HELICOVERPA ZEA (BODDIE) (LEPIDOPTERA: NOCTUIDAE) IMMUNE SYSTEM RESPONSES TO VARIATION IN NUTRITIONAL AND PLANT DEFENSIVE ENVIRONMENTS

A Dissertation

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ABSTRACT

Insects challenged with a pathogen must respond with the appropriate level and type of immune response to maximize the likelihood of survival. They exhibit behavioral defenses, as well as humoral and cellular defenses, which are regulated by signal induction pathways. Extensive research has been done to understand the signaling pathways that elicit different immune responses; however, most of the research has been conducted in model organisms. Little is known about the immune system of *Helicoverpa zea*, possibly the most important crop pest in the New World. Even less is known about the nutritional behavioral changes within this species, or the effects of inducible plant defenses on altering pathogen susceptibility in *H. zea*. The first objective was to assess variation in the immune response of *H. zea* to four different entomopathogenic microbes throughout the infection cycle. The immune response differed based on pathogen type and time post-inoculation. *Bacillus thuringiensis* bacteria induced the strongest immune response, upregulating *Relish*, the transcription factor for the IMD pathway. The second objective was to determine whether nutritional variation affected the immune response to *Helicoverpa armigera* nucleopolyhedrovirus (HearNPV). Insects have been found to alter their intake of proteins and carbohydrates to off-set costs associated with activating immunity, while others have been shown to actively self-medicate to survive the infection. However, in this study *H. zea* did not exhibit compensatory feeding or self-medication; rather the pathogen could be manipulating the host. The final objective was to understand the effects plant defenses have on HearNPV virulence in previously-

infected *H. zea* larvae. Plant defenses had an effect on healthy larvae, while infected larvae were not affected. Control larvae reared on plants with induced systemic resistance (ISR) upregulated did not survive as well as larvae on Control plants or plants with systemic acquired resistance (SAR) upregulated. Together, these data illuminate how *H. zea* responds to pathogenic infections and the constraints imposed by nutritional and plant defensive environments.

DEDICATION

To my wife, Jessica and kids, Arabella, Thaddeus, and Ezra.

CONTRIBUTORS AND FUNDING SOURCES

Contributors

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CHAPTER I

INTRODUCTION AND LITERATURE REVIEW

Introduction

Helicoverpa zea (Boddie) (Lepidoptera: Noctuidae) is one of the most important crop pests in the New World, feeding on a number of wild and cultivated hosts such as cotton, corn, soybean, and sorghum (Quaintance and Brues 1905; Fitt 1989; Musser et al. 2018; Cook 2018). In current commercial agriculture growers have a limited number of options to control this pest. They can either apply an expensive insecticide such as a diamide, plant transgenic crops with effective *Bt* technology, or apply a relatively inexpensive, naturally occurring, host specific biopesticide such as nucleopolyhedrovirus. The use of entomopathogens in agriculture has always been a rarely utilized control strategy due to entomopathogens not providing complete control like synthetic insecticides or *Bt* technology. Most entomopathogens are only effective in the proper environment, with the proper host, don't have a substantial residual period, and can be highly variable in efficacy across crops and cultivars. Due to these inconsistencies most growers and consultants prefer synthetic insecticides that have a known activity period (residual time), are considerably more broad-spectrum, and have historically been cheaper. When possible, growers also often rely on transgenic crops for insect management. However, such current tactics are progressing further away from integrated pest management (Peterson et al. 2018).

Insect Pathogens

In the broadest sense, an insect pathogen is the causal agent of disease within an insect. Under this broad definition, causal agents could include abiotic factors such as environment and a changing climate. However, for the purposes of this review, insect pathogens will be limited strictly to biotic causal agents such as viruses, fungi, bacteria, nematodes and microsporidia. Of these five major pathogen groups we will further exclude microsporidia mainly due to the lack of commercially available formulations of microsporidia (Mitchell and Cali 1994; Solter and Becnel 2007). Thus, remaining are viral, fungal, bacterial, and nematode pathogens. These four groups of pathogens are morphologically and evolutionarily different; however, they all undergo a similar general pathogenic life cycle.

All pathogens, to varying degrees, must be capable of surviving environmental conditions outside the host for a period of time (this is not necessarily true for latent vertically transmitted pathogens that might never leave the host cadaver, but in general it is true especially for non-vertically transmitted pathogens). All pathogens must be able to invade the host tissue, successfully overcome or avoid the host defense system, colonize and disseminate within the host, and reproduce or replicate before releasing progeny back into the environment or into subsequent generations depending on the means of transmission. Pathogens rely on either vertical or horizontal transmission to infect a subsequent host. Vertical transmission is the transmission of a pathogen from parent to offspring and can occur either trans-ovum or trans-ovarian. Trans-ovum transmission is when the pathogen infects the egg surface and the neonate becomes

infected upon ingestion of the eggshell during emergence. Trans-ovarian transmission is when the pathogen is able to infect the embryonic cells prior to the egg formation. Horizontal transmission is movement of a pathogen from an infected host to an uninfected host. This process can occur via mechanical movement, abiotic transmission, vector transmission, or by movement via carriers, both voluntary and involuntary (Black et al. 2019).

Insect Viruses

Viruses in general are a relatively new discovery in biological sciences. Prior to Pasteur, Koch, and Bassi developing and proving Germ Theory, and the development of high-magnification microscopes, pathogens and especially viruses were not known. Viruses in insects were first described based on the disease they caused and have only recently begun to be described based on an actual classification system. The first insect virus described caused jaundice in the silkworm, *Bombyx mori*, an economically important insect used to produce silks (Nysten 1808). Since then, with the continual advancement of microscopes and the advent of genetic tools we have discovered and reported on over 1,100 viruses capable of affecting invertebrates with the majority capable of infecting insects (Adams 1991). The bulk of these described insect viruses have been classified to a single viral family, Baculoviridae. This family of viruses contain protective protein crystalline matrices termed occlusion bodies (OBs) or inclusion bodies (IBs) around the virion (Herniou et al. 2012). A virion consists of a nucleocapsid and, in the case of enveloped viruses, the outer layer of glycoproteins that comprise an envelope. The nucleocapsid consists of the viral genome and any structural

proteins or enzymes unique to the virus. The OB provides limited protection from harsh environmental conditions that could sterilize or inactivate the virus without the structure (Tanada and Kaya 1993). Baculoviruses are among the biggest viruses with a rod-shaped nucleocapsid ranging from 250 to 300 nanometers in length and 30 to 60 nanometers in diameter and have a genetic makeup of double stranded DNA making them ideal study organisms for viral biology (Evans and Entwistle 1987). Other families containing insect viruses include: Ascoviridae, Birnaviridae, Caliciviridae, Dicistroviridae, Hytrosaviridae, Iflaviridae, Iridoviridae, Nodaviridae, Nudiviridae, Parvoviridae, Picornaviridae, Polydnaviridae, Poxviridae, Reoviridae, Rhabdoviridae, and Tetraviridae (Hunter-Fujita et al. 1998; Williams 2018). Each family is classified by commonality within virion shape, size, and structure, presence or absence of an enveloped nucleocapsid, presence or absence of an occlusion (inclusion) body, genome composition (RNA, DNA, ds, ss), size, and arrangement (linear or circular) (Boucias and Pendland 1998; Tanada and Kaya 1993).

Baculoviridae

Baculoviruses are currently the most well understood and speciose family of viruses known to infect insects (Herniou and Jehle 2007). Due to reclassification in 2006, there are four genera within Baculoviridae: Alphabaculvirus (Lepidopteran-specific nucleopolyhedroviruses), Betabaculovirus (Lepidopteran-specific granuloviruses), Gammabaculovirus (Hymenopteran-specific nucleopolyhedroviruses), and Deltabaculovirus (Dipteran-specific nucleopolyhedroviruses) (Jehle et al. 2006). Based on the new classification, baculoviruses are divided into two categories:

nucleopolyhedroviruses (NPVs) and granuloviruses (GVs). NPVs are distinguished from GVs mainly by morphological characteristics of the OBs. In NPVs the OB is polyhedral in shape and contains multiple virions within a single OB; however, GVs contain a granular-shaped OB and only a single virion is encapsulated within (Bilimoria 1991). Further classification of the NPVs occurs by the distinction between single nucleopolyhedroviruses (SNPVs) and multiple nucleopolyhedroviruses (MNPVs). This difference is based solely on the number of nucleocapsids contained in a single envelope, with SNPVs containing one nucleocapsid per envelope, and MNPVs containing multiple nucleocapsids per envelope (Evans and Entwistle 1987). So, while all NPVs contain multiple virions (nucleocapsid + envelope), SNPV virions contain a single nucleocapsid per envelope, while MNPV virions contain multiple nucleocapsids per envelope, but all NPVs contain multiple envelopes (virions) per OB.

Baculoviruses must be ingested in order to induce disease in their host (Tanada and Kaya 1993; Bilimoria 1991). They utilize the conditions of the insect midgut to break down the OB surrounding the virions. While the OB is breaking down, the viral particle also releases chitinases and other enzymes that help degrade the peritrophic matrix surrounding the gut lumen (Matos et al. 1999; Ishimwe et al. 2015). Once the peritrophic matrix is degraded to the point where the virion can cross, it will infect the midgut epithelial cells. After endocytosis occurs, the host cell transports the virus to the nucleus where the viral genome enters into the nucleus and begins producing a dissemination form of the virus termed budded virus (BV) (Kong et al. 2018). This form of the virus does not contain an OB, but is a single nucleocapsid containing an envelope

derived from budding through the host cell's cytoplasmic layer. Following the infection of the midgut epithelial cells the *vfgf* viral gene is activated which causes tracheal cells to migrate close to the basement membrane, allowing BV to infect these tracheal cells, thereby gaining access to the host hemocoel where rapid dissemination can occur (Katsuma et al. 2008; Ikeda et al. 2013). Prior to host mortality, the virus begins forming the reproductive form, or occlusion derived virus (ODV). This form of the virus builds up in the host cells until they lyse, causing the death of the host and releasing the OBs into the environment where horizontal transmission can lead to further infections.

Fungal Pathogens

Insects have been hosts of fungal pathogens dating as far back as 100-110 million years ago (Vega et al. 2012). It wasn't until Agostino Bassi helped prove Germ Theory of Disease by documenting *Beauveria bassiana* killed silkworms that the study of entomopathogenic fungi began developing. Now, there are over 171 entomopathogenic fungal products commercially available (Faria and Wraight 2007). After a major reclassification on fungi the main phyla containing entomopathogenic fungi are Entomophthoromycotina and Ascomycota (Spatafora et al. 2016). Entomophthoromycotina contain the orders Entomophthorales and Neozygitales, both of which contain insect pathogens (Boomsma et al. 2014). Ascomycota is the best described fungal phylum (Blackwell 2011). Within this phylum, Onygenales and Hypocreales are the two orders containing insect pathogens, with Hypocreales containing the vast majority of described or known pathogens (Vega et al. 2012). The most commonly studied genera of insect fungal pathogens are contained within three

families within the order Hypocreales: Clavicipitaceae, Cordycipitaceae, and Ophiocordycipitaceae (Sung et al. 2007).

Ascomycota have a pleomorphic lifestyle containing a distinct asexual (anamorph) and sexual (teleomorph) life stage (Vega et al. 2012). Pathogens such as *Beauveria* and *Meterhizium* can possess relatively broad host ranges, while other fungal pathogens have a much narrower host range (Hu et al. 2014). It is believed that there is also a difference in host range based on the life stage, with teleomorphs having a narrower host range than anamorphs (Boomsma et al. 2014). During reproductive stages, teleomorphs produce ascospores that are energetically released into the environment while anamorphs produce conidia that is passively discharged. Once conidia are disseminated within the environment they will not germinate or penetrate the host until a suitable environment is available, where both a carbon and nitrogen source is present on the host surface (Vega et al. 2012). After penetration of the host cuticle has occurred, hypocreals will produce secondary metabolites which could impair host immune response aiding in the successful colonization of the host (Molnár et al. 2010; Rohlfs and Churchilll 2011). When the host immune system has been suppressed or evaded and entry to the hemocoel is achieved, the fungal pathogen colonizes the host, kills the host, and sporulates, releasing propagules back into the environment. An individual conidium or ascospore is termed a propagule, and these propagules can build up in soil over time resulting in propagule reserves which allow for establishment of infection once a suitable host is present (Hesketh et al. 2010). Fungal pathogens rely mainly on horizontal transmission, with successful transmission being strongly influenced by climate and host

density (Long et al. 2000). Steinkraus (2006) found that fungal pathogens tend to be more successful in gregarious insect species. Outside of the host, the Hypocrealean species are thought to only persist for a few days to over a year if in the soil profile (Vänninen et al. 2000; Jaronski 2007). This short viability in the environment is compensated for by the pathogen in the mass quantities of propagules produced. Also, if no host is present but there is a build-up of detritus, Hypocrealean fungi are capable of being facultative saprophytes (Hu and St. Legar 2002; Wang et al. 2005; Wang et al. 2011).

Bacterial Pathogens

Entomopathogenic bacteria have been discovered in the gram positive Firmicutes, the gram negative Proteobacteria, and in the Tenericutes (Jackson et al. 2018). The first records of bacterial pathogens come from silkworms, honeybees, and stored grain pests (Krieg 1987). Bacteria contain extrachromosomal DNA in the form of plasmids that are shared between species via horizontal gene transfer. This phenomenon allows bacteria to share common toxins or virulence factors; however, the loss of a plasmid could lead to the loss of a toxin production capability or the loss of virulence in a host (Jurat-Fuentes and Jackson 2012; Waterfield et al. 2001; Aronson et al. 1982; Gonzalez and Carlton 1984). The most studied and well-known bacterial insect pathogen is *Bacillus thuringiensis*. It was first recognized from silkworms by Ishiwata in 1906 and has since been determined to have a large number of variants capable of attacking insects in evolutionarily diverse orders (Beegle and Yamamoto 1992; Jurat-Fuentes and Jackson 2012). In general, *B. thuringiensis* is more virulent on foliar herbivorous pests

than on soil-dwelling pests; however, there are variants effective in targeting soil-dwelling Coleopteran pests (Huger et al. 1986). Once a susceptible host encounters a viable bacterial pathogen the pathogen must be ingested or gain entry via a wound in order to begin the infection process. After entering the host, bacterial cells are stimulated based on a conducive environment, leading to colonization. The bacterial cells produce toxins that degrade gut cells resulting in vegetative bacterial growth, and ultimately allowing entry into the hemocoel. Following entry into the hemocoel the bacteria are able to multiply in the nutrient-rich body cavity ultimately causing septicemia and death in a successful infection. Like viruses and fungi, the majority of infections are caused by horizontal transmission with little to no evidence of vertical transmission in bacterial pathogens. As with other pathogens, higher insect densities favor transmission by increasing the transmission potential and likely success (Konecka et al. 2007). Abiotic conditions such as sunlight, temperature, humidity, and chemical properties influence bacterial survival in the environment. Biotic conditions such as competition for nutrients and resources, predation from protozoa, nematodes, or bacteriophages also result in bacterial mortality. Despite these factors, several bacterial entomopathogens are available commercially and have been used successfully in biological control through inundative and inoculative techniques. Inundative biological control would be a tactic that applied a pathogen in high quantities when the insect host was present and thereby gain effective control of the pest for a limited time. Inoculative biological control focuses on establishing the pathogen population within the environment, usually not requiring another establishment of the pathogen even across years. However, pathogen

applications can still occur to boost the population of the established pathogen to control outbreak pest populations.

Insect-Colonizing Nematodes

All nematodes undergo six life stages: the egg, four immature stages, and the adult; and can reproduce within or outside of the host, relying on the host for nutrition. Insect-colonizing nematodes can be separated into two distinct groups: insect-parasitic nematodes and entomopathogenic nematodes. Insect-parasitic nematodes differ from entomopathogenic nematodes in that they will eventually kill the infected host, while entomopathogenic nematodes quickly (within 2 to 3 days) kill the infected host (Shapiro-Ilan et al. 2018). Insect-parasitic nematodes are relegated to three families within Nematoda: Mermithidae, Neotylenchidae, and Rhabditidae. Nematodes in these three families are considered lethal parasites of insects. For parasitic nematodes there is usually one environmentally tolerant immature stage in which the nematode can find a new host, termed the infectious juvenile (IJ). The IJ stage can vary based on the family. For example, the aquatic mosquito larvae parasite *Romanomermis culicivorax* Ross and Smith is in the family Mermithidae and undergoes the IJ stage during the second juvenile stage. During this stage the IJ seeks out an aquatic mosquito larva and penetrates through the cuticle. It will develop within the larva for 7 to 10 days before leaving, sinking to the bottom of the aqueous environment where final development to an adult occurs (Platzer 1981). The family Neotylenchidae are facultative parasites with a very complex life cycle described by Lewis and Clarke (2012). While insect-parasitic nematodes are important, the lack of virulence makes them unsuitable candidates for

commercial agricultural practices, unlike entomopathogenic nematodes. Entomopathogenic nematodes are only found in two nematode families: Heterorhabditidae and Steinernematidae, these nematodes reach the IJ stage during the third juvenile stage. All species are obligate parasites and contain an association with a symbiotic bacterium that is vital to the nematode colonizing insect hosts. Heterorhabditidae contains 21 species of nematodes, while Steinernematidae contains 97 species (Shapiro-Ilan et al 2016). Heterorhabditids are associated with a bacterium in the genus *Photorhabdus*, while steinernematids are associated with a bacterium in the genus *Xenorhabdus*. Once a developing nematode reaches the third juvenile stage it will remain sealed in the second-stage juvenile cuticle which serves as a protective coating from environmental extremes the IJ might encounter outside the host (Rickert-Campbell and Gaugler 1991). No development, feeding, or reproduction will occur during the IJ stage, only seeking out or ambushing an uninfected host. The ability of the IJ to live outside the host is dependent on the resources acquired from the primary host and the ambient temperature of the environment, but a typical life span of an IJ is weeks to months (Lewis et al. 1995). After an IJ is in contact with a susceptible host, it will penetrate the cuticle, or enter by means of natural openings such as spiracles or wounds (Bedding and Molyneux 1982). Following a successful invasion past the cuticle into the hemocoel, the IJ molts and releases the bacteria through regurgitation or defecation (Ciche and Ensign 2003). The bacteria then either avoids or suppresses the immune system and ultimately kills the host by producing toxins and lysing cells which leads to septicemia within a few days. The nematodes continue to develop within the cadaver.

First generation heterorhabditids will develop into hermaphrodites, with following generations producing males, females, and hermaphrodites (Shapiro-Ilan et al. 2018). Steinernematids will develop into males and females and will always be amphimictic. Depending on the quality and size of the host, 1-3 generations can occur within the cadaver across 10-30 days before a reduction in nutritional quality and quantity and a buildup of waste products will drive the nematodes to begin building up IJ populations (Shapiro-Ilan et al. 2018). Once the IJs exit the host they begin the search for a new host. Currently nematodes are only transmitted horizontally, with no form of vertical transmission being documented, although eggs have been found to be an adequate host (Kalia et al. 2014). *Steinernema carpocapsae* (Nematoda: Steinernematidae) has been found to have an experimental host range of over 200 insects across 10 orders; however, the ecological host range may be more limited (Poinar 1979). *S. carpocapsae* is an ambusher, meaning the IJs have low motility and stay near the soil surface waiting for a host to contact them (Campbell and Gaugler 1993). The other tactic to host finding is cruisers which seek out hosts, are highly mobile, and move through the soil profile. However, most nematodes have developed a mixture of both tactics rather than relying on only one. This group is termed intermediate foragers (Lewis 2002).

Pathogen-Host Interactions

Once a susceptible host comes in contact with a pathogenic microbe (viral, fungal, bacterial, or nematode) the insect relies on physical barriers to inhibit the successful infection by the pathogen. For certain pathogens such as viruses and bacteria, the melanized waxy cuticle is an impenetrable barrier. Some pathogens such as

nematodes or fungi have mechanisms to puncture or pierce the cuticle, thereby gaining access to the relatively vulnerable and nutrient rich hemocoel. Viruses and bacteria must be ingested to gain entry into their host, but also must survive the extreme conditions of the midgut including pH extremes and proteases and lipidases capable of killing pathogenic particles (Sparks et al. 2008; Ikeda et al. 2013; Ishimwe et al. 2015; Kong et al. 2018). If the pathogen avoids these enzymes the insect has the peritrophic matrix as a physical barrier between the gut lumen and the midgut epithelial cells (Sparks et al. 2008). Once infection is established in the midgut epithelial cells the insect can slough off these infected cells, negating the infection, before the pathogen spreads across the basement membrane into the hemocoel (Sparks et al. 2008). Bacteria and viruses can also enter the host hemocoel directly through wounds, and bacteria can also infect tracheal cells through spiracles. Fungal pathogens and entomopathogenic nematodes can enter through any opening such as wounds, trachea, oral, or anal; and are capable of penetrating the cuticle directly thereby gaining access to the hemocoel. Once the pathogen reaches the hemocoel, the insect relies on its innate immune system to combat the successful colonization of the pathogen. Pathogens can be either generalists or specialists, and there are some differences between how these types of pathogens interact with hosts. A generalist viral pathogen *Autographa californica* nucleopolyhedrovirus (AcMNPV) can infect *Heliothis virescens* and *H. zea*; however, *H. zea* is more resistant to the pathogen than *H. virescens* (Trudeau et al. 2001). A study by Trudeau et al. (2001) showed *H. zea* were able to melanize and encapsulate virions within 48 hours of infection and their hemocytes were highly resistant to AcMNPV infection. The

hemocytes would take up the virus particle readily and transport it to the nucleus but no expression of viral genome would occur. Therefore, the hemocytes were removing the virus from circulation rather than amplifying the titer of the virus (Trudeau et al. 2001). However, all pathogens that gain entry into a viable host will induce a fitness cost on the host by reducing survival, longevity, and/or reproduction potential (Schmid-Hempel 2011). This is usually thought to occur due to a reallocation or resources within the host possibly to activate or stimulate the immune system to respond.

Innate Immune System

The insect immune system is an innate immune system that is non-specific and assumed to be without memory, yet it is still vitally effective at attacking and overcoming challenges by pathogens or parasitoids (Beutler 2004; Beutler and Hoffmann 2004; Schmidt et al. 2008). Insect immune systems are able to recognize non-self and altered-self molecular structures which allows for a targeted response that has minimal direct impact on the unaltered self (Kato et al. 1994; Lavine and Strand 2001; Janeway and Medzhitov 2002; Beutler 2003; Cheon et al. 2006). It has been observed that cytokines and signaling pathways such as Toll/Spaetzle and JAK/STAT pathways might be important in the identification of non-self/altered self, and the initiation of the immune response (Clark et al. 1997; Clark et al. 1998; Strand and Clark 1999; Lavine and Strand 2003; Clark et al. 2004; Bidla et al. 2007; Strand 2008a). This immune response is accomplished in a two-pronged response: humoral and cellular defense response. The humoral response includes antimicrobial peptides and products produced from complex proteolytic cascades, including phenoloxidase (PO) (Strand 2008a;

Blandin and Levashina 2004; Cornelis and Soderhall 2004; Imler and Bulet 2005; Theopold et al. 2004). Cellular defenses include phagocytosis, encapsulation, nodulation, and clotting which are directly mediated by hemocytes and regulated by eicosanoids (Strand 2008b; Gillespie et al. 1997; Irving et al. 2005; Lackie 1988; Strand and Pech 1995).

