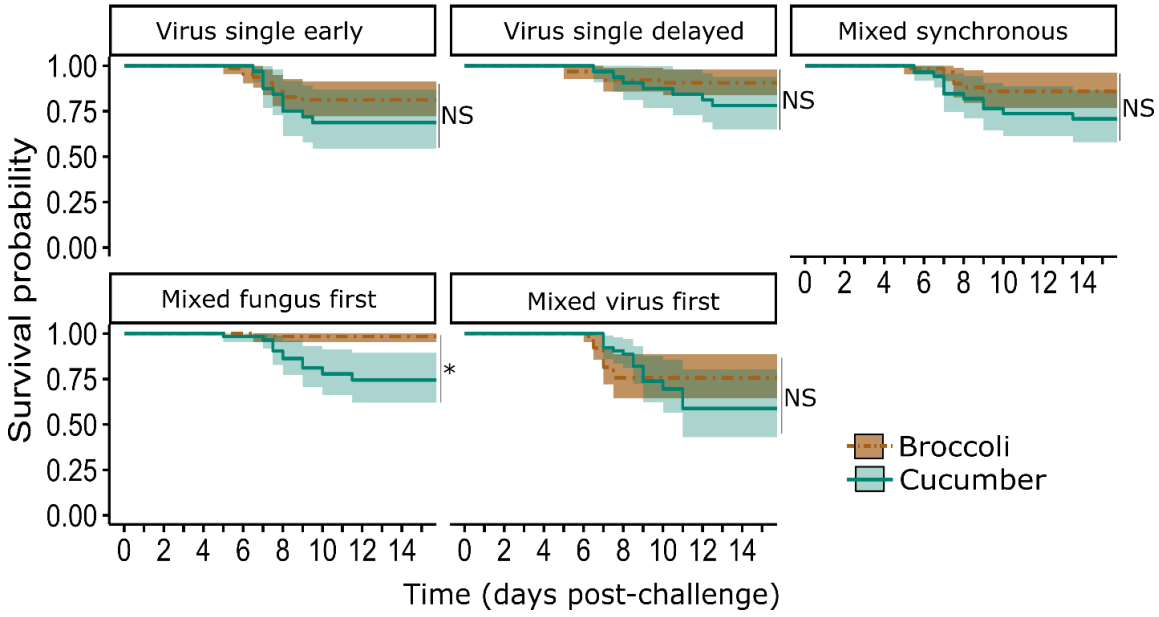

Figure B2: **Kaplan-Meier survival plot (with 95% confidence intervals) for 4th instar *T. ni* larvae that died of viral infection after being challenged with either a single dose of TniSNPV (500 OBs) or both TniSNPV *and B. bassiana* (20,000 spores) and fed broccoli or cucumber leaves post-pathogen challenge. Stars show significant differences between each host plant ($p<0.05$).**

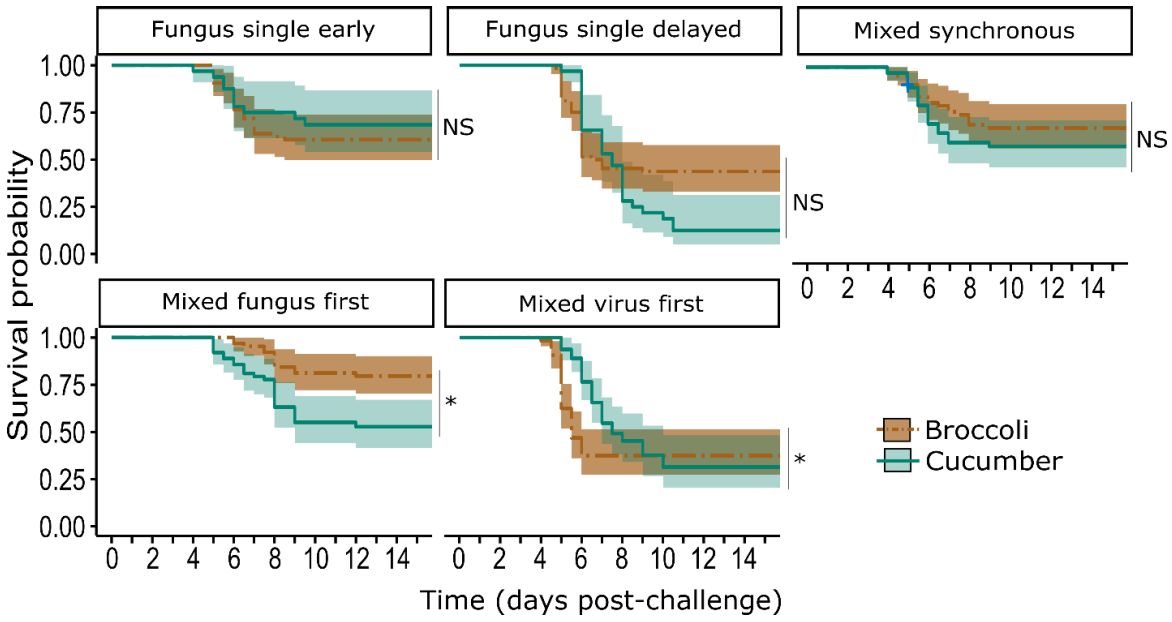

Figure B3: **Kaplan-Meier survival plot (with 95% confidence intervals) for 4th instar *T. ni* larvae that died of fungal infection after being challenged with either a single dose of TniSNPV (500 OBs) or both TniSNPV *and B. bassiana* (20,000 spores) and fed broccoli or cucumber leaves post-pathogen challenge. Stars show significant differences between each host plant ($p<0.05$).**