An insect's response to a pathogen, the level of PO activity and encapsulation response, is variable among identically reared larvae, and has been found to be heritable (Cotter and Wilson 2002). Also, Kurtz et al. (2000) looked at sex-determined immune responses and found female *Panorpa vulgaris* tend to have higher levels of lysozyme-like antibacterial activity and hemolymph phagocytosis ability when compared to males. The authors concluded this was mainly due to an increase in hemocyte count compared to males since there was no difference in the number of particles individual phagocytes were able to phagocytize (Kurtz et al. 2000). Then, McNeil et al. (2010a; 2010b) examined the role of the immune system in inducing systemic resistance to a pathogen by utilizing *Lymantria dispar* and the viral pathogen *Lymantria dispar* nucleopolyhedrovirus (LdMNPV). They measured hemocyte responsiveness, hemolymph PO activity, FAD-glucose dehydrogenase activities, and melanization. Mid-instar larvae had higher PO levels, and hemocyte responsiveness when infection progressed past the midgut. Ultimately, this research showed innate immune responses can be indicative of antiviral defenses, and successful clearing of viral pathogens can occur independent of host age (McNeil et al. 2010a; McNeil et al. 2010b). Finally, in 2008, Haine et al. monitored the progression of the immune response to bacterial

pathogens (*E. coli*, *Bacillus subtilis*, peptidoglycan, and LPS) across a temporal landscape and compared it to a healthy *Tenebrio molitor* host. The authors determined that magnitude and temporal variation in PO and antimicrobial activity were present, with antimicrobial response being long-lasting and PO activity only lasted a short time (Haine et al. 2008).

Recognition Pathways

The insect immune system is able to distinguish self from non-self and altered-self by means of lipid particles and pathogen-associated molecular patterns. Pathogen-associated molecular patterns (PAMP) include lipopolysaccharides, peptidoglycans, and glucans common to pathogens. The host hemocytes contain pattern recognition receptors structured to bind to a specific PAMP, and once activated, initiates phagocytosis (Franc et al. 1999; Lavine and Strand 2001; Ramet et al. 2001; Ramet et al. 2002; Kocks et al. 2005; Rao et al. 2018; Wang et al. 2019a; Wang et al. 2019b). Lipid particles may function as lipid carriers but also as sensor particles involved in recognition and detoxification of lipopolysaccharides, other toxins, and reactive oxygen species (Kato et al. 1994; Vilcinskas et al. 1997; Arakawa et al. 1996). Lipid metabolism and lipid carrier proteins have been shown to be vital to systemic immune responses to parasites and pathogens, and lipid particles have also been shown to be important in the PO cascade (Cheon et al. 2006; Mullen and Goldsworthy 2003). Lipid particles carry PO, their activating proteases, and recognition proteins (Rahman et al. 2006). Immune-induced lipid particles can become associated with immune proteins and become adhesive particles able to form cell-free aggregates or interact with cells (Ma et al. 2006; Rahman

et al. 2006). Lipids can adhere to pathogens and become opsonized and interact with opsonin-specific receptors on the insect cells inducing a Velcro-like endocytosis (Swanson and Baer 1995; Cho et al. 1999; Levashina et al. 2001; Moita et al. 2005; Dong et al. 2006; Garver et al. 2006; Terenius et al. 2007).

Humoral Defense

Antimicrobial Peptides

The humoral response includes production of antimicrobial peptides (AMPs) and other products from complex proteolytic cascades such as the phenoloxidase (PO) cascade (Strand 2008a; Blandin and Levashina 2004; Cornelis and Soderhall 2004; Imler and Bulet 2005; Theopold et al. 2004). Most AMPs are synthesized as inactive precursor proteins or pro-proteins and activated by limited proteolysis during a pathogen invasion (Yi et al. 2014). Most are small cationic molecules with activity against bacteria and/or fungi with some showing activity against parasites and viruses (Yi et al. 2014). The Toll pathway is activated when PAMPs such as Lys-type PGN or β-1,3-glucan are bound by PRRs, stimulating an enzymatic reaction resulting in the cleavage and proteolytic activation of the Spätzel protein (Spz) (Schneider et al. 1994). This activated Spz binds to Toll receptors in the transmembrane, triggering a conformational change resulting in an active Toll dimer. The Toll dimer interacts with a signaling complex consisting of a signaling transducer protein (MyD88), an adapter protein (Tube), and a protein kinase (Pelle) (Drier and Steward 1997). This signaling complex initiates phosphorylation and degradation of Cactus, which retains the transcription factor Dorsal in the cytoplasm by masking its nuclear localization signal (Wasserman 2000). Once Cactus is degraded,

Dorsal is free to enter the nuclei (Belvin and Anderson 1996). Once in the nuclei, Dorsal begins transcribing loci specific to the Toll pathway and/or loci inducible by both Toll or IMD, resulting in the production of AMPs that have activity against most gram positive bacterial and fungal pathogens (De Gregorio et al. 2001; De Gregorio et al. 2002; Hedengren-Olcott et al. 2004; Imler et al. 2004; Irving et al. 2001; Lemaitre et al. 1997).

The IMD pathway is activated by bacterial PGN that contain *meso*-diaminopimelic acid (DAP) at the third position of the stem peptide (Choe et al. 2002; Kaneko and Silverman 2005; Kaneko et al. 2006). This DAP-type PGN is characteristic of all gram negative bacteria and some gram positive genera, including *Bacillus* and *Clostridium*. Upon binding DAP-type PGN, the transmembrane receptor PGRP-LC recruits immune deficiency protein (Imd) (Choe et al. 2005; Ferrandon et al. 2007; Georgel et al. 2001). Imd signals through a branching signaling complex to the transcription factor Relish, activating it, resulting in the transcription of loci specific to the IMD pathway, and/or loci inducible by both Toll or IMD (Erturk-Hasdemir et al. 2009; Meinander et al. 2012; Stoven et al. 2003). The AMPs produced by both Toll and IMD are effective at killing bacterial cells by binding to the cell wall and distorting the osmotic pressure, lysing the cell (Wu et al. 2018; Mackintosh et al. 1998; Hoffmann and Hetru 1992).

Phenoloxidase Cascade

The PO cascade is a major humoral immune response that is also important in cellular immune responses. The quinones formed from the initial enzymatic activity of PO, triggered by wounding or infection, undergo a series of enzymatic and non-

enzymatic reactions that lead to polymerization and melanin synthesis with reactive oxygen species being produced as a by-product (Cerenius et al. 2008; González-Santoyo and Córdoba-Aguilar 2011; Bidla et al. 2009). Phenoloxidase is synthesized as prophenoloxidase (PPO) which is activated by site-specific proteolytic cleavage upon recognition of infection or wounding (Gorman et al. 2007). Active PO catalyzes the formation of quinones which form melanin (Cerenius and Soderhall 2004; Nappi and Christensen 2005). The melanin produced during the PO cascade can be deposited in nodules and is frequently observed in the cellular defense mechanism as encapsulation, whereby pathogens are surrounded and encapsulated by hemocytes (Strand and Pech 1995; Schmidt et al. 2001; Wertheim et al. 2005). Cytotoxic reactive oxygen species and nitrogen intermediates are formed during the melanin synthesis process and are toxic to pathogens, including viruses (Nappi and Christensen 2005). The PO cascade, PPO activation, and subsequent melanin and reactive oxygen species formation are thought to be an important component of the insect immune system and its ability to defend against many forms of pathogens including viral infections (Ourth and Renis 1993; Shelby and Popham 2006; Popham et al. 2004; Trudeau et al. 2001). However, there is debate over whether the PO cascade actually contributes to the antiviral immune response (Shelby and Popham 2006; Saejeng et al. 2010; Popham et al. 2004; Trudeau et al. 2001; Scholefield et al. 2019; Pan et al. 2020). Trudeau et al. (2001) identified encapsulation and melanization as important immune responses of *H. virescens* and *H. zea* to AcMNPV, implementing the phenoloxidase cascade as an important contributor in antiviral defense. This was further corroborated by Popham et al. (2004) and Shelby and

Popham (2006), where potent virucidal activity was discovered in *H. virescens* plasma with activity against *Helicoverpa zea* single nucleopolyhedrovirus (HzSNPV). This virucidal activity was attributed to phenoloxidase. However, no antiviral activity was found for phenoloxidase in *Plodia interpuncetella* infected with *Plodia interpuncetella* granulovirus (Saejeng et al. 2010), *Trichoplusia ni* infected with *Trichoplusia ni* single nucleopolyhedrovirus (Scholefield et al. 2019), or in *H. zea* infected with AcMNPV or HzSNPV (Pan et al. 2020).

Cellular Defense

The second primary component of an insect's innate immune system is the cellular response to infection involving encapsulation, nodulation, phagocytosis, and apoptosis of pathogens mediated by hemocytes and regulated by eicosanoids (Strand 2008b; Gillespie et al. 1997; Irving et al. 2005; Lackie 1988; Strand and Pech 1995). In the majority of insects, the most prominent hemocytes in circulation are granulocytes, plasmatocytes, spherule cells, and oenocytoids (Lavine and Strand 2002; Ribeiro and Brehelin 2006). In Lepidoptera, granulocytes are the most abundant, and are able to strongly adhere and spread symmetrically on foreign surfaces (Strand 2008b). Plasmatocytes are usually larger than granulocytes, spread asymmetrically on foreign surfaces, and are the major capsule-forming hemocyte (Strand 2008b). Oenocytoids are non-adhesive hemocytes containing PO cascade components. Spherule cells are non-adhesive and are a potential source of cuticular components (Lavine and Strand 2002). The number of hemocytes in circulation can rapidly increase in response to stress, wounding, or infection (Lackie 1988; Ratcliffe et al. 1985). Some studies attribute this to

rapid differentiation in the hematopoietic organ (Sorrentino et al. 2002; Wertheim et al. 2005), while others indicate already differentiated hemocytes are often sessile and weakly adhere to surface of internal organs, but rapidly enter circulation following an immune challenge (Castillo et al. 2006; Elrod-Erickson et al. 2000; Gardiner and Strand 2000; Lanot et al. 2001; Moita et al. 2005).

Coagulation

Coagulation of hemolymph occurs at external wound sites (Bidla et al. 2005; Theopold et al. 2004). Coagulation begins with soft clots of a fibrous matrix that is embedded with hemocytes, primarily granulocytes or plasmatocytes, that hardens by cross-linking of proteins and the melanization process (Scherfer et al. 2006; Theopold et al. 2004).

Phagocytosis

Phagocytosis is a widely conserved humoral defensive pathway where individual cells internalize and destroy small targets. This process depends on receptor-mediated recognition via opsonins or other pathogen associated microbial patterns (PAMPs) leading to cell binding which induces the formation of a phagosome, and engulfment of the foreign material via actin polymerization-dependent mechanisms (Stuart and Ezekowitz 2005; Stroschein-Stevenson et al. 2006). The phagosome then matures through a series of fission and fusion events to a phagolysosome (Stuart and Ezekowitz 2005; Stroschein-Stevenson et al. 2006). Insect hemocytes are capable of phagocytizing many different types of bacteria, fungi, and protozoans (Nazario-Toole and Wu 2017).

Encapsulation

Encapsulation refers to the envelopment of large invaders such as nematodes or parasitoids. The process of enveloping bacteria is termed nodulation (Ratcliffe and Gagen 1976; Ratcliffe and Gagen 1977; Satyavathi et al. 2014). Plasmatocytes are the main hemocyte responsible for encapsulation, but some pathogen species will not be encapsulated without the added immune response of granulocytes in capsule formation (Lavine and Strand 2001; Pech and Strand 1996; Pech and Strand 2000). When granulocytes are required, they initially recognize the invading pathogen and bind to it forming a monolayer. Then plasmatocytes are recruited and activated by the granulocytes through the release of PSP and other cytokines (Clark et al. 1998). This specific type of encapsulation ends when a final monolayer of granulocytes attaches to the periphery of the capsule and produce a basement membrane-like layer (Grimestone et al. 1967; Liu et al. 1998; Pech and Strand 1996). Encapsulation without granulocytes occurs through the aggregation of plasmatocytes onto the invading pathogen and the deposition of melanin within and around the capsule in order to form a barrier through which nutrients cannot pass and the invading organism cannot survive (Schmidt et al. 2001).

Apoptosis

Apoptosis, programmed cell death, is important for the insect host during early stages of infection to lyse host cells infected with pathogens to keep the pathogen from replicating. In later stage infections, it becomes important for the pathogen to break free from the host cell (Ikeda et al. 2013; Kong et al. 2018; Nguyen et al. 2012; Nguyen et al.

2013). Apoptosis as an immune response has been shown to be inducible via the Jun-N-terminal kinase (JNK) signaling pathway (Kockel et al. 2001). The JNK pathway is a highly conserved mitogen-activated protein kinase (MAPK) pathway. Jun-N-terminal kinase is a MAP kinase that phosphorylates the Jun and Fos transcription factors that activate transcription of target genes (Horton et al. 2011; Boutros et al. 2002; Chen et al. 2002; Sluss et al. 1996). JNK in *Drosophila* is involved in embryonic development, apoptosis, stress response, cell proliferation and differentiation, and immunity (Kockel et al. 2001). TAK1, a part of the signaling complex in the IMD pathway, can activate JNK, as can LPSs, revealing a glimmer of how these multiple immune pathways work in tandem to orchestrate a robust immune response specific to the invading pathogen (Silverman et al. 2003; Park et al. 2004; Garcia-Lara et al. 2005).

Eicosanoids

Eicosanoids were first proposed to act in immune functions by Stanley-Samuelson et al. (1991). This research supported a broad hypothesis that eicosanoids act in insect bacterial clearance. Dunn and Drake (1983) identified nodule formation cleared bacterial disease, and from this it was hypothesized that eicosanoids mediated micro-aggregation and nodulation formation reactions to bacterial infection (Miller et al. 1994). Eicosanoids are also responsible for mediating cell spreading in primary hemocytes (Miller 2005). Stanley and Miller (2006) showed eicosanoids are mediators in chemotaxis processes between hemocytes and bacterial peptides. From the growing amount of literature on eicosanoids it can be concluded that they are key cellular response mediators and can even mediate some humoral responses (Stanley et al. 2009).

Behavioral Defense

Along with the humoral and cellular defenses, insects are capable of generating or cultivating behavioral defenses utilized to increase survival, longevity, and reproduction potential. Some behavioral tactics are avoidance tactics such as the ability of *Anthocoris nemorum* to detect and avoid leaf surfaces inoculated with *B. bassiana* (Meyling and Pell 2006). Or perhaps the insect population has evolved to exhibit density-dependent prophylaxis as in the case of the desert locust (Wilson et al. 2002). Behavioral defenses include fever induction, avoidance tactics, excessive grooming, density-dependent prophylaxis, anorexia, and self-medication. These behaviors can be classified into two categories: proactive and reactive behaviors. Proactive behaviors decrease the likelihood of an infectious pathogen from successfully penetrating the host's natural defenses, while reactive behaviors alter or induce changes in the humoral and cellular mechanisms of the host's innate immune response after an infection occurs.

Avoidance and Grooming

There are many examples of avoidance in arthropod studies (Behringer et al. 2006; Parker et al. 2010). Gypsy moths have been found to avoid infected cadavers, thus altering the successful implementation of a pathogen as a biological control tactic (Eakin et al. 2015). *A. nemorum* were able to detect and avoid *B. bassiana* infected leaf surfaces; however, they were not able to detect spores in the soil possibly because the soil is an unfamiliar environment (Meyling and Pell 2006). Mole crickets will avoid tunneling in soils contaminated with *B. bassiana*, and when total avoidance is not possible the mole cricket will abandon the soil (Thompson et al. 2007). Ants and

termites have been known to wall off infected portions of the nest (Oi and Pereira 1993; Yanagawa et al. 2010). Grooming is another proactive behavioral defense implemented by insects that is preventative. Cockroaches are capable of grooming pathogenic nematodes from their legs and antennae (Koehler et al. 1992). Ants groom each other, secret antibiotics, keep the nest hygienic, use avoidance tactics, and utilize an altruistic mentality in order to avoid pathogen outbreaks (Oi and Pereira 1993). *Lasius japonicus* will increase allogrooming to improve survivorship when exposed to *Meterhizium anisopliae* (Okuno et al. 2012). *Coptotermes formosanus* will mutually groom to remove fungal spores and can utilize olfaction to identify at least three species of fungi (Yanagawa et al. 2010).

Density-Dependent Prophylaxis

Insects in high densities tend to invest relatively more resources into pathogen resistance than those existing in low densities because the probability is higher the denser populations will contact a pathogen (Wilson et al. 2001). This is the primary theory behind density-dependent prophylaxis, which was determined to be true for *Spodoptera littoralis* when Wilson et al. (2001) linked resistance melanism and PO activity. However, some insects undergo phases of solidarity and gregarious natures (Cotter and Wilson 2002). It was therefore hypothesized that species that have large fluctuations in population densities should have plasticity in their immune systems where the investment matches the risk of infection (Cotter and Wilson 2002). This was further supported in insects in high-density populations having an increased resistance to pathogens. Many of these insects showed an increase in cuticular melanin. In *S.*

littoralis, dark larvae exhibited higher hemolymph and cuticular PO and stronger encapsulation response compared to pale larvae (Cotter et al. 2004). In this study larval density, rather than larval color had little effect except by capsule melanization, and antibacterial activity was higher in solitary insects and lower in gregarious insects. Therefore, they concluded variation in immune function cannot be explained solely by condition-dependence (Cotter et al. 2004). *Schistocerca gregaria* that were reared under crowded conditions were more resistant to *M. anisopliae* var. *acridum* than solitarily reared (Wilson et al. 2002). They also had elevated antimicrobial activity in crowded compared to solitary but did not show a thermal preference difference or behavioral fever difference (Wilson et al. 2002).

Behavioral Fever

Probably the most well-known example of a behavioral defense is that of the locust sun-bathing upon infection in order to induce pyrexia that is non-conducive to pathogen development. The grasshopper, *Oedaleus senegalensis*, is capable of altering its thermoregulatory behavior to a temperature at which the pathogen, *Metarhizium flavoviride*, cannot survive (Blanford et al. 1998). Therefore, induction of a fever is an example of an induced reactive behavioral defense as this behavior is not observed in healthy individuals, only in infected conspecifics.

Starvation and Anorexia

In *Manduca sexta*, starvation and food limitation can have a restructuring effect on the immune system responses with some immune responses being decreased while others are increased (Adamo et al. 2016). This restructuring can be a useful tool for

insects to actively manipulate the primary components of their immune response to combat a specific pathogen. However, in honeybees challenged with lipopolysaccharides and micro-latex beads to simulate parasitoids and bacterial pathogens, those that were under starvation conditions had a reduced survival compared to the controls (Moret and Schmid-Hempel 2000). Thus, even though starvation can be beneficial in some insects, it can be detrimental in others such as the honeybee. Under the assumption that starvation is an imposed environmental condition and anorexia is a behavioral choice, Adamo et al. (2010) proposes that changes in feeding behavior is consistent with the insect's need to reduce lipid transport in order to maximize the immune function. Therefore, illness-induced anorexia is one method animals can bias physiological pathways to enhance immune function, by reducing the lipid concentration ingested they reduce the level of lipid transport occurring. This allows for the reallocation of nutrients to be freely utilized by the immune system rather than normal physiological pathways. Povey et al. (2013) found that *Spodoptera exempta* increased their intake ratio of proteins to carbohydrates not by consuming more protein but rather through reduction of carbohydrate intake which reduced the overall diet consumption consistent with illness-induced anorexia.

Self-Medication

Behavioral changes regarding ingestion of toxins, food sources, nutrition, or the regulation of diet intake must meet four criteria in order to be classified as self-medication (Shikano and Cory 2016). First, only infected individuals engage in the behavior. Second, the behavior must alleviate the potential fitness loss of the infected individual. Third, uninfected individuals must suffer a fitness cost when engaging in the

behavior. Fourth, the parasite cannot benefit from the behavior (Clayton and Wolfe 1993; Singer et al. 2009; Karban and English-Loeb 1997; Lefevre et al. 2009; Lefevre et al. 2010). Abbot (2014) defined self-medication as having four qualifications three of which are the same; however, Abbot adds that the substance must be deliberately contacted rather than by chance. From these qualifications we can determine self-medication can be quantitative, such as protein consumption or diet consumption alteration, and qualitative (Singer et al. 2009). Also, self-medication can be innate, but in order to distinguish between self-medication, diet choice/compensation, and host manipulation all four criteria must be met (de Roode et al. 2013). The third criteria address the adaptive plasticity of diet choice in that the substance or behavior must have a detrimental effect on the host in the absence of the pathogen. This has been further studied in *T. ni* larvae infected with AcMNPV where infected larvae increased their protein intake but did not alter their carbohydrate intake. The increase protein did not benefit the virus but also did not increase the probability of the larvae to survive the infection at 24˚C, and from this the authors concluded the four criteria were not met and that this was an example of compensatory feeding (Shikano and Cory 2016). In *S. exempta* infected with *Spodoptera exempta* nucleopolyhedrovirus (SeNPV), infected larvae that fed on higher protein content diets were more likely to survive the infection than larvae reared on lower protein diets. When given a choice, the larvae challenged with the infection chose higher protein diets compared to non-infected larvae which points to a possible self-medication, however the authors did not test all four criteria (Povey et al. 2013). From these studies we can determine that it is relatively difficult to

distinguish between compensatory feeding, host manipulation, and self-medication, and very few studies have actually attempted to address all four criteria of self-medication.

Self-medication is a regulatory pathway of the immune system, and it is important to note that the function of the immune system is to regulate all interactions with microorganisms, not exclusively pathogenic organisms but also limiting the cost of responding to organisms that can be tolerated and allowing beneficial microbes to grow. The immune response is modulated by host genetics as seen in the innate responses and how some activity levels can be hereditary, and also by host nutrition and the regulation of nutritional intake (Lazzaro and Little 2009; Schmid-Hempel 2011). Thus, it would stand to reason that to better understand the immune system we must first understand nutrition and how the regulation of nutrition can alter the immune response (Ponton et al. 2013).

Insect Nutrition in a Heterogenous Landscape

In 1993, Simpson and Raubenheimer proposed the concept of a geometric framework to explain nutrition using functional, mechanistic, ontogenetic, and comparative aspects of nutrition to develop a multi-dimensional nutritional space with each relevant nutrient forming a dimension. This integrative framework must be able to accurately represent the animal of interest, the environment, and the nutritional basis for the interaction between the animal and environment (Raubenheimer et al. 2009). From the study of insects within this framework it was determined that most insect herbivores have an optimal nutritional space termed the intake target (IT) that is reached by ingesting diet composed of carbohydrates (C) and proteins (P), and that achieving this

optimal IT can maximize fitness (Behmer et al. 2001; Lee et al. 2006; Behmer 2009; Roeder and Behmer 2014). However, an IT cannot always be reached in every environment (Simpson and Raubenheimer 2012). An improper ratio of dietary protein and carbohydrates can result in sub-optimal growth, reproduction, and fitness (Thompson and Simpson 2009). Most insects offset these costs by the Rule of Compromise usually associated with either the Equal Distance Rule or the Closest Distance Rule. The Equal Distance Rule says an insect will regulate their diet to a point in the nutritional landscape that is equally distant from the protein target as from the carbohydrate target, while the Closest Distance Rule is where the insect regulates to the closest point within the nutritional framework to the desired IT regardless of balancing or unbalancing P:C ratios (Simpson and Raubenheimer 2012; Simpson et al. 2004).

The environment in which an insect interacts must also be accounted for in a nutritional landscape. The environment is constructed of many food components, and the consequences of an insect's behavior and physiological responses to the nutritional environment must be represented. If we know the nutritional needs of the insect and the nutritional environment available, we can make predictions about which food it will eat knowing it will attempt to optimize the P:C ratio to the IT via optimal foraging (Simpson et al. 2004; Simpson and Raubenheimer 2012). Insects exist in a highly heterogenous nutritional landscape, where even within plant distribution and sequestration of proteins and carbohydrates can be highly variable (Elser et al. 2000; Deans et al. 2016a; Deans et al. 2018). Within cotton it was determined that P and C concentrations and total macronutrient content was significantly different across plant tissues, plant ages, and

environments (Deans et al. 2016a). Cotton foliar tissues had higher total P+C content compared to reproductive tissues, except seeds and flowers, which had twice the P+C content as the leaves (Deans et al. 2016a). This shows that even agricultural monocultures provide a heterogenous nutritional landscape for insects to optimize foraging to reach the IT (Deans et al. 2016a). Furthermore, insect herbivores are capable of assessing the nutrients present in plant tissue and thereby regulate specific nutrients (Raubenheimer and Simpson 1999; Simpson and Raubenheimer 1999; Simpson et al. 2015). Utilizing the geometric framework, Deans et al. (2015) empirically determined *H. zea* P:C IT to be slightly protein biased at 1.6:1, which was vastly different from the previously published IT of 4:1 (Waldbauer et al. 1984). Diet quality availability was shown to alter *M. sexta* immune response, revealing diet quality mediates relationship between performance and immune function and results in trade-offs within immune functions (Wilson et al. 2018).

Insect Nutrition Affecting Susceptibility

Insect immune systems react differently based on the nutritional diet the individual insect has acquired (Ponton et al. 2011). Due to the insect actively regulating its nutritional intake, it was hypothesized that an insect confronted with a disease-inducing pathogen can and would alter their intake to maximize survival (see self-medication section). *S. exempta* that were infected with *B. subtilis* increased survivability by ingesting higher quantities of protein, suggesting a protein cost associated with bacterial resistance (Povey et al. 2009). The larvae that fed on higher quantities of protein had a higher hemolymph PO activity, a higher antimicrobial

activity, and higher hemolymph protein levels (Povey et al. 2009). The infected larvae were shown to increase their protein intake while not altering their carbohydrate intake, implying an increase in protein as a compensatory mechanism (Povey et al. 2009). Deans et al. (2016b) proposed the idea that achieving the intake target for *H. zea* could be a factor in the development of *Bt*-toxin resistance. Following this idea, *H. zea* have been shown to have varying susceptibility to the *Bt*-toxin Cry1Ac based on the P:C ratio of diet ingested (Deans et al. 2017). The authors found a 100-fold increase in LD50 values for larvae on optimal versus carbohydrate-biased diets, implying a significant variation in survival could be mediated by diet nutrition (Deans et al. 2017). *H. armigera* and *H. punctigera* susceptibility to *Bt*-toxins under differing nutritional regimes was similarly affected by dietary P:C ratios (Tessnow et al. 2018). The authors looked at a resistant and susceptible strain and tested susceptibility against Cry1Ac, Cry2Ab, and Vip3Aa. *H. armigera* was less susceptible to Cry1Ac when reared on diet conducive of the larval IT (Tessnow et al 2018). However, *H. punctigera* was not affected by diet nutritional content for any of the three toxins tested, implying nutritional P:C ratios can be important factors to consider for some toxins but can have no apparent effect on larval susceptibility for others (Tessnow et al. 2018).

Insect Nutrition and Viral Susceptibility

Dietary nutrition has also been shown to have an important role in insect's susceptibility to viral pathogens. *H. virescens* and *T. ni* larvae infected with a generalist viral pathogen, AcMNPV and that ingested higher quantities of protein had shorter LT50 values than infected larvae that did not increase protein intake (Hoover et al. 1998). The

authors concluded virus production was faster in larvae that developed faster, leading to potential host manipulation on the premise that the virus benefited from diet alteration (Hoover et al. 1998). Another study utilizing *T. ni* infected with a low dose of AcMNPV and provided diet in a choice-assay showed virus-challenged larvae developing slower and accruing lower protein levels in the hemolymph resulting in fewer hemocytes compared to healthy larvae (Shikano et al. 2016). The virus-challenged larvae laid fewer eggs once adulthood was reached, and there was no evidence of increased resistance in the offspring of infected adults implying no transgenerational immune priming (Shikano et al. 2016). Shikano and Cory (2015) found specialist and generalist viral pathogens exert different costs on their hosts. They determined that overall virus-challenged *T. ni* performed better on protein-biased diets over carbohydrate-biased diets, but that environmental conditions favoring host performance differed from TnSNPV and AcMNPV. TnSNPV inflicted fitness costs while AcMNPV did not. Performance of TnSNPV-challenged insects increased with increasing P:C ratios across all temperatures utilized, while in AcMNPV-challenged insects the temperature modulated the optimal P:C ratio implying nutrition moderates the temperature-size rule (Shikano and Cory 2015). In *S. littoralis*, dietary protein influenced both resistance to viral pathogen attack and constitutive immune function to a greater extent than dietary carbohydrates, indicating higher protein costs to resistance when infected with *Spodoptera littoralis* nucleopolyhedrovirus (SlNPV) (Lee et al. 2006). Insects that survived the virus challenge increased protein intake compared to uninfected conspecifics and infected conspecifics that succumbed to the infection, demonstrating compensation of protein

costs, revealing the host's nutritional demand to fight the infection induces a compensatory shift in feeding behavior (Lee et al. 2006). However, *Grammia incorrupta* larvae injected with beads to simulate a parasitoid egg reduced intake of high-protein foods, favoring a carbohydrate-heavy diet (Mason et al. 2014). The observed carbohydrate-biased intake was further corroborated in a no-choice test, and when melanization response was determined, carbohydrate increase resulted in an increase in melanization activity (Mason et al. 2014). *Plodia interpunctella* challenged with *Plodia interpunctella* granulovirus exhibited a diet-effect when reared on high- and low-quality foods, with higher quality diet having lower LD50 values similar to Hoover et al. (1998) (McVean et al. 2002). However, infected larvae survived longer on higher quality foods compared to lower quality foods. From these conclusions the authors determined dietary stress from low quality food does not cause an increase in susceptibility to infection but there is a more complex effect occurring (McVean et al. 2002). Caloric restrictions can affect immune function, both constitutive and induced immune responses are not limited by the total quantity of nutrients consumed but rather different traits respond differently to variations in the ratios of macronutrients and peak in different regions of nutritional space (Cotter et al. 2011). Therefore, the preferred dietary composition represents a compromise between nutritional requirements and immune response. Some immune-challenged insects modify allocation of nutrients to improve immune response rather than solely altering diet choice. No diet can optimize all components of the immune response (Cotter et al. 2011).

Host Plant Effects on Insect Susceptibility

The insect's host plant or source of nutrition can have a major effect on the insect's susceptibility to pathogens. Host plant quality can affect immune response and disease resistance of *T. ni* larvae, which implies an important function of understanding bottom-up effects in insect-entomopathogen interactions (Shikano et al. 2010). *S. litura* infected with *Spodoptera litura* nucleopolyhedrovirus (SlMNPV) have a lower LT50 value reared on tomatoes compared to cauliflower due to the effect of phytochemicals found in tomato plants on the larvae (Monobrullah et al. 2007). Certain herbivore-induced plant volatiles might not just attract predators and parasitoids but are also capable of increasing herbivore susceptibility to pathogens (Gasmi et al. 2019). *S. exigua* infected with *Spodoptera exigua* nucleopolyhedrovirus (SeMNPV) and exposed to indole or linalool resulted in an increase in susceptibility to SeMNPV; however, exposure to (Z)-3-hexenyl acetate did not increase susceptibility. Exposure to indole also increased *S. exigua* susceptibility to *B. thurengiensis* (Gasmi et al. 2019). Host plant also effects the viral production of the pathogen during the infection, with increasing OB production resulting in slower speed of kill and decreased viral dose in *S. frugiperda* infected with *Spodoptera frugiperda* Multiple Nucleopolyhedrovirus (SfMNPV) (Shikano et al. 2017). Also, herbivory-induced defensive responses can prolong an insect's susceptibility to pathogens via the slow-growth, high-mortality hypothesis in *S. frugiperda* and the associated baculovirus, SfMNPV (Shikano et al. 2018). Chen et al. (2018) determined host plant association can also affect the levels of transcription for chitinase and chitin deacetylase genes, altering the peritrophic matrix thickness. *T. ni*

reared on potato leaves had lower transcription levels resulting in a thicker peritrophic matrix compared to *T. ni* reared on cabbage (Chen et al. 2018). Similarly, in *H. zea,* susceptibility to Elcar, HearNPV, was reduced in larvae reared on cotton compared to tomato or artificial diets (Forschler et al. 1992). Sorghum also had a reduction in susceptibility, which was contributed to the presence of tannins (Forschler et al. 1992). Black soldier fly larvae (*Hermetia illucens*) express a much-expanded spectrum of antimicrobial peptides when allowed to feed on diet containing high bacterial loads which reveals a diet-dependent expression of antimicrobial peptides (Vogel et al. 2018).

Plant genotypes within a crop can alter the susceptibility of an herbivorous insect to a pathogen (Shikano et al. 2017). Plant genotypes produce various phenolic levels which can have a negative impact on a baculovirus' ability to infect the insect host systemically (Shikano et al. 2017). High oxidation of plant-derived phenolics cause midgut cells to be sloughed quicker resulting in lower probability of successful viral infection and establishment. Some plant genotypes can resist herbivory while maintaining low levels of phenolic content which implies baculoviruses can be used in tandem with certain genotypes (Shikano et al. 2017). These genotypes are thought to have evolved in areas where the pest and pathogen are at high levels the plant will undergo selective pressure to invest in defensive strategies that do not affect the pathogen or inhibit efficacy (Shikano et al. 2017). One crop where plant genotype might play a crucial role on the successful implementation of entomopathogens for insect management is in cotton where there is a dramatic decrease in host susceptibility to NPVs compared to other crops. *H. virescens* infected with AcMNPV were more

susceptible to the pathogen on lettuce or diet compared to cotton when infected orally, but not when inoculum was injected into the hemocoel, implying an issue with the pathogen initiating infection rather than inhibition of systemic spread of the pathogen (Hoover et al. 2000). The reduction in infection was contributed to an increased sloughing of infected midgut cells due to ingestion of cotton generating reactive oxygen species within the midgut lumen damaging epithelial cells (Hoover et al. 2000). Other issues such as unfavorable environment for the pathogen have also been explored (McLeod et al. 1977; Young et al. 1977).

Integrated Pest Management of *Helicoverpa zea*

Integrated Pest Management (IPM) incorporates multiple areas of science into an overarching applied approach at reducing crop losses from insects. IPM has seven foundational areas of knowledge that should be researched for the pest of interest prior to making an informed decision on control. The life cycle and behavior of the insect, its seasonal cycle (univoltine or multivoltine), population dynamics, proper identification, rearing or culturing data, sampling procedures, and the bio-economics (relates pest density to economic losses) are all areas of knowledge that should be explored before control tactics can be established (Pedigo and Rice 2009). Once these areas are explored, pesticide efficacy, natural enemies, host plant resistance, effects of modifying the environment, attempting to exclude the pest, or impacting the pest's reproductive capacity are all control tactics that can be explored (Pedigo and Rice 2009). Unfortunately, in modern agriculture we have relied predominantly on pesticide development and host plant resistance through genetic manipulation to produce

genetically modified organisms, leaving natural enemies, host plant resistance through tolerance, alteration of environment, exclusion, and reproduction impacts largely unexplored which was recently lamented in the IPM review authored by Peterson et al. (2018).

Insect of Study

Helicoverpa zea (Boddie) (Lepidoptera: Noctuidae) is a polyphagous insect that feeds on several wild and cultivated host plants. It is one of the most important crop pests in the New World feeding on soybeans, sorghum, cotton, and corn (Quaintance and Brues 1905; Fitt 1989; Musser et al. 2018; Cook 2018). In commercial crops, *H. zea* infestations are typically controlled by the application of an insecticide; however, they have become resistant to many insecticide classes including Pyrethroids (Abd-Elghafar et al. 1993; Kanga et al. 1996; Musser et al. 2015). Currently, the only viable options are to apply an expensive insecticidal chemistry such as diamides or utilize a natural entomopathogenic virus that is specific to Heliothines, *Helicoverpa armigera* Nucleopolyhedrovirus (HearNPV) (Adams et al. 2016; Black 2017). While much more cost effective, HearNPV has several limitations that must be considered before use. Few larvae will succumb to an infection once they reach the 4th instar (Ignoffo et al. 1978; Luttrell et al. 1982; Alam et al. 1987). The virus is degraded by ultraviolet light and quickly loses efficacy after foliar applications in crop fields, especially if the pest is not present (Ignoffo et al. 1972; Young and Yearian 1974; McLeod et al. 1977). Also, temperature and humidity can cause variability in control efficacy, and continuous generations are important to increase the likelihood of inducing an epizootic (Ignoffo et

al. 1976; Black et al. 2019). Due to these factors and others discussed previously, the efficacy of HearNPV is notorious for being highly variable, especially in cotton. The increase in variability in cotton relative to other crops such as soybeans was suggested to be caused by the pH of dew on the cotton leaves when it evaporated reaching a pH around 9.3, high enough to kill the viral particles (McLeod et al. 1977; Young et al. 1977). While this might explain some of the variability, with so many other potential factors effecting *H. zea* susceptibility to HearNPV unexplored, we cannot rule out the possibility that the nutritional composition of cotton compared to other crops might intrinsically increase survivorship of *H. zea* when exposed to HearNPV.

In today's conventional commercial cotton, the majority of the fields planted are now planted with a *Bt*-technology (Fleming et al. 2018; USDA 2019). However, recently one of the targeted pests, *H. zea*, has begun developing resistance to the toxins utilized (Tabashnik et al. 2013; Fleming et al. 2018). This has led to some technologies being managed like conventional non-*Bt* cotton (Little et al. 2017). To compound the problem, the leading foliar insecticide for this pest, diamides, are also beginning to show signs of resistance development which will ultimately lead to a need for alternative controls (Adams et al. 2016).

Historically, entomopathogens in cotton have not performed well; however, an understanding of the nutritional effects on *H. zea* immune response to the pathogens, and an understanding of differing dietary regimes on *H. zea* survival of pathogens might lead to a more thorough understanding of the underpinning mechanisms associated with this reduction in susceptibility displayed in cotton (McLeod et al. 1977; Young et al. 1977).

Although much is known about the insect immune system, nutrition, and nutritional effects on the immune system, little to nothing is known about one of the most important crop pests in the New World, *H. zea*, and its immune response to entomopathogens or the nutritional and ecological variables affecting its response. Elucidating these mechanisms involved in *H. zea* immunity could result in more effective use of pathogens as a biological control tactic in agroecosystems, possibly initiating the first step away from the "silver bullet" mentality current-day IPM programs tend to follow (Peterson et al. 2018).

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CHAPTER II

HELICOVERPA ZEA PHYSIOLOGICAL AND TRANSCRIPTIONAL IMMUNE RESPONSES TO INFECTION WITH DIFFERENT ENTOMOPATHOGENIC GROUPS

Introduction

The insect innate immune system is non-specific and assumed to be without memory, yet it still can be vitally effective at attacking and overcoming challenges by pathogens or parasitoids (Beutler 2004; Beutler and Hoffmann 2004; Schmidt et al. 2008). Insect immune systems are able to recognize non-self and altered-self molecular structures by means of pathogen recognition receptors (PRRs) that bind to lipid particles and pathogen-associated molecular patterns (PAMPs). PAMPs include lipopolysaccharides (LPSs), peptidoglycans (PGNs), and glucans common to pathogens. This identification mechanism allows for a targeted pathogen response that has minimal direct impact on the uninfected portions of the host (Kato et al. 1994; Lavine and Strand 2001; Janeway and Medzhitov 2002; Beutler 2003; Cheon et al. 2006). Cytokines and signaling pathways such as Toll, IMD, JNK and JAK/STAT are important in the identification of non-self/altered self, and the initiation of an immune response (Clark et al. 1997; Clark et al. 1998; Strand and Clark 1999; Lavine and Strand 2003; Clark et al. 2004; Bidla et al. 2007; Strand 2008a). The resulting immune response to invading pathogens is a two-pronged response consisting of humoral and cellular defenses. The humoral defense consists of antimicrobial peptide (AMP) production via the IMD and Toll signaling pathway, and the formation of other effector molecules through complex

proteolytic cascades such as the phenoloxidase (PO) cascade (Reviewed in Strand 2008a; Cornelis and Soderhall 2004; Yi et al. 2014; González-Santoyo and Córdoboa-Aguilar 2011; Wu et al. 2018). The second primary component of an insect's innate immune system is the cellular response to infection involving encapsulation, nodulation, phagocytosis, and apoptosis of pathogens mediated by the JNK signaling pathway, hemocyte signaling, and regulated by eicosanoids (Reviewed in: Strand 2008b; Satyavathi et al. 2014; Castillo et al. 2011).

Responses to Specific Pathogen Groups

Insect defense mechanisms against viral pathogens appear to utilize several different immune pathways, but are still relatively limited compared to other pathogen responses. There are many families of viruses that are pathogenic to insects; however, baculoviruses are the most widely studied and best understood. They also are currently being used in commercial agricultural production as biopesticides. Baculoviruses are large viruses with a circular dsDNA genome. Most baculoviruses are highly host specific, and only capable of replicating in a narrow range of related hosts. Baculoviruses must be ingested or gain entry to the hemocoel through a wound in order to initiate cellular invasion and replication. If ingested, the primary infection is in the midgut epithelial cells, and within 2 hours post-infection (hpi), a secondary infection can be established in the tracheal system (Engelhard et al. 1994). Once the infection is established in the trachea, the infection moves to the hemocoel and becomes systemic. Cellular and humoral defense mechanisms such as nodule formation, phagocytosis, and PO-derived reactive oxygen species production have been observed as potential antiviral

defense mechanisms; however, the primary antiviral defense appears to be RNA interference (RNAi) (Bronkhorst and van Rij 2014; Hillyer 2016; Trudeau et al. 2001; Popham et al. 2004; Shelby and Popham 2006; Jayachandran et al. 2012; Tsakas and Marmaras 2010). Currently, there are three types of known RNAi: small-interfering RNA (siRNA), microRNA (mRNA), and piwi-RNA (piRNA). Of these three, during a viral infection, the siRNA pathway is the more potent antiviral defense mechanism (Bronkhorst and van Rij 2014; Mueller et al. 2010; Van Rij et al. 2006; Wang et al. 2006; Campbell et al. 2008; Bronkhorst et al. 2012). The siRNA pathway functions as an RNA-degrading mechanism, with dsRNA being the central trigger for activation. These dsRNA strands are bound to dsRNA-binding proteins Loquacious PD isoform and R2D2, which are cofactors of Dicer-2, an RNase III enzyme. Dicer-2 processes the dsRNA into 21-nt siRNA duplexes (Sabin et al. 2013; Galiana-Arnoux et al. 2006). These duplexes are then loaded onto Argonaute-2 (AGO2) protein which uses the template strand to degrade sequence specific strands of RNA through cleavage (Czech et al. 2009). AGO2 is contained within the RNA induced silencing complex (RISC) (Bronkhorst and van Rij 2014). Although this defense mechanism specifically targets RNA, and has been shown to be effective against (-) RNA viruses, (+) RNA viruses, and dsRNA viruses, recent research has demonstrated that DNA viruses are also targets of the antiviral RNAi response (Mueller et al. 2010; Wang et al. 2006; Keene et al. 2004; Campbell et al. 2008; Bronkhorst et al. 2012; Jayachandran et al. 2012; Bronkhorst et al. 2013; Marques et al. 2013).

Bacterial entomopathogens, like viruses, must gain entry to the host either through oral ingestion through orifices such as spiracles or wounds. Once the physical barriers have been surmounted, the insect host relies on cellular and humoral immune responses to clear the infection. Once PRRs recognize and bind to the PAMPs produced by the bacterial pathogen, an immune response is elicited (Wang et al. 2019b; Wang et al. 2019c; Li et al. 2014). Cellular responses to bacterial invasion include phagocytosis and nodule formation, while humoral responses consist of activation of the PO cascade, and AMP production via the activation of either the Toll or IMD pathway, depending on the type of the LPSs in the bacterial cell wall (Myllymäki et al. 2014; Lindsay and Wasserman 2014; Xiong et al. 2015; Ratcliffe and Gagen 1976; Haine et al. 2008; Satyavathi et al. 2014).

Fungal entomopathogens are capable of directly penetrating the host cuticle through the deployment of an appressorium, or invade through orifices such as spiracles, wounds, or oral ingestion (Talbot 2019). Once the infection reaches the hemocoel, hemocytes will begin encapsulating spores, and the PO cascade melanizes the capsule, fumigating it with reactive oxygen species (Hung and Boucias 1996; Hung and Boucias 1992; Xiong et al. 2015; Lee et al. 2005). Furthermore, β-1,3-glucan is a major component of the fungal cell wall that functions in insects as a PAMP that will induce the activation of the Toll pathway, producing AMPs with activity against fungi (Lindsay and Wasserman 2014; Hillyer 2016; Hou et al. 2014).

Entomopathogenic nematodes are also capable of penetrating the insect cuticle or invade orifices similarly to fungal pathogens. However, recent evidence suggests that the

cuticle of some nematodes does not elicit an immune response from the insect host, thereby evading detection until farther into the infection (Binda-Rossetti et al. 2016; Jiao et al. 2018; Wiesner 1992; Wang et al. 2019a). During nematode infections, the primary immune response is one of encapsulation and melanization (Castillo et al. 2011). Once the nematode's bacterial symbiont is detected, the host will respond with AMP production based on the signaling pathway activated (Wang et al. 2019a; Castillo et al. 2011).

Expanding Immune Studies to Non-Model Insects

Currently, we have a fairly robust understanding of insect developmental signaling pathways, and how duplication and modification of those pathways has given rise to complex immune signaling pathways (Sackton et al. 2007; Palmer and Jiggins 2015). We also understand the basic mechanisms by which these signaling pathways are triggered, and the effector molecules they produce. However, most of the established immune pathway-pathogen associations have been studied in model organisms such as *Drosophila melanogaster*, and are rarely expanded to include broader insect taxa. The increasing availability of genomic data along with tools to study gene expression such as real time quantitative polymerase chain reaction provides new opportunities to expand in the understanding of insect immune responses to a range of socially or economically important insects, either for their control or conservation. Currently, most hypothesis-driven immune-related studies continue to employ physiological analyses to measure immune responses, and typically focus on one pathogen and one time point post-infection (Lee et al. 2006). While some more recent papers have begun to utilize

transcriptional analyses to understand regulation of the known pathways, these studies are typically exploratory in nature usually fail to incorporate physiological measurements (Zhao et al. 2013; Hou et al. 2014; Xiong et al. 2015). As with physiological analyses, they usually fail to incorporate more than one pathogen or time point into the analysis.

We set out to broaden our knowledge of immunity in economically important non-model insects by conducting a systematic assessment of multiple insect defensive responses over the course of infection when challenged by several major pathogen groups. We utilized *Helicoverpa zea* larvae, one of the most important agricultural pests in the Western Hemisphere (Musser et al. 2019; Musser 2019; Reisig et al. 2019; Black et al. 2019). These larvae were separately exposed to four different pathogens, and immune responses were measured via physiological and transcriptional analyses at three different points across the infection cycle. This study provides a unique understanding, withing a single organismal system, of how a highly destructive pest copes with invasion by broad range of different pathogens. Furthermore, this study provides the foundation for future studies investigating the ecology and evolution of pathogen resistance in *Helicoverpa* species.

Materials and Methods

Insects and Pathogens

Helicoverpa zea caterpillars were purchased from Benzon Research Inc. (Carlisle, PA) as eggs and were reared on artificial diet purchased from Southland Products Inc. (Lake Village, AR) until reaching the targeted instar. Larvae were

maintained in rearing chambers under a constant temperature of 25°C, relative humidity of 70%, and light-dark ratio of 14:10 for all experiments. The strain of *Helicoverpa armigera* nucleopolyhedrovirus (HearNPV) was provided by AgBiTech LLC (Fort Worth, TX), and is listed under the trade name Heligen®. To get to the desired concentration for inoculation, the highly concentrated viral solution was serially diluted from 7.5×10^9 occlusion bodies/mL to 7.5×10^5 occlusion bodies/mL. The *Beauveria bassiana* strain GHA was isolated from BotaniGard Maxx® purchased from BioWorks Inc. (Victor, NY). The spores were extracted by placing 30mL of the solution into a 50mL Falcon tube and centrifuging at 3000 rpm for 10 minutes, then removing the supernatant and adding 30mL of sterile water. The solution was then vortexed, and this process was repeated three times before the final pellet was resuspended in sterile water and stored at 4°C until needed. Spore suspension viability was tested prior to use by making a serial dilution and plating the 5^{th} and 6^{th} dilution. The plates were allowed to incubate at room temperature for three days and then colonies were counted and multiplied by the dilution factor to determine viable spore concentration in the spore suspension. The *Bacillus thuringiensis* pathogen was diluted from Thuricide BT® purchased from Southern AG Insecticides, Inc. (Hendersonville, NC) to 7.5×10^5 CFUs/mL. *Steinernema carpocapsae* was purchased from ARBICO Organics (Oro Valley, AZ), and serially diluted to 7.5×10^5 nematodes/mL.

Inoculation Procedures

Once *H. zea* larvae molted to 3^{rd} instar, they were inoculated with one of five treatments determined prior to initiation of the experiment. This was done by pipetting a

10µL drop of liquid containing either the treatment pathogen dosage or a control of sterile deionized water onto a fresh piece of artificial diet, approximately 50mg, where it was absorbed. Inoculation time zero was defined as this point of pathogen introduction into the individual larva's environment, which was a sterilized 2oz deli cup (ULINE, Pleasant Prairie, WI). The larvae were then allowed to feed on the infested diet and only those that consumed the inoculated diet cube were utilized in each of the two experiments described below.

Experiment 1: Temporal Physiological Immune Response

This experiment utilized five pathogen treatments (Control, HearNPV, *B. bassiana*, *B. thuringiensis*, and *S. carpocapsae*), and subdivided each treatment into sample times. Three temporal sampling points of 4 hours, 24 hours, and 48 hours post-inoculation were implemented to develop an understanding of how the *H. zea* immune response changes during a pathogen invasion. Later temporal sample points were not possible because most larvae succumbed to the pathogens by three days and survivors across all pathogens were too few for meaningful analysis. Individual larvae were sampled by extracting their hemolymph at the designated sample times as described below. Hemolymph from two larvae were pooled to make one biological replicate. Each pathogen × time-treatment had 32-34 biological replicates collected across three independent trials.

Hemolymph Extraction

Hemolymph extraction occurred by sterilizing the larva with an ethanol wash, weighing the larva, and then chilling the larva on ice before piercing the larva with a

sterile 27-gauge needle between the second pair of prolegs. The insect hemolymph was allowed to drain directly into an Eppendorf tube on ice and placed into a -20°C freezer immediately upon completion of the extraction. While extracted volumes varied across biological samples, each sample had at least 70µL to complete all the physiological assays described below.

Hemolymph Phenoloxidase and Prophenoloxidase Assay

An 8µL aliquot of hemolymph was added to 360µL of sodium cacodylate (NaCac) in a 2mL microcentrifuge tube. The sample was then evenly divided into two 2mL microcentrifuge tubes. One tube had the prophenoloxidase (PPO) activated by adding 20µL of 20mg/mL chymotrypsin suspended in NaCac buffer, while the other tube served as the spontaneously activated phenoloxidase (PO) control with 20µL NaCac added. Samples were incubated at 25°C for one hour to allow the PPO time to be activated prior to microplate reader analysis. All analyses were run in duplicate using Costar® 96 well flat bottom plates and analyzed in a Infinite M200 Pro microplate reader (Tecan, Männedorf, Switzerland). Plates were first loaded with 90µL of the sample solution per well, and then 90µL of 4mM dopamine was pipetted into each well. Once all wells had both the sample solution and dopamine, the plate was placed into the microplate reader and the absorbance was measured at 492nm. The amount of phenoloxidase in the sample was calculated in phenoloxidase units, where one unit is the amount of enzyme required to increase the absorbance by 0.001 per minute.

Hemolymph Protein Assay

Protein was measured using a BCA Protein Assay Kit II (BioVision Inc., Milpitas, CA) by adding 25µL of the hemolymph solution to 200µL of the BCA working reagent in each of the Costar® 96 well flat bottom plate wells. The plate was covered and incubated at 37°C for 30 minutes. After the incubation the absorbance was measured at 562nm. A standard curve using the provided standards was utilized to determine protein concentration (µg/mL). Once protein concentrations were known, phenoloxidase units were expressed as phenoloxidase units per mg of protein.

Hemocyte Count

Hemocyte counts were determined using an improved Neubauer hemocytometer. The hemocytometer was loaded with 8µL of pure hemolymph, allowed to settle for 20 minutes and the five non-adjacent squares were counted on each side of the hemocytometer to give an estimate of hemocyte density.

Antimicrobial Activity Assay

Lytic activity against the bacterium *Micrococcus lysodeikticus* was determined using a lytic zone assay. Agar plates were made prior to the assay by mixing 10mL of agar suspension containing the following: 1.5g agar, 0.75g *M. lysodeikticus* in 50mL 0.2 M potassium phosphate buffer, 0.1mg/mL streptomycin sulphate, and 67mM potassium phosphate buffer (pH 6.4) and pouring the mixture into a plastic petri dish and stored in a -4°C refrigerator. For each plate, approximately 13 holes with a diameter of 2mm were punched into the agar and filled with 1µL of hemolymph, with two technical replicates per sample. The plates were incubated at 32˚C for 24 hours, photographed, and the

diameter of the clear zones calculated with ImageJ imaging software. Standard curves were obtained using a serial dilution of egg white lysozyme, and concentration of egg white lysozyme equivalents were calculated. Standard curves were developed for each batch of plates. Based on the logarithmic connection to lysozyme concentration, diameters of lytic zones obtained from the hemolymph samples were converted to HLAs (ng/µL – equivalents of hen egg white lysozyme activity).

Encapsulation Response Assay

Immediately after the hemolymph extraction, a 3mm long piece of nylon monofilament was inserted completely into the puncture wound of each larva in such a way to minimize the potential of rupturing the midgut. Surviving larvae were returned to diet for 24 hours. After that time, the surviving larvae were frozen and upon death the nylon monofilament was dissected out, mounted on a slide and photographed. The level of melanization and area of cell cover was quantified using ImageJ (Reuden et al. 2017) imaging software distributed by Fiji (Schindelin et al. 2012). One larva in each pooled biological sample was subjected to an encapsulation assay, however; frequently the gut was ruptured and the larva discarded, or the nylon filament was not recovered during the dissection.

Statistical Analysis

Pair-wise MANOVAs were conducted for each Pathogen × Control pairing, with Treatment and Time as main effects, and the measured immune responses as dependent variables. The average pooled larval weight was used as a covariate since all dependent variables were analyzed for all samples. Pillai's trace statistic was used to compare

differences from the Control. Then, each immune response was subjected to an ANOVA and Tukey's HSD. All data were checked for conformity and normalcy. All analyses were conducted in R Studio (R Core Team, 2020).

Experiment 2: Temporal Transcriptional Immune Response

This experiment utilized the same five pathogen treatments as in Experiment 1, but each treatment was subdivided into three different sample times: 24 hours, 48 hours, and 72 hours post-inoculation. Changes in the expression of genes involved in the major immune pathways were measured as opposed to physiological immune responses. Twenty-five larvae were reared for each pathogen × sample time treatment combination. Five larvae were pooled for each biological replicate, resulting in five biological replicates per pathogen x sample time treatment combination. Hemolymph was extracted as described above, except immediately following extraction, the 2mL microcentrifuge tubes were flash frozen in liquid nitrogen then stored in a -80°C freezer. RNA was extracted from the hemolymph samples using the RNeasy Mini Kit (Qiagen, Hilden, Germany). RNA concentrations were determined by using a NanoView Plus (General Electric, Boston, MA). RNA concentrations were then standardized to 100ng/μL before being converted to cDNA using iScript gDNA Clear cDNA Synthesis Kit (Bio-Rad Laboratories, Hercules, CA). The resulting DNA concentrations were determined with a NanoView Plus, and diluted to 100ng/μL by adding RNase and DNase free water. Once sample DNA concentrations were standardized, Quantitative Real-Time PCR (qPCR) was conducted using primers targeting specific immune genes, *Actin* as a housekeeping gene and *RPS3* as a verification gene that *Actin* was not differentially expressed across

treatments (Table II.1). Transcript-specific primers were designed by first extracting putative transcript sequences from the published *H. zea* draft genome and associated annotation file using gffread (Pearce et al. 2017; Pertea and Pertea 2020). This generated a sequence file of parsed mRNA and coding sequences (CDSes) from which we ran BLASTn searches against *Helicoverpa armigera* CDSes of *Actin*, *RPS3*, *PPO2*, *Argonaute-2*, *JNK*, *Dorsal*, and *Relish* as the query. The obtained *H. zea* transcripts were then secondarily validated through BLAST searches of the NCBI database to confirm sequence identifications. The obtained transcripts were passed through the PrimerQuest™ Tool provided by Integrated DNA Technologies to generate qPCR primers. Conventional PCR products were obtained from each primer pair and purified using a Monarch DNA Gel Extraction Kit (T1020S) and submitted for Sanger Sequencing to validate their specificity to the desired transcripts. qPCR was conducted using SYBR™ Green (Bio-Rad Laboratories, Hercules, CA) and Precision Blue Real-Time PCR Dye (Bio-Rad Laboratories, Hercules, CA) in a C1000 Touch Thermal Cycler with the CFX384 Real-Time System attachment (Bio-Rad Laboratories, Hercules, CA). Data was then exported into CFX Maestro (Bio-Rad Laboratories, Hercules, CA) software, and analyzed using ANOVA and Tukey's HSD post hoc test in R Studio (R Core Team, 2020). All target genes were previously determined to be differentially expressed during pathogenic infection in *H. armigera* and *S. frugiperda*, two species closely related to *H. zea* (Xiong et al. 2015; Karamipour et al. 2018).

Table II.1: Forward and Reverse primers used in the transcriptional analyses.

Gene of Interest		Primer Sequence	Annealing Temp. (°C)	Size (bp)
Actin	Forward	ATGGGACAGAAGGACTCGTA	54.9	100
	Reverse	GGTGCCAGATCTTCTCCATATC	54.8	
PPO2	Forward	GATTACTCCGAAGGGTGACAAA	54.6	785
	Reverse	ACGGTGAACTGAGGGTATCT	55.2	
JNK	Forward	GAATGTCGCCATCAAGAAGTTG	54.4	751
	Reverse	ACGCGTTTAGAAGACCGATTAT	54.1	
Dorsal	Forward	TGTCACCAAAGATGAGCCTTAC	54.9	543
	Reverse	CGAGGTTCTTGAACTGGTACTC	54.6	
Relish	Forward	TGTGATTGACTGTGCGTGATA	54.2	750
	Reverse	GGAGAACTATGAGGAGGAGAGT	54.9	
Argonaute-2	Forward	TCAGGGCCTACTCCTGTATT	54.9	107
	Reverse	GGTGGCATAGCAGTAGAAGTAG	54.8	
Ribosomal Protein S3	Forward	CGGCTGTCCAATAGGATCTTC	54.8	219
	Reverse	CAGCCTCTTCATCTCATCCTTG	54.9	

Results

Experiment 1: Physiological Immune Response

Viral Entomopathogen: *Helicoverpa armigera* nucleopolyhedrovirus

The physiological responses of prophenoloxidase (PPO) and phenoloxidase (PO) levels, lysozyme concentrations, number of hemocytes, and encapsulation ability for Control larvae and larvae infected with HearNPV were analyzed using a MANOVA with main effects being Treatment and Time. There was a significant Treatment effect (Pillai = 0.153, $F_{5, 143} = 5.18$, $p < 0.001$) and a significant Time effect (Pillai = 0.435, $F_{10, 288} = 8.00$, $p < 0.001$); however, the Treatment × Time interaction was not significant. Analysis of variance revealed at 4 hours post-inoculation (hpi), there was a significant increase in the number of hemocytes in larvae infected with HearNPV compared to the Control ($\alpha = 0.05$, $F_{1, 65} = 4.21$, $p = 0.0403$), but all other physiological measurements

were not significantly different from the Control group (Figure II.1). By 24 hpi, only encapsulation ability was significantly lower for HearNPV-infected larvae ($\alpha = 0.1$, $F_{1, 44} = 3.94$, $p = 0.053$), which was continued at 48 hpi ($\alpha = 0.1$, $F_{1, 53} = 3.95$, $p = 0.052$) (Figure II.1). Also, at 48 hpi, PO concentrations were significantly lower in HearNPV-infected larvae compared to the Control ($\alpha = 0.1$, $F_{1, 66} = 3.19$, $p = 0.079$), while all other physiological responses were not significantly different from the Control (Figure II.1).

Bacterial Entomopathogen: Bacillus thuringiensis

The MANOVA results showed there was a significant Treatment × Time interaction (Pillai = 0.207, $F_{10, 260} = 3.01$, $p < 0.001$), and both Treatment and Time were independently significant. ANOVAs revealed there was no significant differences across all physiological responses measured at 4 hpi; however, by 24 hpi, PO and PPO levels had increased in *B. thuringiensis*-infected larvae compared to Control larvae ($F_{1, 62} = 6.33$, $p < 0.05$; $F_{1, 62} = 3.19$, $p < 0.1$) (Figure II.2). At 48 hpi, encapsulation ability, PO levels, and PPO levels were significantly higher in *B. thuringiensis*-infected larvae compared to Control larvae ($F_{1, 47} = 5.43$, $p <0.05$; $F_{1, 66} = 13.8$, $p < 0.001$; $F_{1, 66} = 15.4$, $p < 0.001$), and hemocyte number was significantly lower in *B. thuringiensis*-infected larvae compared to Control larvae ($F_{1, 66} = 8.63$, $p < 0.01$) (Figure II.2).

Fungal Entomopathogen: Beauveria bassiana

MANOVA results comparing *B. bassiana*-infected larvae to Control larvae were not significant for Treatment or Treatment × Time interaction, but were significant for Time, showing that the physiological response changes over time, but that it is not necessarily an immune response change (Pillai = 0.342, $F_{10, 264} = 5.45$, $p < 0.001$). When

the α-value was reduced to $\alpha = 0.1$, only one physiological response variable was significantly different across treatments (Figure II.3). PPO levels in *B. bassiana*-infected larvae was significantly lower than Control larvae 24 hpi ($F_{1, 62} = 2.97$, $p < 0.1$) (Figure II.3).

Entomopathogenic Nematode: Steinernema carpocapsae

The MANOVA results showed a significant Treatment × Time interaction effect (Pillai = 0.142, $F_{10, 280} = 2.14$, $p < 0.05$), and a significant Time effect (Pillai = 0.414, $F_{10, 280} = 7.30$, $p < 0.001$), but no significant effect by Treatment. ANOVAs revealed no significant differences in physiological responses between Control larvae and larvae infected with *S. carpocapsae* at 4 hpi. By 24 hpi, encapsulation ability was decreased for *S. carpocapsae*-infected larvae compared to Control larvae ($F_{1, 45} = 3.59$, $p < 0.1$) (Figure II.4). At 48 hpi, hemocyte numbers were significantly lower in *S. carpocapsae*-infected larvae ($F_{1, 64} = 3.32$, $p < 0.1$) (Figure II.4).

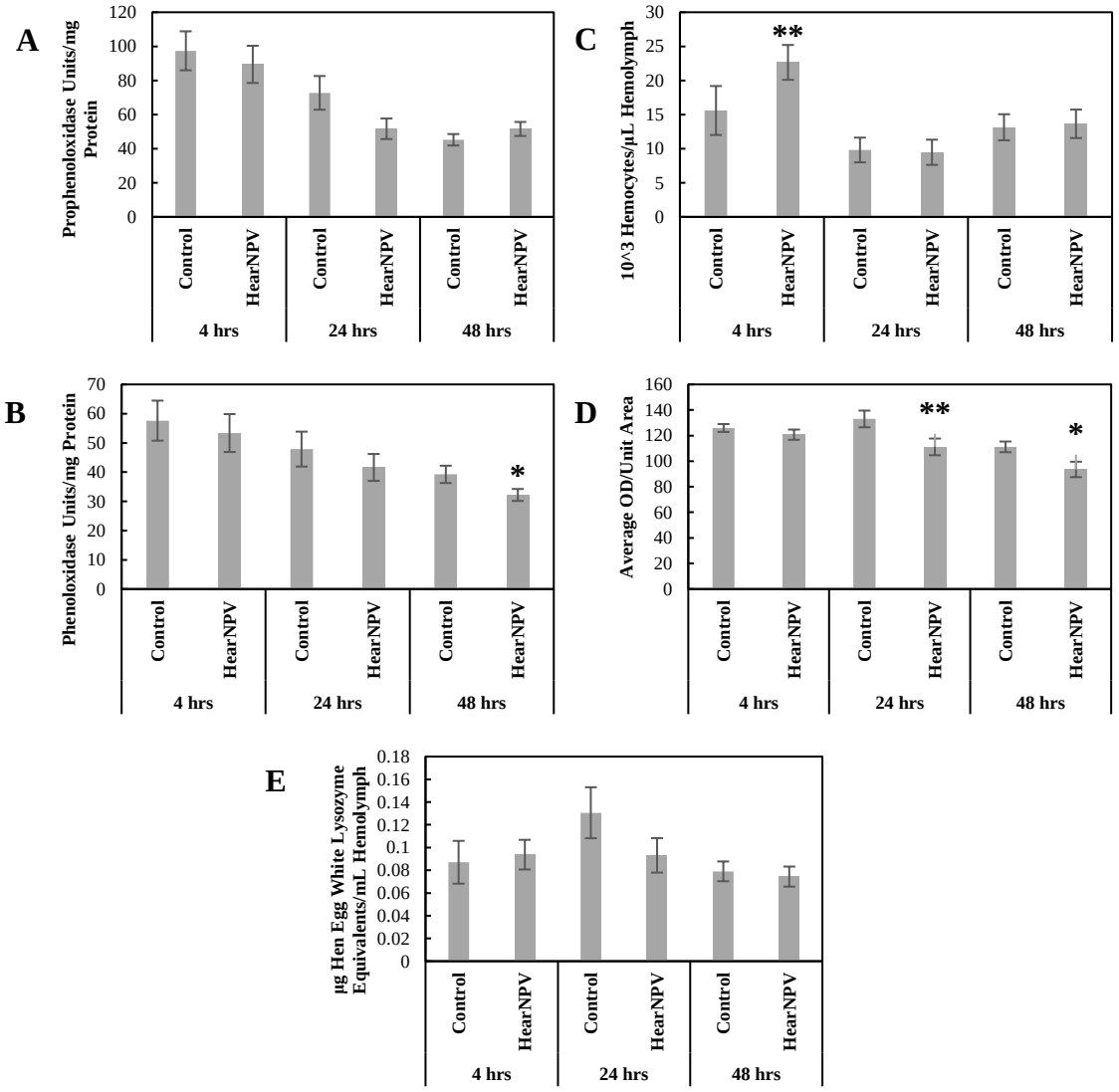

Figure II.1: Comparison of physiological immune responses between Control and *Helicoverpa armigera* nucleopolyhedrovirus-infected *Helicoverpa zea* over time. (A) Prophenoloxidase levels as a function of hemolymph protein levels (Mean ± SE), (B) Phenoloxidase levels as a function of hemolymph protein levels (Mean ± SE), (C) Total hemocyte numbers (103) per µl of hemolymph (Mean ± SE), (D) Encapsulation activity reported in ocular density per unit area (Mean ± SE), and (E) Lysozyme concentrations reported in hen egg white lysozyme equivalent per ml of hemolymph (Mean ± SE). A single asterisk indicates a marginally significant effect (ANOVA, $P < 0.1$) of treatment relative to the Control at that time point. Double asterisks indicate a stronger significant effect (ANOVA, $P < 0.05$) of treatment relative to the Control at that time point (n = 74-100 insects per treatment).

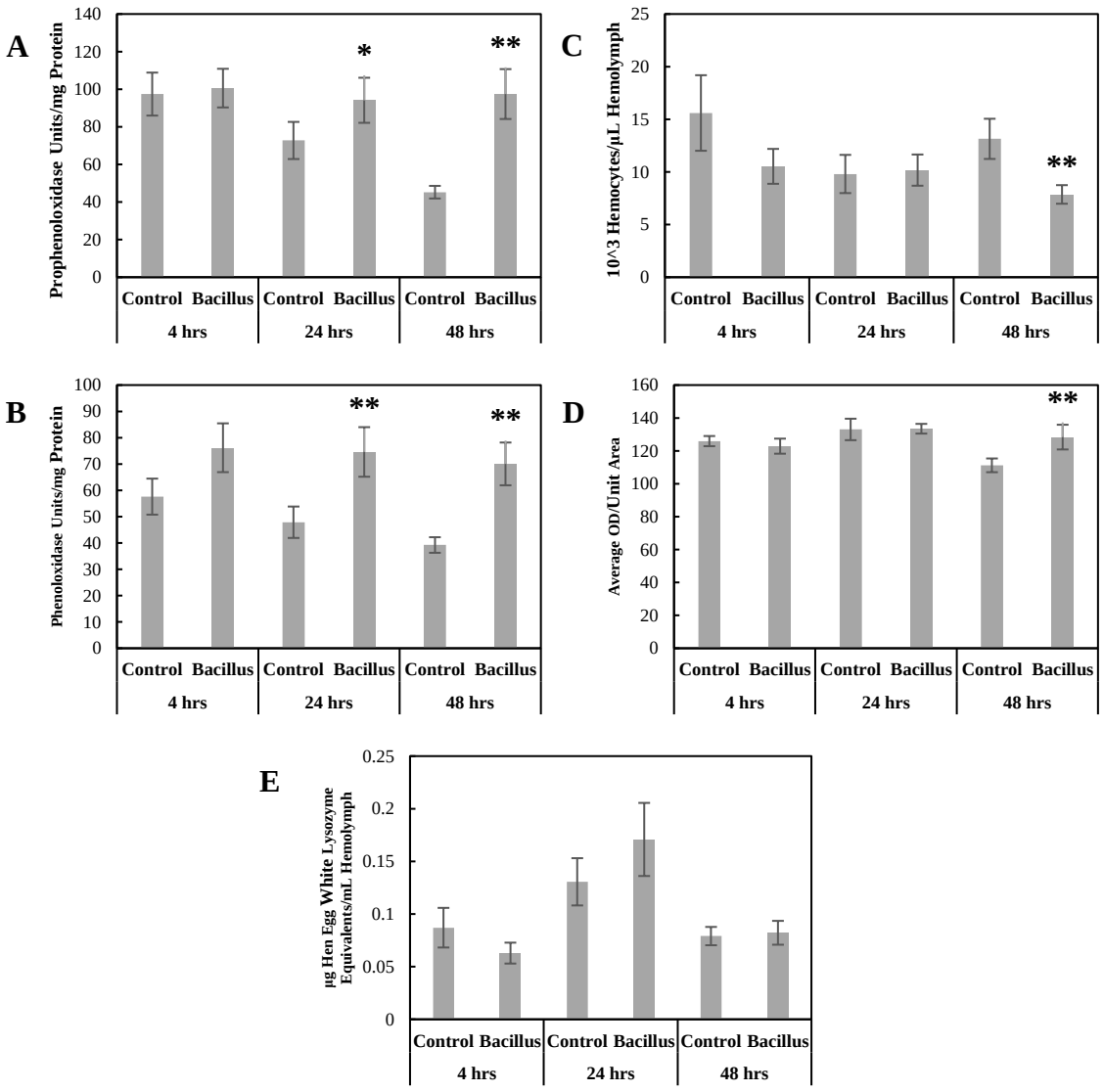

Figure II.2: Comparison of physiological immune responses between Control and *Bacillus thuringiensis*-infected *Helicoverpa zea* over time. (A) compares prophenoloxidase levels as a function of hemolymph protein levels (Mean ± SE), (B) compares phenoloxidase levels as a function of hemolymph protein levels (Mean ± SE), (C) compares total hemocyte numbers (103) in hemolymph (Mean ± SE), (D) compares encapsulation activity (Mean ± SE), and (E) Lysozyme concentrations reported in hen egg white lysozyme equivalent per ml of hemolymph (Mean ± SE). The single asterisk indicates a significant effect (ANOVA, $P < 0.1$) of treatment relative to the Control at that time point. The double asterisk indicates a stronger significant effect (ANOVA, $P < 0.05$) of treatment relative to the Control at that time point (n = 60-99 insects per treatment).

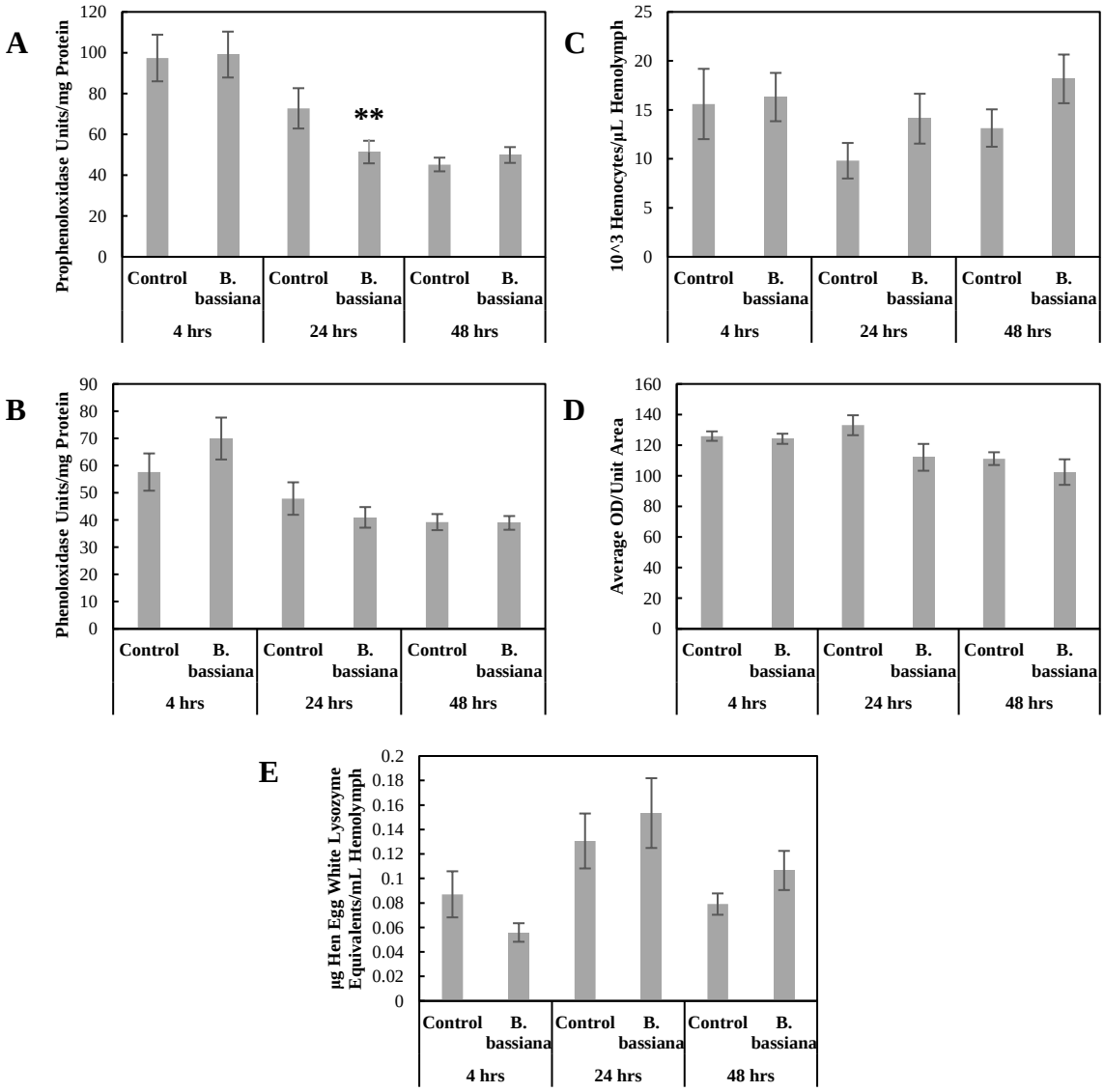

Figure II.3: Comparison of physiological immune responses between Control and *Beauveria bassiana*-infected *Helicoverpa zea* over time. (A) compares prophenoloxidase levels as a function of hemolymph protein levels (Mean ± SE), (B) compares phenoloxidase levels as a function of hemolymph protein levels (Mean ± SE), (C) compares total hemocyte numbers (103) in hemolymph (Mean ± SE), (D) compares encapsulation activity (Mean ± SE), and (E) Lysozyme concentrations reported in hen egg white lysozyme equivalent per ml of hemolymph (Mean ± SE). The single asterisk indicates a significant effect (ANOVA, $P < 0.1$) of treatment relative to the Control at that time point. The double asterisk indicates a stronger significant effect (ANOVA, $P < 0.05$) of treatment relative to the Control at that time point (n = 62-100 insects per treatment).

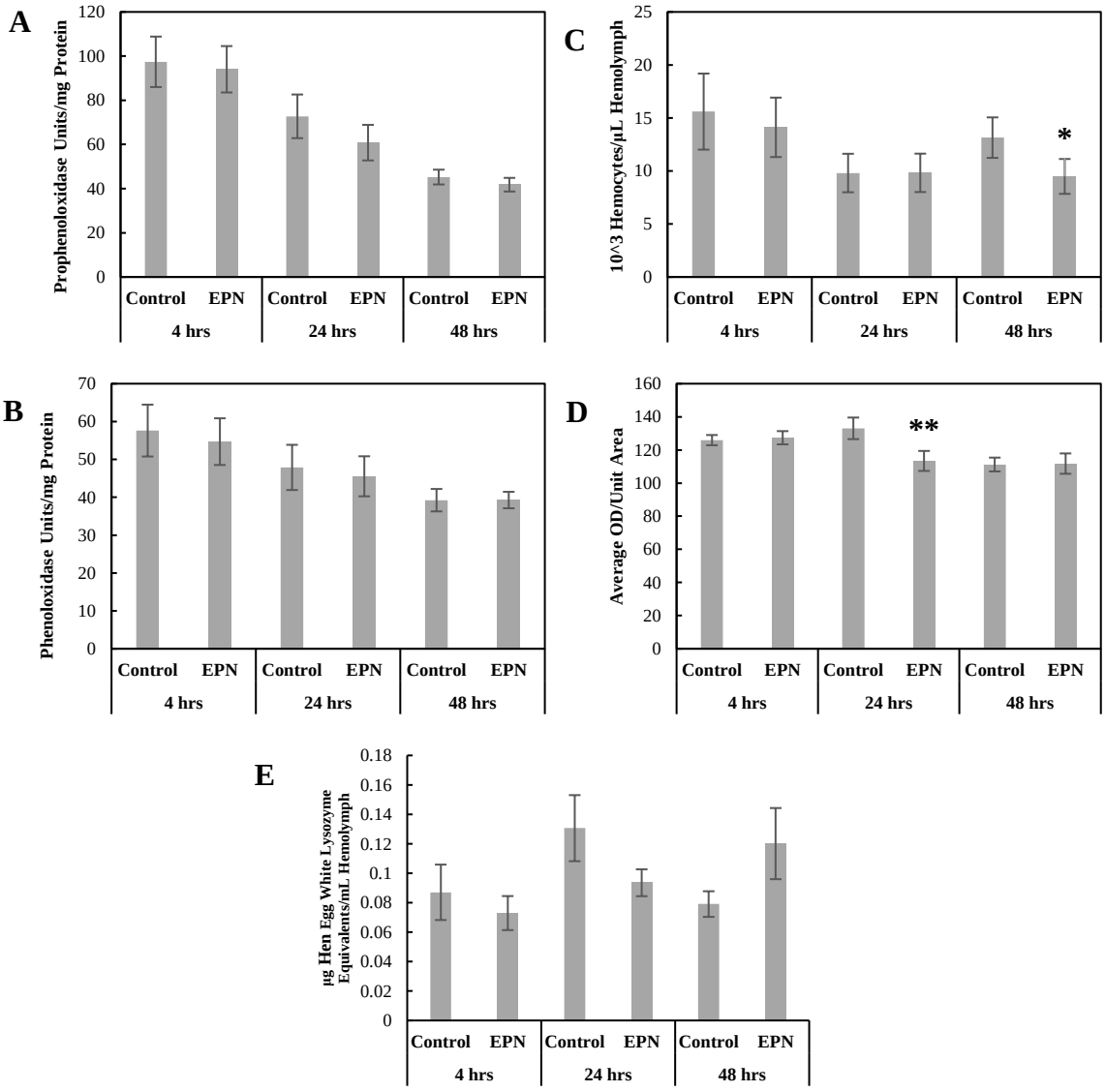

Figure II.4: Comparison of physiological immune responses between Control and *Steinernema carpocapsae*-infected *Helicoverpa zea* over time. (A) compares prophenoloxidase levels as a function of hemolymph protein levels (Mean ± SE), (B) compares phenoloxidase levels as a function of hemolymph protein levels (Mean ± SE), (C) compares total hemocyte numbers (10^3) in hemolymph (Mean ± SE), (D) compares encapsulation activity (Mean ± SE), and (E) Lysozyme concentrations reported in hen egg white lysozyme equivalent per ml of hemolymph (Mean ± SE). The single asterisk indicates a significant effect (ANOVA, $P < 0.1$) of treatment relative to the Control at that time point. The double asterisk indicates a stronger significant effect (ANOVA, $P < 0.05$) of treatment relative to the Control at that time point (n = 70-99 insects per treatment).

Experiment 2: Temporal Transcriptional Immune Response

Viral Entomopathogen: *Helicoverpa armigera* nucleopolyhedrovirus

The relative gene expression levels for several different immune response signaling pathways were analyzed in a MANOVA with Treatment and Time as main effects, and relative gene expression levels of *Dorsal*, *Argonaute-2*, *PPO-2*, *JNK*, and *Relish* as variables. MANOVAs comparing HearNPV-infected larval gene expression to Control larval gene expression revealed a significant interaction effect of Treatment × Time (Pillai = 0.974, $F_{10, 42} = 3.99$, $p < 0.001$), and significant effects by both Treatment and Time independently (Pillai = 0.591, $F_{5, 20} = 5.79$, $p < 0.001$; Pillai = 1.49, $F_{10, 42} = 12.26$, $p < 0.001$). ANOVAs revealed no significant differences across immune gene expression levels 24 hpi. By 48 hpi, only *Dorsal* gene expression differed from the Control, with significantly lower expression levels in HearNPV-infected larvae ($F_{1,8} = 3.15$, $p < 0.1$) (Figure II.5). At 72 hpi, *Argonaute-2*, *Dorsal*, *PPO-2*, and *Relish* genes were all differentially expressed compared to the Control, with significantly lower levels of expression ($F_{1, 8} = 15.2$, $p < 0.05$; $F_{1, 8} = 3.83$, $p < 0.1$; $F_{1, 8} = 41.7$, $p < 0.001$; $F_{1 ,8} = 14.2$, $p < 0.05$) (Figure II.5).

Bacterial Entomopathogen: Bacillus thuringiensis

The MANOVA results revealed a significant effect by Treatment (Pillai = 0.719, $F_{5, 20} = 10.26$, $p < 0.001$), Time (Pillai = 1.54, $F_{10, 42} = 13.98$, $p < 0.001$), and Treatment × Time interaction (Pillai = 1.09, $F_{10, 42} = 4.99$, $p < 0.001$). At 24 hpi, ANOVAs revealed a significant increase in *Relish* gene expression levels in *B. thuringiensis*-infected larvae compared to Control gene levels ($F_{1, 8} = 6.15$, $p < 0.05$), and a significant decrease in

PPO-2 expression levels ($F_{1, 8}$ = 12.5, p < 0.05), with no differences in expression levels for *Argonaute-2*, *JNK*, or *Dorsal* (Figure II.6). At 48 hpi, *Argonaute-2*, *Dorsal*, and *JNK* expression levels were significantly lower in *B. thuringiensis*-infected larvae ($F_{1, 8}$ = 15.8, p < 0.05; $F_{1, 8}$ = 6.5, p < 0.05; $F_{1, 8}$ = 9.04, p <0.05), with *Relish* and *PPO-2* expression levels not being significantly different between treatments (Figure II.6). By 72 hpi, *Argonaute-2* and *PPO-2* expression levels were significantly reduced in *B. thuringiensis*-infected larvae ($F_{1, 8}$ = 7.24, p < 0.05; $F_{1, 8}$ = 31.3, p < 0.05), and *Relish* expression levels were significantly higher than the Control group ($F_{1, 8}$ = 18.6, p < 0.05) (Figure II.6).

Fungal Entomopathogen: Beauveria bassiana

The MANOVA results showed Time as the only significant main effect (Pillai = 1.61, $F_{10, 42}$ = 17.21, p < 0.001), and both Treatment and the Treatment × Time interaction were not significant; therefore, no immune response was observed. Univariate ANOVAs showed no significant differences in gene expression across all genes tested at 24 hpi (Figure II.7). However, by 48 hpi, *Argonaute-2* expression was significantly reduced compared to Control expression levels ($F_{1, 8}$ = 4.14, p < 0.1) (Figure II.7). At 72 hpi, *JNK* and *Relish* expression levels were significantly higher than the Control ($F_{1, 8}$ = 26.4, p < 0.001; $F_{1, 8}$ = 6.8, p < 0.05) (Figure II.7). This difference between MANOVA and univariate ANOVA results could be due to a small sample size (n=5) affecting the statistical power of the MANOVA.

Entomopathogenic Nematode: Steinernema carpocapsae

The MANOVA results showed both Treatment and Treatment × Time interaction did not have a significant effect on the data, only Time was a significant effect (Pillai = 1.53, $F_{10, 42} = 13.82$, $p < 0.001$); therefore, no immune response was detected. ANOVA results showed no significant differences in gene expression for either the 24-hpi or the 48-hpi samples (Figure II.8). However, the 72-hpi samples revealed *JNK* and *Dorsal* expression levels were significantly elevated compared to the Control treatment ($F_{1, 8} = 27.2$, $p < 0.001$; $F_{1, 8} = 5.94$, $p < 0.05$) (Figure II.8). This difference between MANOVA and univariate ANOVA could be due to a small sample size (n=5), reducing the statistical power of the MANOVA.

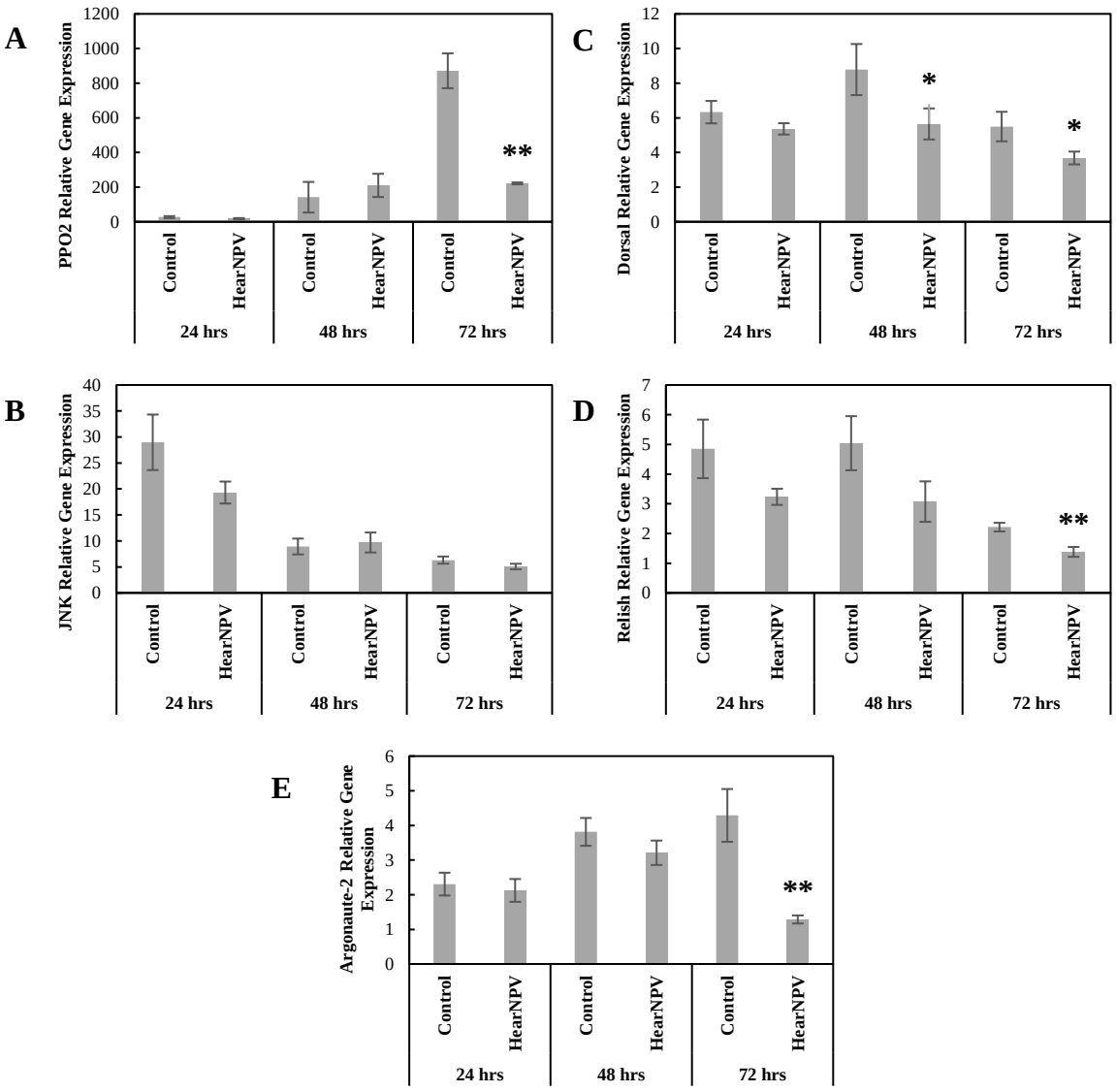

Figure II.5: Comparison of relative expression levels of immune-related genes in Control and *Helicoverpa armigera* nucleopolyhedrovirus-infected *Helicoverpa zea*. (A) *PPO-2* (Mean ± SE), (B) *JNK* (Mean ± SE), (C) *Dorsal* (Mean ± SE), (D) *Relish* (Mean ± SE), and (E) *Argonaute-2* (Mean ± SE). *Actin* was used as the housekeeping gene. The single asterisk indicates a significant effect (ANOVA, $P < 0.1$) of treatment relative to the Control at that time point. The double asterisk indicates a stronger significant effect (ANOVA, $P < 0.05$) of treatment relative to the Control at that time point ($n = 5$ insects per treatment).

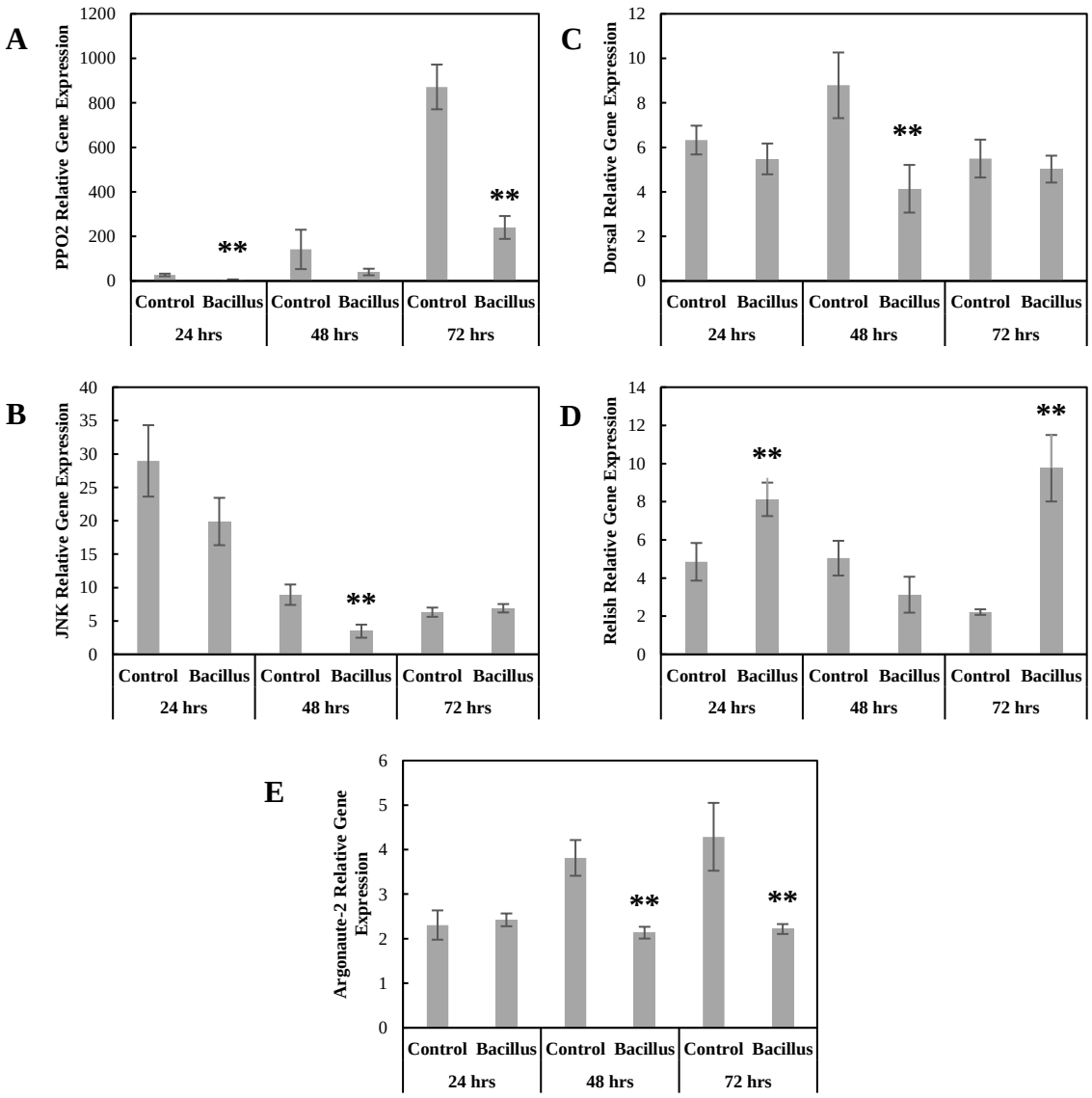

Figure II.6: Comparison of relative expression levels of immune-related genes in Control and *Bacillus thuringiensis*-infected *Helicoverpa zea*. (A) *PPO-2* (Mean ± SE), (B) *JNK* (Mean ± SE), (C) *Dorsal* (Mean ± SE), (D) *Relish* (Mean ± SE), and (E) *Argonaute-2* (Mean ± SE). *Actin* was used as the housekeeping gene. The single asterisk indicates a significant effect (ANOVA, $P < 0.1$) of treatment relative to the Control at that time point. The double asterisk indicates a stronger significant effect (ANOVA, $P < 0.05$) of treatment relative to the Control at that time point ($n = 5$ insects per treatment).

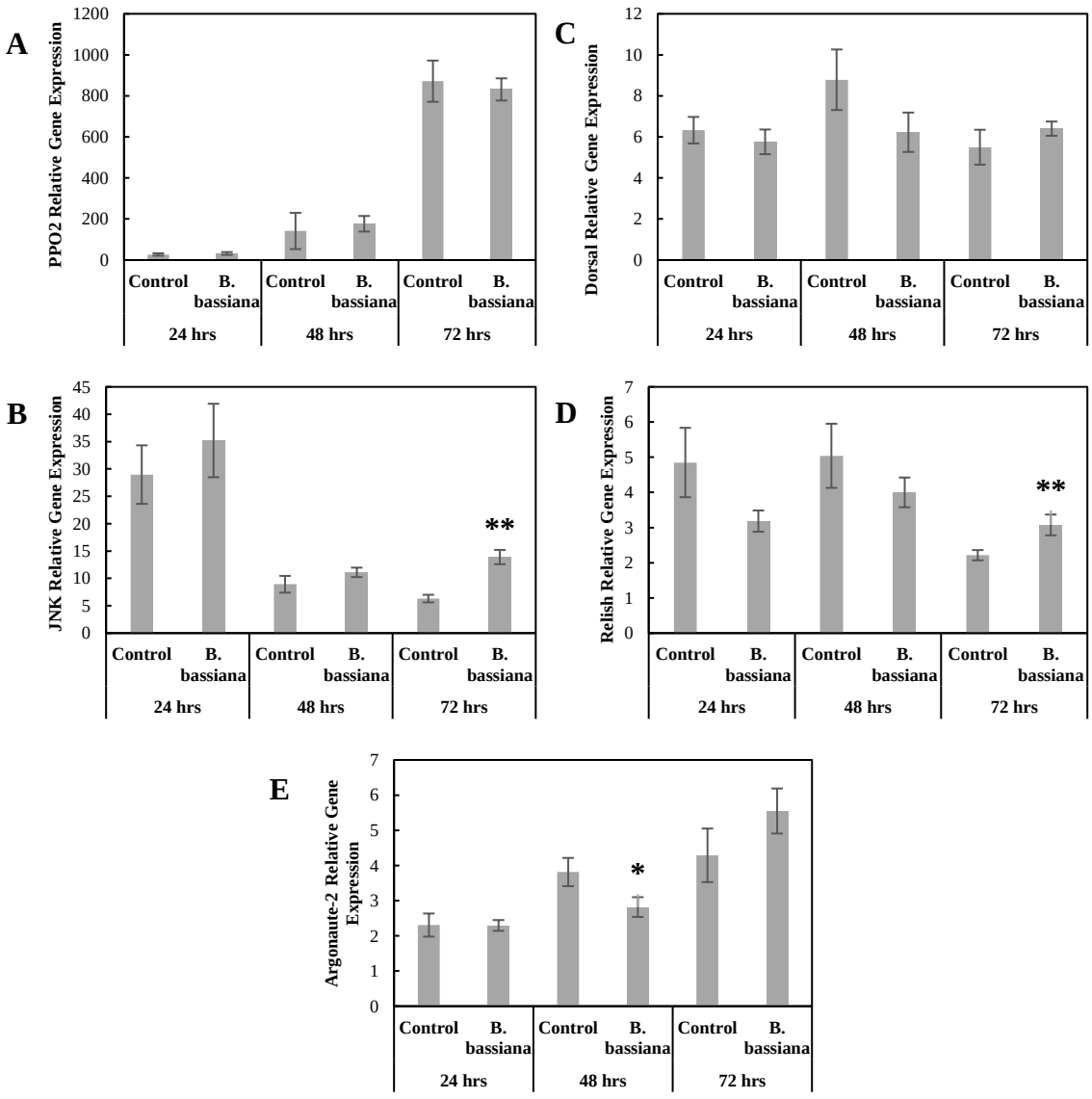

Figure II.7: Comparison of relative expression levels of immune-related genes in Control and *Beauveria bassiana*-infected *Helicoverpa zea*. (A) *PPO-2* (Mean ± SE), (B) *JNK* (Mean ± SE), (C) *Dorsal* (Mean ± SE), (D) *Relish* (Mean ± SE), and (E) *Argonaute-2* (Mean ± SE). *Actin* was used as the housekeeping gene. The single asterisk indicates a significant effect (ANOVA, $P < 0.1$) of treatment relative to the Control at that time point. The double asterisk indicates a stronger significant effect (ANOVA, $P < 0.05$) of treatment relative to the Control at that time point ($n = 5$ insects per treatment).

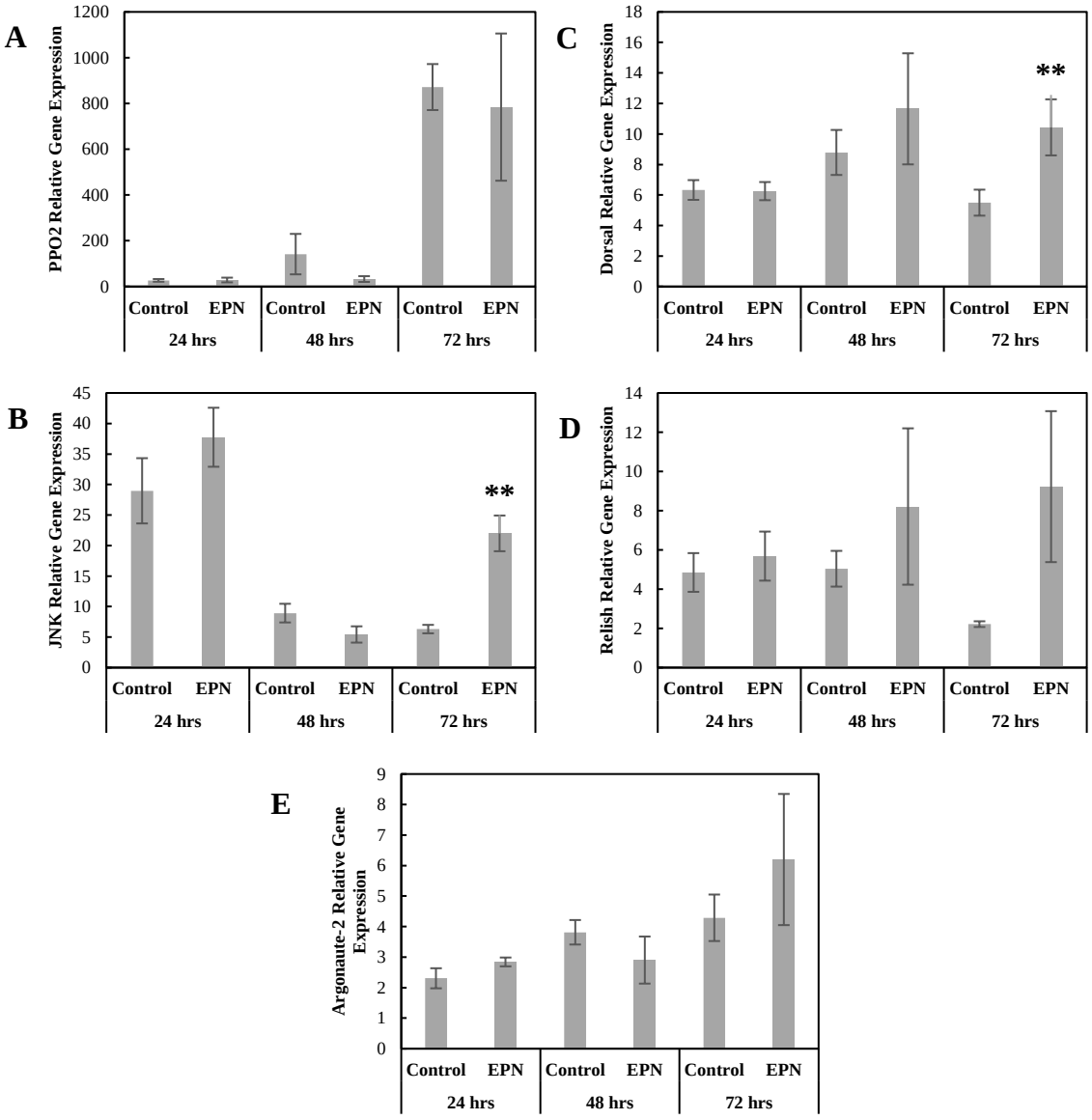

Figure II.8: Comparison of relative expression levels of immune-related genes in Control and *Steinernema carpocapsae*-infected *Helicoverpa zea*. (A) *PPO-2* (Mean ± SE), (B) *JNK* (Mean ± SE), (C) *Dorsal* (Mean ± SE), (D) *Relish* (Mean ± SE), and (E) *Argonaute-2* (Mean ± SE). *Actin* was used as the housekeeping gene. The single asterisk indicates a significant effect (ANOVA, $P < 0.1$) of treatment relative to the Control at that time point. The double asterisk indicates a stronger significant effect (ANOVA, $P < 0.05$) of treatment relative to the Control at that time point ($n = 5$ insects per treatment).

Discussion

Our findings clearly demonstrate *H. zea* responds differently to each major pathogen group at different stages of infection, both physiologically and transcriptionally. The *H. zea* immune response when infected with *Bacillus thuringiensis* was the most robust of all the pathogens we tested. Both PPO and PO concentrations were significantly higher in *B. thuringiensis*-infected larvae at 24 and 48 hpi compared to Control larvae. This increase in PPO and PO levels could be a significant indicator of increased nodule formation, supported by the significant increase in encapsulation ability of infected larvae over the Control and the reduction in hemocytes simultaneously observed at 48 hpi (Satyavathi et al. 2014). Interestingly, there were no significant differences in lysozyme-like activity between treatments for any sample point; however, gene expression of *Relish* was significantly higher at 24 and 72 hpi. This increase in expression of the IMD transcription factor should indicate an increase in AMPs with activity against *Bacillus thuringiensis* due to the DAP-type PGNs (Park and Lee 2012). Concurrently, there was a significant reduction in *Dorsal* expression at 48 hpi, indicating a reduction in AMPs with activity against most gram positive and fungal pathogens. Furthermore, *Argonaute-2* gene expression was significantly down-regulated compared to the Control at 48 and 72 hpi, possibly due to resource allocation away from antiviral activity. *Bacillus thuringiensis* induced the most robust immune response, which resulted in the up-regulation of *Relish* and the down-regulation of *Dorsal*, and also showed evidence of nodulation formation.

Helicoverpa zea larvae infected with HearNPV demonstrated an initial increase in hemocytes at 4 hpi compared to the Control larvae, possibly revealing the importance of hemocytes in an antiviral role as described by Trudeau et al. (2001) and McNeil et al. (2010a, b). Unlike *T. ni* infected with TnSNPV, total hemocytes peaked early during the infection at 4 hpi rather than 48 hpi as observed by Scholefield et al. (2019). Our physiological data is similar to Pan et al. (2020), in that we did not see a prolonged induced response of hemocyte counts or PO concentrations. We also observed an overall decrease in encapsulation ability compared to the Control even when hemocyte counts remained the same. This could be indicative of host hemocytes being exploited and controlled by the viral pathogen (Ikeda et al. 2013; Kong et al. 2018). Transcriptionally, HearNPV-infected larvae had significantly lower *PPO-2* gene expression compared to the control at 72 hpi, further indicating a lack of viricidal activity or active silencing by HearNPV. Both *Dorsal* and *Relish* were downregulated compared to the Control, possibly revealing a diversion of resources away from AMP production; however, JNK expression never altered significantly from the Control. This implies that HearNPV infections are not inducing apoptosis via the JNK pathway. Interestingly, *Argonaute-2*, the gene encoding the cleavage protein in the siRNA antiviral pathway was not differentially expressed from the Control until 72 hpi, when it was counterintuitively downregulated, possibly implying a silencing effect by HearNPV. The lack of a substantial immune response by *H. zea* to HearNPV infection is likely due to *H. zea* being a fully-permissive host of HearNPV, while a semi-permissive or non-permissive host might mount an effective antiviral response (Ikeda et al. 2013; Kong et al. 2018).

We did not find evidence of effective up-regulation of the siRNA pathway against HearNPV contrary to Jayachandran et al. (2012). This difference in results could be attributed to the differences between utilizing cell lines and whole organism studies. In our study, *Argonaute-2* was not differentially expressed until late in the infection, when it was down-regulated. This down-regulation suggests the potential of HearNPV to silence the siRNA pathway in *H. zea*, possibly due to *H. zea* being a fully-permissive host (Ikeda et al. 2013; Kong et al. 2018).

Beauveria bassiana did not elicit an immune response in our analyses. All physiological measurements were not significantly different from the Control, except PPO concentrations at 24 hpi which were marginally lower than the Control. This data, coupled with no differences between *B. bassiana*-infected and Control larval *PPO-2* expression levels indicate PPO and PO are not important *H. zea* immune responses to *B. bassiana*. The only genes that were differentially expressed with *B. bassiana* infection were *JNK* and *Relish* at 72 hpi, with both being significantly up-regulated compared to the Control. Once again, these data indicate a surprising lack of an immune response by *H. zea* towards *B. bassiana*, even at a transcriptional level, which is startling considering the wide host range *B. bassiana* is capable of infecting (Uma Devi et al. 2008). It remains to be seen if this lack of response extends to other fungal pathogens.

Steinernema carpocapsae-infected larvae did not differ from Control larvae in PPO or PO concentrations, or in *PPO-2* expression levels indicating that the PO cascade does not contribute significantly to the immune response of *H. zea* to *S. carpocapsae*. Furthermore, there was a significant reduction in encapsulation ability in infected larvae

at 24 hpi, but no difference by 48 hpi. However, there was a significant decrease in hemocytes in infected larvae by 48 hpi. The only two genes differentially expressed were *JNK* and *Dorsal* at 72 hpi. This upregulation and associated physiological response of reduced hemocytes is consistent with the host immune response towards the bacterial symbiont carried by *S. carpocapsae*. These data are further evidence of a potential immune-masking ability by the nematode's cuticle, with little to no evidence of encapsulation occurring, but subsequent up-regulation of genes associated with an immune response against Lys-type gram positive bacteria by 72 hpi (Binda-Rossetti et al. 2016; Castillo et al. 2011). Therefore, *S. carpocapsae* did not appear to elicit an immune response; however, the bacterial symbiont does appear to have elicited an immune response indicative of a Lys-type gram positive bacteria.

In conclusion, this study provided a novel assessment of the immune response of a non-model organism at both the physiological and transcriptional levels, to multiple pathogen groups at multiple times during the infection. It provides the foundation for future studies investigating the ecology and evolution of pathogen resistance in *Helicoverpa zea*. Our findings indicate that the *H. zea* immune system responds differently depending on the pathogen invading and the specific time course of an infection. We also highlight the lack of importance for the PO cascade in *H. zea* immune response to all pathogens utilized except *B. thuringiensis*. While this study gives heretofore unknown information about *H. zea* immunity, future studies are necessary to explore the differences between semi-permissive and fully-permissive hosts of HearNPV or other viral pathogens, and exploration into differences between cell lines and larval

immune assays. Furthermore, insects used in the current study were from a domesticated strain acquired from Benzon Research Inc. (Carlisle, PA). The possibility of wildtype populations exhibiting different immune responses compared to highly domesticated lineages should be explored (Rolff et al. 2004; Tessnow et al. 2018). Further studies should realize the benefits of utilizing both physiological and transcriptional analyses, and implement multiple pathogens and sampling points to gain a clearer picture of how the insect is responding. Furthermore, non-model insect immune assessments are infrequent but necessary to fill key knowledge gaps such as understanding the importance of the PO cascade in immunity. The revelation of a complete lack of immune response to *B. bassiana* is startling, and further promotes the need for studies in non-model organisms.

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CHAPTER III

THE NUTRITIONAL ECOLOGY OF VIRAL INFECTION IN *HELICOVERPA ZEA*

Introduction

Organisms are regularly bombarded with invasions by parasites and pathogens in nature. When a pathogen invades a host, the immune response exhibited is key to determining the outcome in terms of both pathogen and host survival. The immune response in insects is an innate response that is capable of responding differently to specific types of pathogens (Black et al. unpublished). These responses are mediated through several complex signaling pathways which lead to the formation of effector molecules such as phenoloxidase or antimicrobial peptides, or to cellular responses such as encapsulation or phagocytosis (Reviewed in: Park and Lee 2012). All of these immune signaling pathways require metabolic energy, proteins, and carbohydrates to respond throughout the course of a pathogenic invasion. These nutritional resources are diverted from normal metabolic activities, or derived from reserves such as fat bodies (Lazzaro and Little 2009; Schmid-Hempel 2011). This diversion of resources can lead to altered nutritional regulation in the host that ultimately manifests as changes in foraging behavior (Lee et al. 2006; Moret and Schmid-Hempel 2000; Povey et al. 2009).

Many organisms, including several insect species, have been shown to actively regulate their nutritional macronutrient intake to a specific ratio and quantity of accessible proteins (p) and carbohydrates (c) (Deans et al. 2015; Cotter et al. 2011; Simpson et al. 2004). This ratio is termed the intake target, which is a single point in a geometric framework for nutrition first described by Simpson and Raubenheimer (1993),

and updated in 2012 (Simpson and Raubenheimer 2012). The geometric framework for nutrition models how an organism navigates a nutritionally heterogenous landscape to achieve an optimal balance of nutrients. When a pathogen invades, certain insects have been shown to alter their intake target (Lee et al. 2006; Povey et al. 2009; Shikano and Cory 2015; Shikano and Cory 2016; Adamo et al. 2010; Tessnow et al. 2018; Povey et al. 2013). During an infection, altered feeding behavior can be caused by either pathogen manipulation of the host, compensatory feeding, or self-medication (Abbott 2014; Shikano and Cory 2016). Certain pathogens are capable of altering the behavior of their host, and when the host intake target is altered in a manner that benefits the pathogen, or is detrimental to the host, it is believed this is a result of host manipulation by the pathogen. When the host responds to a pathogenic invasion, the response is nutritionally costly. Compensatory feeding is when the host alters their intake target in order to recover the resources lost during the immune response. Compensatory feeding is, therefore, not a direct response to the pathogen, but a simple recovery of resources expended during activation of the immune response to an infection or invasion. However, self-medication is a direct response to a pathogenic invasion, and is therefore a part of the innate immune system (Abbott 2014; Shikano and Cory 2016).

Self-medication was defined by Singer et al. (2009) as a disease-induced change in behavior or phenotype that improves and individual's probability to survive and reproduce. In order to distinguish self-medication from compensatory feeding or host-manipulation by the pathogen, five criteria must be met: (1) only infected individuals should engage in the altered behavior; (2) the behavior must alleviate the potential

fitness loss of infection; (3) if an uninfected individual engages in the behavior, they should suffer a fitness loss; (4) the pathogen cannot benefit from the behavior; and (5) the substance must be deliberately contacted rather than by chance (de Roode et al. 2013; Shikano and Cory 2016; Abbott 2014; Clayton and Wolfe 1993; Singer et al. 2009; Karban and English-Loeb 1997; Lefevre et al. 2009; Lefevre et al. 2010). Self-medication is most easily observed in organisms that use ingested plant secondary metabolites as a direct defense against pathogens. However, self-medication has also been observed in organisms that alter their macronutrient intake target or maintain similar p:c ratios but reduce overall consumption (illness-induced anorexia) to mediate infections (Lee et al. 2006; Povey et al. 2009; Adamo et al. 2010; Adamo et al. 2016; Tessnow et al. 2018; Povey et al. 2013; Mason et al. 2014). In *S. exempta* infected with *Spodoptera exempta* multiple nucleopolyhedrovirus (SeMNPV), infected larvae that fed on higher protein content diets were more likely to survive the infection than larvae reared on lower protein diets. When given a choice, the larvae challenged with the infection chose higher protein diets compared to non-infected larvae which points to a possible self-medication, however the authors did not test all criteria (Povey et al. 2013).

Food limitation, as observed in illness-induced anorexia, can have a restructuring effect on immune system responses, with some responses declining while others are increased (Adamo et al. 2016). This restructuring can be a useful tool for insects to actively manipulate the primary components of their immune response to combat a specific pathogen. Under the assumption that starvation is an imposed environmental condition and anorexia is a behavioral choice, Adamo et al. (2010) proposes that changes

in feeding behavior are consistent with the insect's need to reduce lipid transport to reallocate resources to maximize the immune function. Therefore, illness-induced anorexia is one way in which animals can bias physiological pathways to enhance immune function; by reducing lipid ingestion they reduce the level of lipid transport occurring. This allows for the reallocation of nutrients to be freely utilized by the immune system rather than normal physiological pathways. Povey et al. (2013) found that *S. exempta* increased their intake ratio of proteins to carbohydrates not by consuming more protein but rather through reduction of carbohydrate intake which reduced the overall diet consumption consistent with illness-induced anorexia.

As illustrated by these prior studies, it is relatively difficult to distinguish between compensatory feeding, host manipulation, and self-medication, and very few studies have actually attempted to simultaneously address all criteria of self-medication. Self-medication is able to regulate the response of the innate immune system, while it is the function of the immune system to regulate all interactions with microorganisms, not exclusively pathogenic organisms, limiting the cost of responding to organisms that can be tolerated and allowing beneficial microbes to grow. The immune response is modulated by host genetics as seen in the innate responses and how some activity levels can be hereditary, and also by host nutrition and the regulation of nutritional intake (Lazzaro and Little 2009; Schmid-Hempel 2011). Thus, it would stand to reason that to better understand the immune system we must first understand nutrition and how the regulation of nutrition can alter the immune response (Ponton et al. 2013).

Our objective in this study was to understand the effects of different nutritional regimes on susceptibility of an agriculturally important crop pest, *Helicoverpa zea*, to its host-specific baculoviral pathogen, *Helicoverpa armigera* nucleopolyhedrovirus (HearNPV). This was accomplished with a series of dosage-response, no-choice susceptibility, and intake alteration choice tests. A set of follow-up caloric restriction experiments were conducted to simulate illness-induced anorexia and test hypotheses about self-medication versus host manipulation by HearNPV as an explanation for observed changes in foraging by infected caterpillars.

Materials and Methods

Insects and Virus

Helicoverpa zea caterpillars were purchased from Benzon Research Inc. (Carlisle, PA) as eggs and were kept individually in sterilized 2oz deli cups and reared in an incubator at 25°C with a 14:10 (L:D) on artificial diet until reaching the targeted instar specified in each experiment described below. Diets were checked daily to ensure fresh diet was always available to the larvae. Diet was replaced as needed with a minimum of every three days until the larvae died or reached the prepupal stage. The strain of *Helicoverpa armigera* nucleopolyhedrovirus (HearNPV) was provided by AgBiTech LLC (Fort Worth, TX), and is listed under the trade name Heligen®. To get to the desired working concentrations for each experiment, the highly concentrated viral solution was serially diluted in sterile deionized water from an initial concentration of 7.5×10^9 occlusion bodies/mL.

Artificial Diets

All experiments were conducted using artificial diet described by Ritter and Nes (1981), with modifications by Jing et al. (2013). All diets used differed in their ratio of soluble proteins (p) and digestible carbohydrates (c). These diets were made by altering the amounts of casein and sucrose, while maintaining the same concentration of all other ingredients. All diets had a total macronutrient concentration (p + c) of 42%.

Dosage-Response Experiment

This experiment utilized 1st instar neonates. There were 7 artificial diets and 5 HearNPV dosage treatments resulting in 35 total treatments. A single trial was conducted using ten larvae allocated to each diet × HearNPV dosage treatment for a total of 350 larvae. The seven diet treatments spanned a range of low to high p:c concentration ratios and were 35% protein 7% carbohydrate (p35:c7), p30:c12, p26:c16, p21:c21, p16:c26, p12:c30, and p7:c35. The viral dosages were 0 OBs, 7.5 OBs, 75 OBs, 750 OBs, and 7500 OBs. Once eggs hatched, neonates were allowed to feed on uninfected diet for 24 hours before experiment initiation to ensure natural mortality effects were minimized. After the 24-hour period, a fresh piece of diet, approximately 50 mg, was inoculated with 10µL of solution containing the proper dosage of HearNPV. As diet ingestion for a 1st instar *H. zea* larva is miniscule, no larvae ingested the entire cube; however, larvae that rejected the diet cube and failed to feed at all were removed from the experiment. After infection, larvae were allowed to feed on uninoculated diet and were monitored daily for mortality or pupation. Data were analyzed using Kaplan-Meier

survival curves in JMP 15 with Log-Rank tests to determine significant differences between survival curves.

No-Choice Susceptibility Experiment

This experiment utilized both 1st and 3rd instar larvae reared on seven artificial diets of varying macronutrient ratios: 35p:7c, 28p:14c, 26p:16c, 21p:21c, 16p:26c, 14p:28c, and 7p:35c. Only two viral dosage treatments were utilized (a Control and a 750 OB dosage) based on the results of the dosage-response experiment where 750 OBs induced 80.6% mortality and Kaplan-Meier curves were significantly different from other dosages used. Both 1st and 3rd instar larvae were inoculated as described in the dosage-response experiment, and monitored for mortality or pupation daily. The no-choice susceptibility experiment for 1st instars was repeated twice, with 15 biological replicates per treatment for the first trial, and 30 for the second, for a total of 45 biological replicates per treatment. The no-choice susceptibility experiment for 3rd instars was repeated twice, with 30 biological replicates per treatment for each trial. However, due to larvae failing to accept and develop on the p7:c35 diet, and due to extra molts or larvae failing to consume the entire diet cube, sample sizes varied across treatments, and are provided in Table III.1. All 3rd instar larvae were weighed to the nearest 0.001g prior to inoculating with a dosage. Mortality and pupation data were recorded for all experiments. Kaplan-Meier curves with Log-Rank tests for significant differences between them were conducted using JMP 15. However, daily diet consumption data was only recorded for the second trial of 3rd instar larvae. Daily diet consumption was recorded by weighing the diet prior to feeding and vacuum freeze-

drying the final mass. A regression curve was established from known wet weight values and known dry weight values. This was done to verify larvae were feeding on the diets. Differences in feeding were analyzed with an ANOVA and Tukey's HSD post hoc test in R Studio (R Core Team, 2020).

Intake Target Deviation Choice Test

Upon molting to 3rd instar, larvae in this experiment were given a pair of diet cubes with differing macronutrient ratios: (1) p35:c7 and p7:c35, (2) p28:c14 and p14:c28, and (3) p35:c7 and p14:c28, with the freedom to feed on either cube. Two HearNPV dosages were used (0 OBs, or 750 OBs) to inoculate the larvae as described in the dosage-response experiment prior to placing the larvae in a petri dish containing the two diet cubes of one of the three treatments. The choice arenas were kept at 25˚C with a 14:10 (L:D) and the arena was checked twice daily to ensure both diets were always available to the larvae. Diets were changed as needed but at most, every three days. The total amount of protein and carbohydrates consumed was calculated as the difference between the initial and final dry mass of the diet blocks. The initial wet mass of the diet was converted to dry mass using linear regression. Mortality and pupation were monitored daily. Kaplan-Meier curves with Log-Rank tests for significant differences between them were conducted using JMP 15. A *t*-test comparing ingestion of the protein-rich diet cube to the carbohydrate-rich diet cube was utilized to ensure non-random feeding, with a significant difference indicating some level of regulation between diets. Differences in daily and total consumption, and average intake targets

were determined from ANOVAs and Tukey's HSD post hoc test using R Studio (R Core Team, 2020).

Caloric Restriction Mortality Experiment

Once the empirically-determined intake target of 1.2 (p:c) and daily protein and carbohydrate consumption was identified for control and HearNPV-infected larvae during the Intake Target Deviation Choice Test, the effects of illness induced anorexia could be explicitly tested. This was done by restricting the amount of diet provided to infected individuals. This experiment utilized four treatment groups, with all larval groups receiving a dosage of 750 OBs of HearNPV. The four treatment groups were all fed the same 1.2 p:c intake target ratio diet, but in different amounts: the average amount ingested by a healthy 3rd instar *H. zea* larva (42 mg/day), the average amount ingested by a HearNPV-infected 3rd instar larva (25.5 mg/day), one quarter the amount ingested by a healthy 3rd instar larva (12.6 mg/day), and one-eighth the amount ingested by a healthy 3rd instar larva (6.3 mg/day). There were 30 3rd instar larvae per treatment, and larvae were observed daily for mortality or pupation. Kaplan-Meier survival curves were established in JMP 15, and the Log-Rank test was used to determine differences between curves.

Caloric Restriction Gene Expression Experiment

This experiment utilized the same 4 diet treatments from the caloric restriction mortality experiment. Thirty 3rd instar larvae were reared for each treatment, and five larvae were pooled for each biological replicate. Therefore, each treatment had six biological replicates. Hemolymph extraction occurred by sterilizing the larva with an

ethanol wash, weighing the larva, and then chilling the larva on ice before piercing the larva with a sterile 27-gauge needle between the second pair of prolegs. The insect hemolymph was allowed to drain directly into an Eppendorf tube and immediately placed into liquid nitrogen, before being stored in a -80°C freezer immediately upon completion of the extraction. RNA was extracted from hemolymph samples with the RNeasy Mini Kit (Qiagen, Hilden, Germany). RNA concentrations were determined using a NanoView Plus (General Electric, Boston, MA), and then standardized to 100ng/μL before being converted to cDNA using iScript gDNA Clear cDNA Synthesis Kit (Bio-Rad Laboratories, Hercules, CA). Resulting DNA concentrations were determined with a NanoView Plus, and diluted to 100ng/μL by adding RNase and DNase free water. Once sample DNA concentrations were standardized, qPCR was conducted using novel primers targeting specific immune genes in *H. zea* (Table III.1; Black et al. unpublished). Quantitative Real-Time PCR was conducted using SYBR™ Green (Bio-Rad Laboratories, Hercules, CA) and Precision Blue Real-Time PCR Dye (Bio-Rad Laboratories, Hercules, CA) in a C1000 Touch Thermal Cycler with the CFX384 Real-Time System attachment (Bio-Rad Laboratories, Hercules, CA). Data were then exported into CFX Maestro (Bio-Rad Laboratories, Hercules, CA) software where melting curves were checked for amplification specificity, and quantitative analysis was done by a comparative C_T method using *Actin* as the reference gene. Data were further analyzed using univariate ANOVA and Tukey's HSD post hoc test in R Studio (R Core Team, 2020).

Table III.1: List of forward and reverse primers used in qPCR analysis. *Actin* was the reference gene used to standardize expression level.

Gene of Interest		Primer Sequence	Annealing Temp. (°C)	Size (bp)
Actin	Forward	ATGGGACAGAAGGACTCGTA	54.9	100
	Reverse	GGTGCCAGATCTTCTCCATATC	54.8	
JNK	Forward	GAATGTCGCCATCAAGAAGTTG	54.4	751
	Reverse	ACGCGTTTAGAAGACCGATTAT	54.1	
Relish	Forward	TGTGATTGACTGTGCGTGATA	54.2	750
	Reverse	GGAGAACTATGAGGAGGAGAGT	54.9	
Argonaute-2	Forward	TCAGGGCCTACTCCTGTATT	54.9	107
	Reverse	GGTGGCATAGCAGTAGAAGTAG	54.8	

Results

Dosage-Response Experiment

Larvae started to die from HearNPV 4 days post-inoculation, and the trial was terminated after all larvae either succumbed to the viral infection or pupated. The last viral death occurred 14 days post-inoculation. Dosage-dependent mortality rates are provided in Figure III.1 for each of the viral concentrations used. As dosage increased, percent mortality increased as well. Viral concentration had a significant overall effect on larval survival (Figure III.2). However, the assigned diet did not have a significant overall effect on survival (Figure III.3). Each viral concentration affected survival consistently across all diet treatments (Table III.2), except the 7.5 OB concentration, which had significantly reduced survival in the p16:c26 diet choice compared to the other diets (Figure III.4). Therefore, the main factor underlying susceptibility in this experiment was viral concentration, but at lower concentrations, diet composition may

have an important role. Based on these results, we utilized 750 OBs as the concentration in subsequent experiments due to the mortality rate of 81%, and the almost linear relationship between survival and time exhibited in the survivorship curves providing a balance of survival, mortality and time to potentially observe treatment effects.

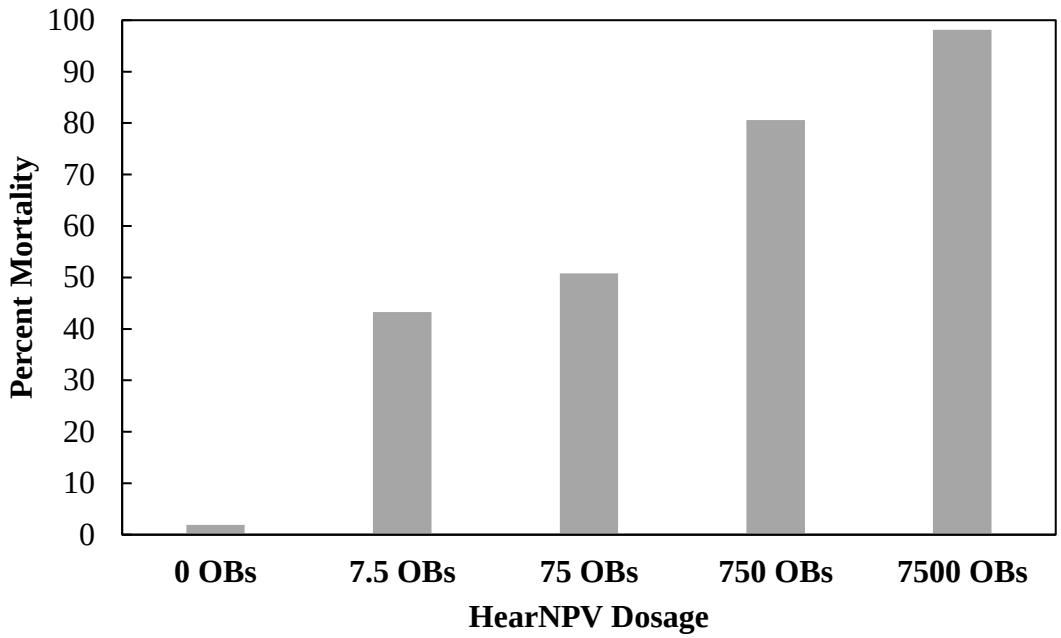

Figure III.1: *Helicoverpa zea* mortality when infected with varying dosages of *Helicoverpa armigera* nucleopolyhedrovirus. There was a 1.9% mortality for 0 OBs, 43.3% for 7.5 OBs, 50.8% for 75 OBs, 80.6% for 750 OBs, and 98.1% for 7500 OBs.

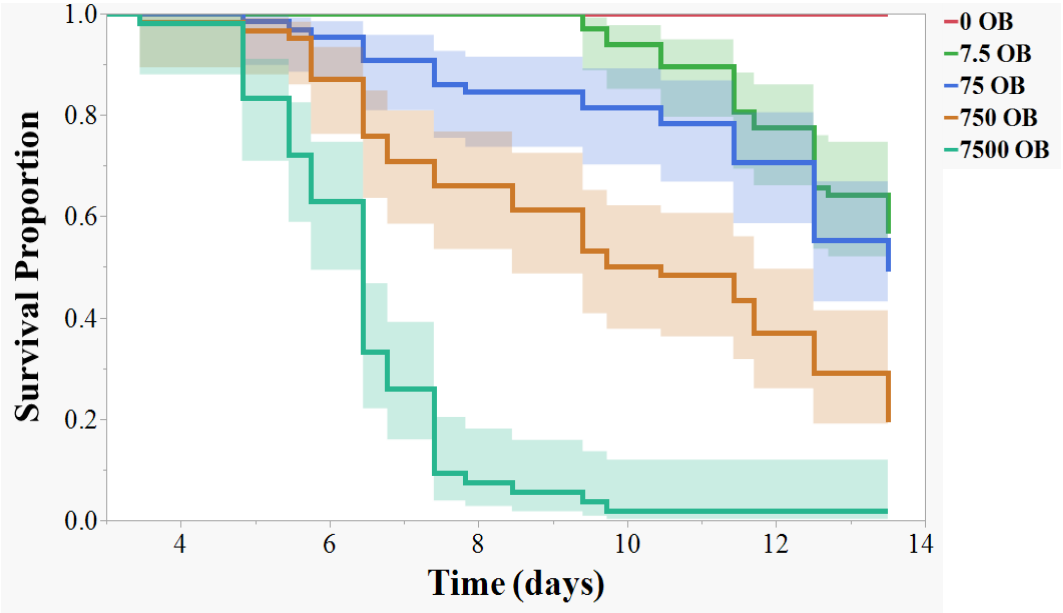

Figure III.2: *Helicoverpa zea* Kaplan-Meier survival curves for each *Helicoverpa armigera* nucleopolyhedrovirus dosage. Not accounting for differences in survival among diets. Log Rank test: $\chi^2 = 279.65$, $p = 0.0001$.

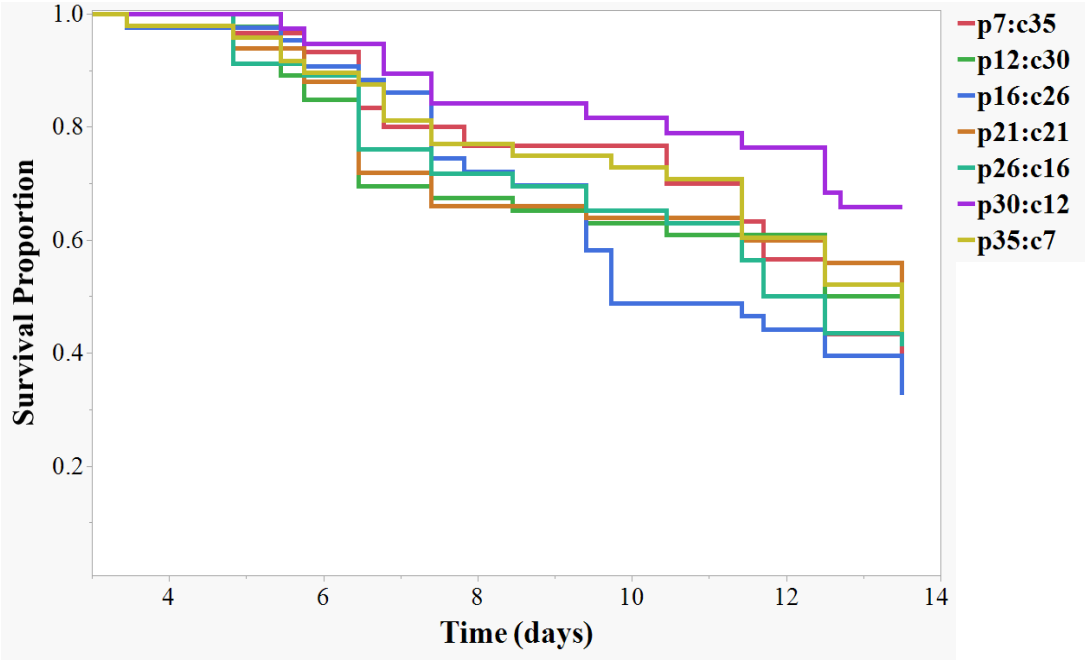

Figure III.3: *Helicoverpa zea* Kaplan-Meier survival curves for each nutritionally distinct diet. Not accounting for differences in survival among the different *Helicoverpa armigera* nucleopolyhedrovirus dosages. Log-Rank test: $\chi^2 = 9.15$, $p = 0.1655$.

Table III.2: Comparison between differences in *Helicoverpa armigera* nucleopolyhedrovirus dosage (Control, 7.5 OBs, 75 OBs, 750 OBs, and 7500 OBs) Kaplan-Meier survival curves for each nutritionally different diet used, based on the Log-Rank test. These data show all viral dosages had significantly different survival curves when diet was consistent across treatments. Both Chi-squared values and P-values are reported.

	HearNPV Dosage	
Diet	X^2	*P-value*
p35:c7	66.92	< 0.0001
p30:c12	13.16	0.0043
p26:c16	46.59	< 0.0001
p21:c21	45.22	< 0.0001
p16:c26	38.91	< 0.0001
p12:c30	31.26	< 0.0001
p7:c35	36.53	< 0.0001

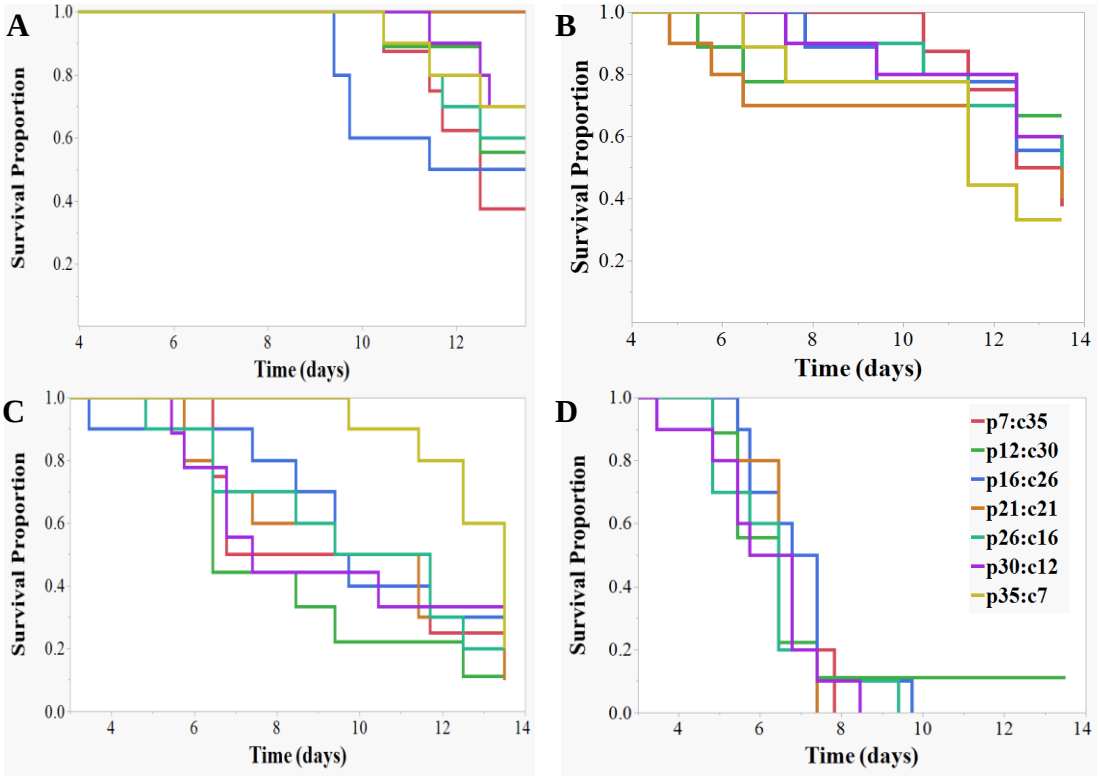

Figure III.4: *Helicoverpa zea* Kaplan-Meier survival curves for each nutritionally different diet, grouped by *Helicoverpa armigera* nucleopolyhedrovirus dosage. (A) 7.5 occlusion bodies (OBs), (B) 75 OBs, (C) 750 OBs, and (D) 7500 OBs. Only 7.5 OBs had significant differences between Kaplan-Meier survival curves as determined by the Log-Rank test (χ^2 = 14.25, *P-value* = 0.027).

No-Choice Susceptibility Experiment

Larvae began dying from HearNPV by 2 days post-inoculation, and all larvae had either died or pupated by 19 days post-inoculation. Uninfected larvae had a lower likelihood of survival when reared on extreme carbohydrate-biased diets p7:c35, and p14:c28 compared to all other diets (Figure III.5). Infected larvae did not exhibit greater survival on protein-biased diets compared to infected conspecifics on carbohydrate-biased diets (Figure III.6). However, as expected, viral presence did significantly decrease larval survival compared to uninfected larvae, regardless of diet (Table III.3; Figure III.7), except for p7:c35, where no differences occurred (Figure III.7). Within virally-infected treatment groups, only p14:c28 and p35:c7 had significantly different survival curves, with larvae on the protein-biased p35:c7 diet having significantly higher survival than larvae on p14:c28 (Figure III.7). Larvae infected with HearNPV consumed significantly less diet than uninfected conspecifics in all bit one diet treatment group (Figure III.8). When compared amongst assigned diets, larvae assigned to p7:c35, p14:c28, and p26:c16 did not consume different amounts between infected and uninfected groups; however, larvae assigned to p16:c26, p21:c21, p26:c16, or p35:c7 ingested significantly less when infected compared to uninfected conspecifics (Figure III.8).

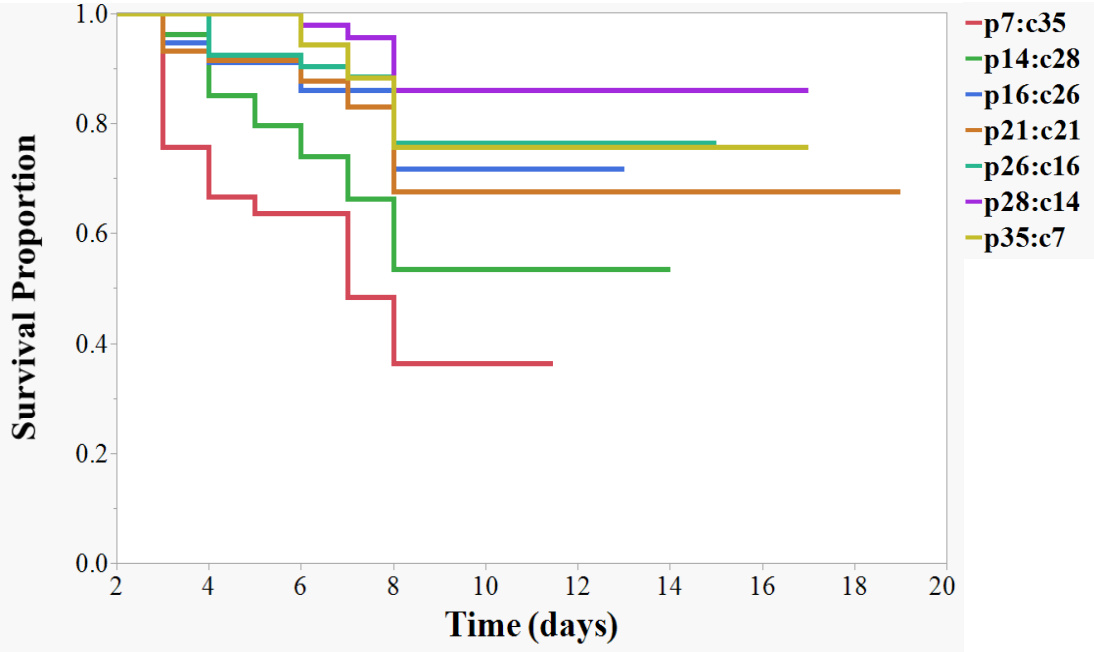

Figure III.5: *Helicoverpa zea* No-Choice Susceptibility Experiment Kaplan-Meier survival curves for Control *H. zea* larvae across each nutritionally different diet. Log-Rank test: $\chi^2 = 43$, $p < 0.0001$.

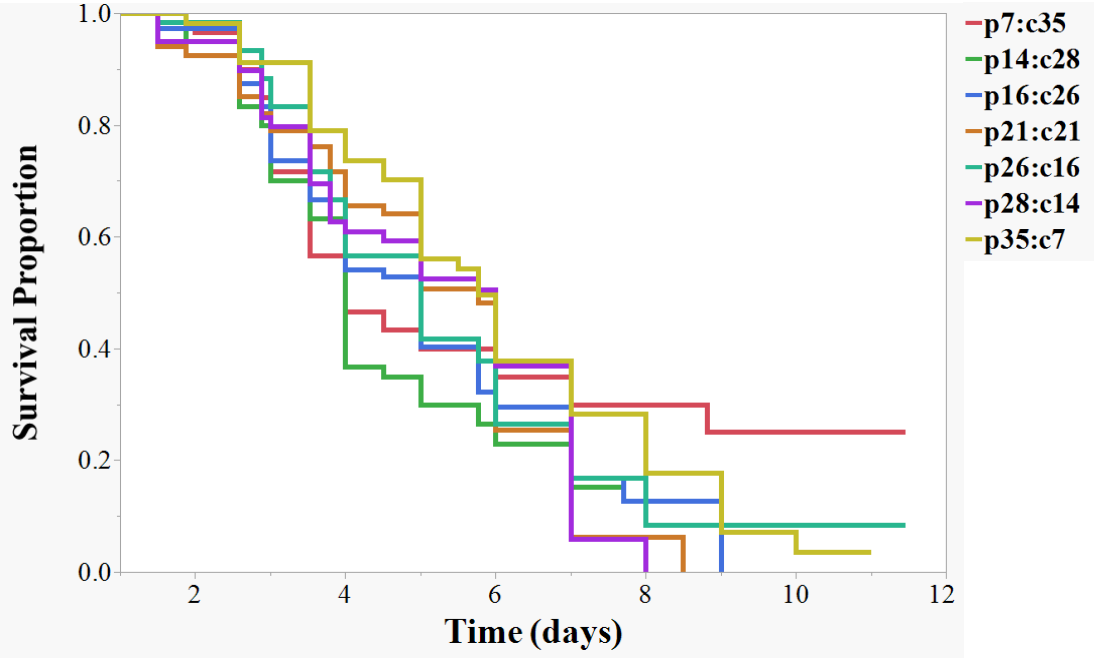

Figure III.6: *Helicoverpa zea* No-Choice Susceptibility Experiment Kaplan-Meier survival curves for *Helicoverpa armigera* nucleopolyhedrovirus-infected *H. zea* larvae across each nutritionally different diet. $\chi^2 = 10.1$, $p = 0.1221$.

Table III.3: Comparison of differences between *Helicoverpa armigera* nucleopolyhedrovirus-infected and Control *Helicoverpa zea* larval Kaplan-Meier survival curves for each nutritionally different diet used, based on the Log-Rank test. Both Chi-squared values and P-values are reported.

	Viral Effect	
Diet	X^2	*P-value*
p35:c7	57.89	< 0.0001
p28:c14	86.58	< 0.0001
p26:c16	59.42	< 0.0001
p21:c21	58.03	< 0.0001
p16:c26	52.05	< 0.0001
p14:c28	40.6	< 0.0001
p7:c35	2.98	NS

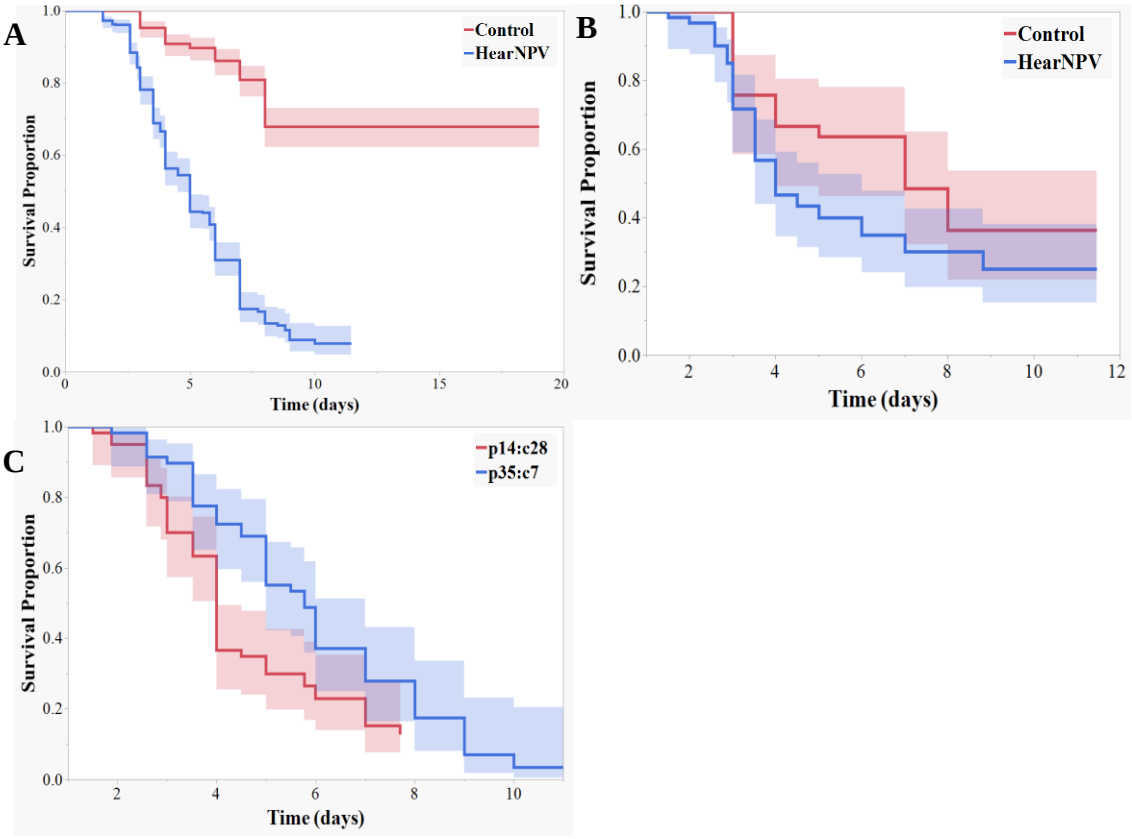

Figure III.7: *Helicoverpa zea* No-Choice Susceptibility Experiment Kaplan-Meier survival curves. (A) Survival curves for *Helicoverpa armigera* nucleopolyhedrovirus-infected and Control *H. zea* larvae, not accounting for differences in survival among diets (χ^2 = 317.71, *P-value* < 0.0001). (B) Survival curves for *Helicoverpa armigera* nucleopolyhedrovirus-infected and Control *H. zea* larvae reared on p7:c35 diets (χ^2 = 2.98, *P-value* = 0.0841). (C) Survival curves for *Helicoverpa armigera* nucleopolyhedrovirus-infected *H. zea* larvae reared on p14:c28 compared to p35:c7 (χ^2 = 7.59, *P-value* = 0.0059).

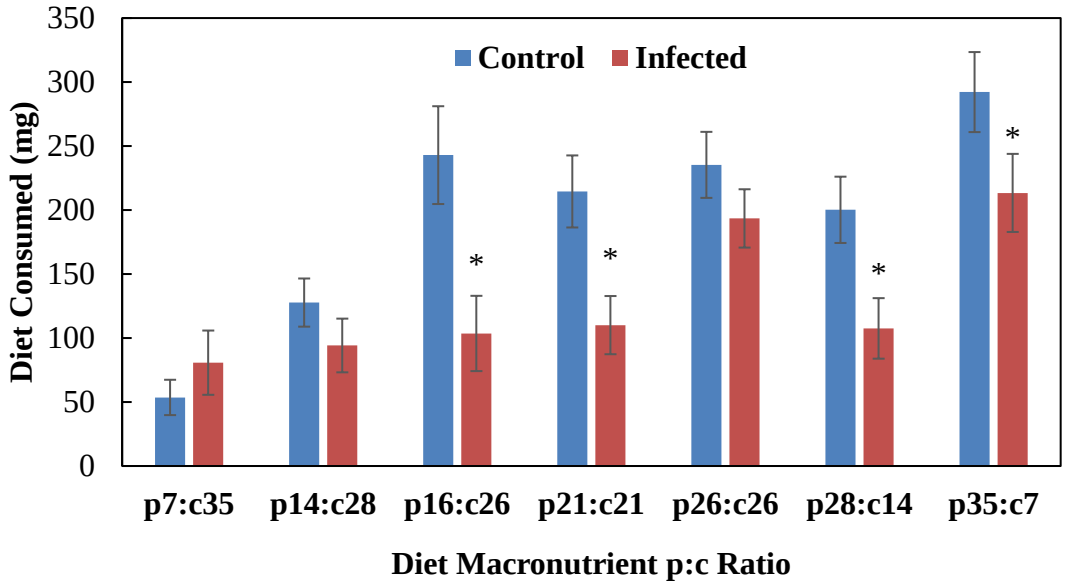

Figure III.8: *Helicoverpa zea* No-Choice Susceptibility Experiment total diet consumption. Consumption data for both *Helicoverpa armigera* nucleopolyhedrovirus-infected and Control larvae on each nutritionally different diet was utilized. Infected larvae ate significantly less overall ($F_{1,380} = 19.019$, $p = 1.67 \times 10^{-5}$), and on p16:c26, p21:c21, p28:c14, and p35:c7 in pairwise comparisons. This reduction in feeding compared to Control larvae suggests a potential self-medicating behavior of illness-induced anorexia.

Intake Target Deviation Choice Test

Control and infected larvae were provided access to one of three diet pairings: (1) p35:c7 w/ p7:c35, (2) p28:c14 w/ p14:c28, and (3) p35:c7 w/ p14:c28 to test whether viral infection caused a shift in macronutrient regulation. Larvae began dying from HearNPV by 3 days post-inoculation, and all larvae had either died or pupated by 12 days post-inoculation. Uninfected larvae survived significantly longer than infected larvae, and there were no differences in survival between diet pairings for either infected or uninfected groups (Figure III.9). Only one larva died while all others pupated in the uninfected groups. Infected larvae on any pairing did not differ in survival. Both Control and infected larvae fed non-randomly on at least one diet pairing indicating actively selecting between two diets (Table III.4). Control larvae on the (2) pairing showed a significant preference for the p28:c14 diet choice. Infected larvae on the (1) and (3) pairings showed a significant preference for the carbohydrate-biased choice (Table III.4). Infected larvae did not significantly alter their p:c intake target (Figure III.10), but rather, reduced overall ingestion of the diet (Figure III.11) compared to uninfected conspecifics. While intake target was not significantly altered by viral infection, it was significantly altered by assigned diet pairing (Figure III.10).

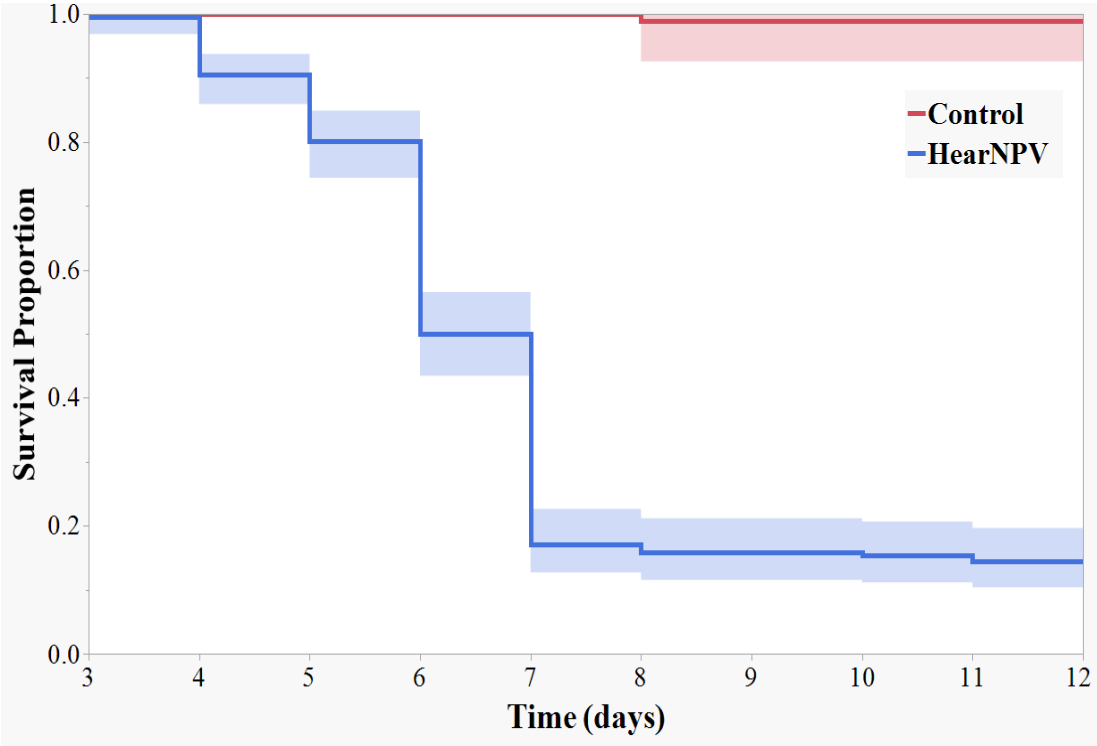

Figure III.9: Intake target deviation choice test Kaplan-Meier survival curves. Curves for both Control and *Helicoverpa armigera* nucleopolyhedrovirus-infected *Helicoverpa zea* larvae, regardless of diet pairing, as diet pairing did not significantly affect survival, while viral presence did (χ^2 =161.42, $p < 0.0001$).

Table III.4: Intake target deviation choice test student t-test results showing larvae in both Control and *Helicoverpa armigera* nucleopolyhedrovirus-infected groups fed non-randomly on at least one of the diet pairings utilized. Therefore, we can assume non-random feeding resulting from regulation of nutritional intake.

	p35:c7 w/ p7:c35			p28:c14 w/ p14:c28			p35:c7 w/ p14:c28		
	t	df	P-value	*t*	df	P-value	*t*	df	P-value
Control	1.405	70	NS	8.38	60	1.05 × 10^-11	0.74	70	NS
HearNPV	-2.22	90	0.0289	1.09	82	NS	-4.75	88	7.71 × 10^-6

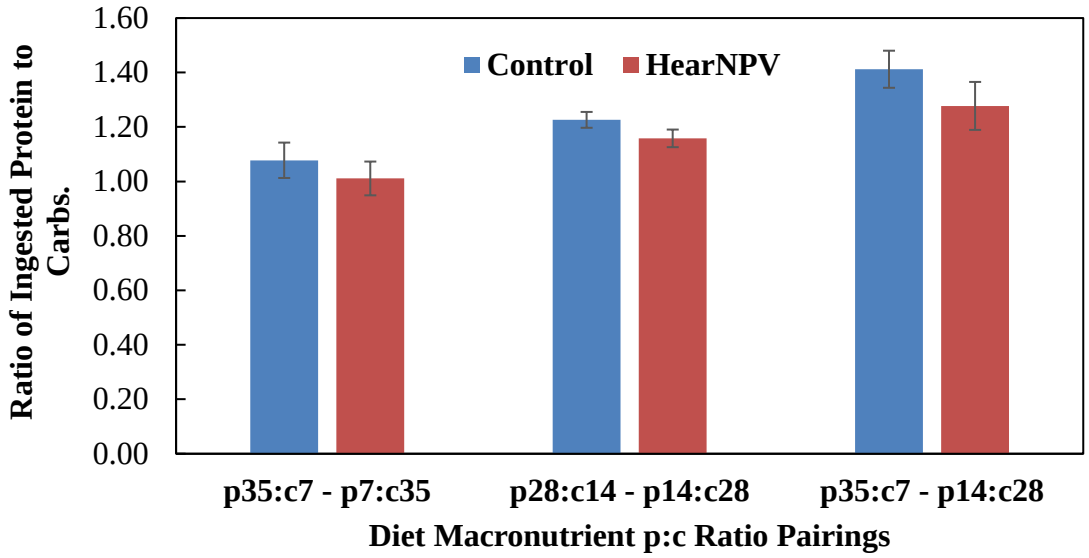

Figure III.10: Intake target deviation choice test results for *Helicoverpa zea* larvae regulating their nutritional intake to a specific point in the nutritional geometric framework. While each diet pairing resulted in slightly different intake targets ($F_{2,307}$ = 11.55, $p = 1.45 \times 10^{-5}$), overall, the intake targets did not differ between Control and *Helicoverpa armigera* nucleopolyhedrovirus treatments ($F_{1,311}$ = 2.616, p = 0.107). Actual intake target averages are reported above each bar in the figure.

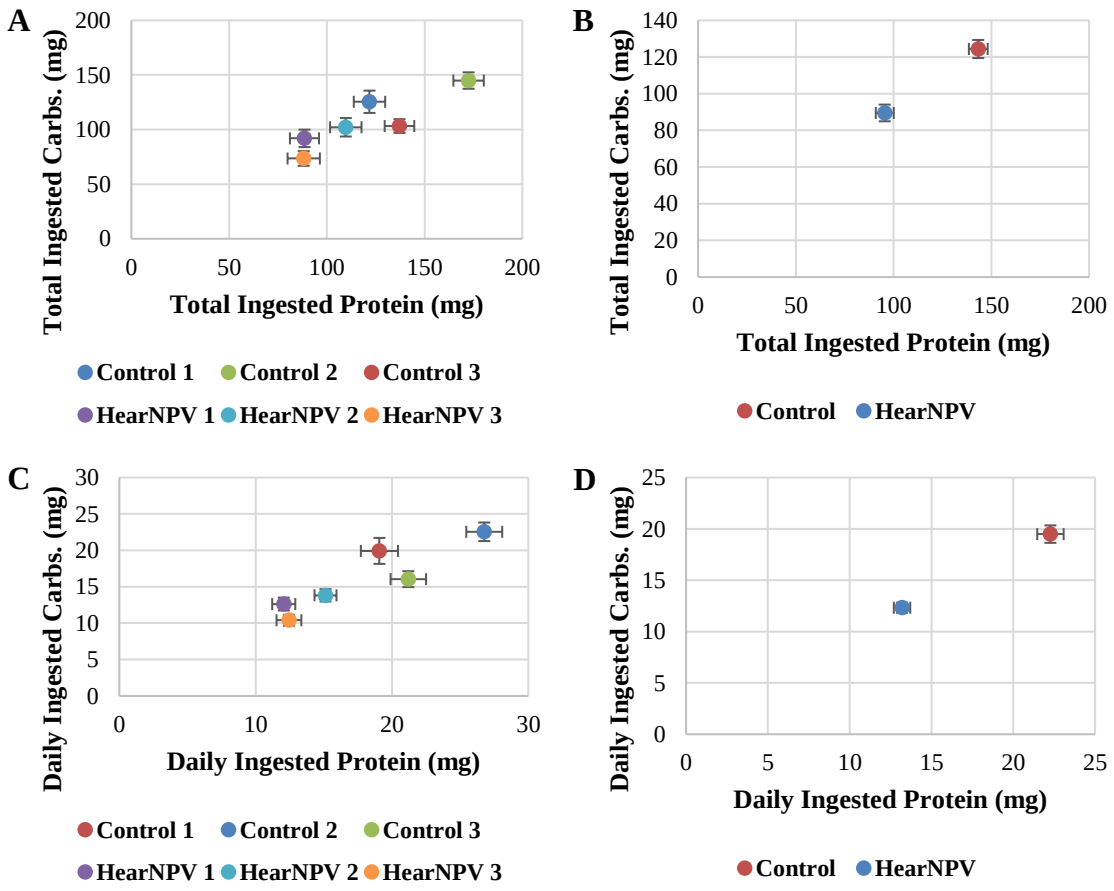

Figure III.11: Daily and total intake targets for Control larvae and *Helicoverpa armigera* nucleopolyhedrovirus-infected *Helicoverpa zea* larvae. (A) Total ingested proteins plotted against total ingested carbohydrates for each diet pairing and viral treatment individually. This gives the total intake target for each diet pairing. (B) Total intake target for Control and *Helicoverpa armigera* nucleopolyhedrovirus-infected *Helicoverpa zea* larvae, regardless of diet pairing. (C) Daily ingestion of protein and carbohydrates, resulting in an average daily intake target for each diet pairing and viral treatment. (D) Average daily intake target for Control and HearNPV-infected larvae, regardless of diet pairing. Overall, consumption was reduced significantly in HearNPV-infected larvae compared to the Control ($F_{1,311} = 205.47$, $p = 3.97 \times 10^{-36}$).

Caloric Restriction Experiment

Larvae began dying from HearNPV 2 days post-inoculation, and all larvae had either died or pupated by 14 days post-inoculation. There was a significant effect of restricting diet on survival, with less diet resulting in lower survival (Figure III.12). Larvae feeding on 42 mg/day differed in survival from larvae feeding on 25.5 mg/day early in the experiment, but was no different later, as expressed by the differences between Log-Rank and Wilcoxon values (Figure III.12). The larvae provided a full healthy diet of 42 mg/day survived significantly better than larvae provided 12.6 mg/day and 6.3 mg/day (Figure III.12). Infected larvae given 25.5 mg/day did not differ in survival from larvae provided 12.6 mg/day, but did significantly increase survival compared to larvae provided 6.3 mg/day ($\chi^2 = 0.0746$, $p = 0.7847$; $\chi^2 = 4.75$, $p = 0.0293$). Larvae assigned to 12.6 mg/day did not differ in survival compared to larvae assigned to 6.3 mg/day ($\chi^2 = 1.32$, $p = 0.2512$). Furthermore, there were no differences between calorically restricted treatment groups in gene expression levels for any immune response genes measured: *Argonaute-2*, *JNK*, or *Relish* (Figure III.13).

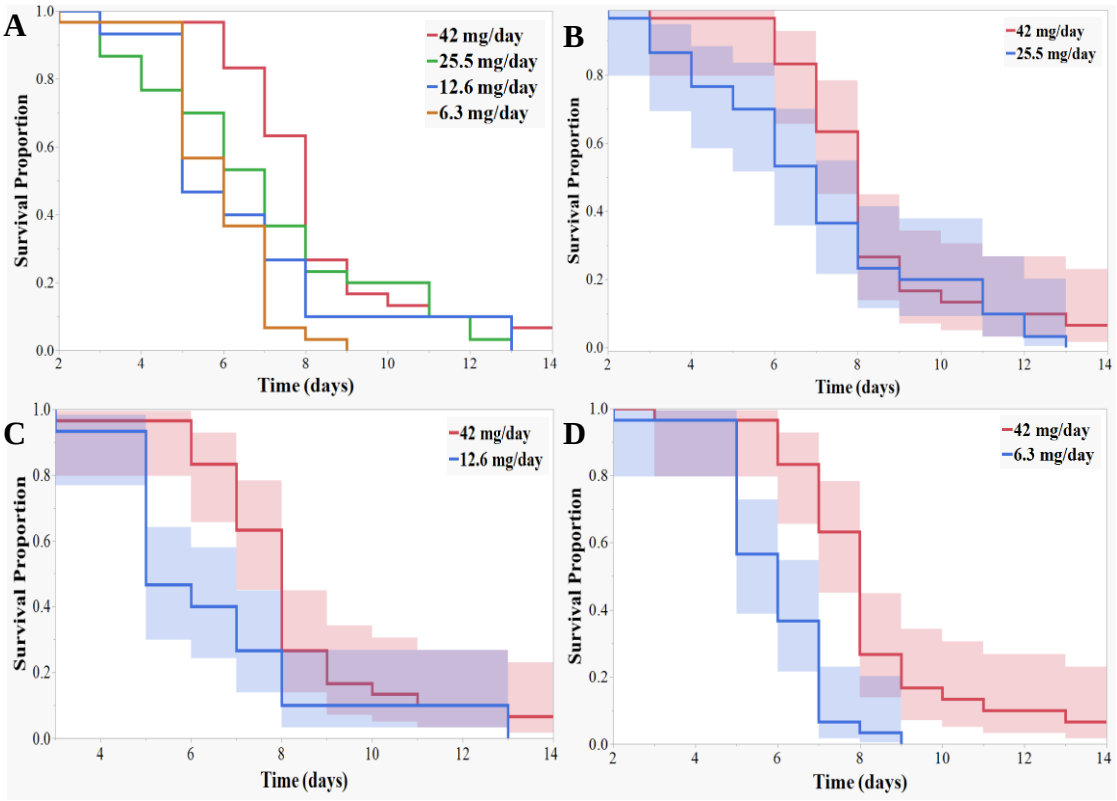

Figure III.12: Kaplan-Meier survival curves for calorically restricted *Helicoverpa armigera* nucleopolyhedrovirus-infected 3rd instar *Helicoverpa zea* larvae. (A) comparison of Kaplan-Meier survival curves for infected larvae fed 42 mg optimal diet per day, infected larvae fed 25.5 mg optimal diet per day, infected larvae fed 12.6 mg optimal diet per day, and infected larvae fed 6.3 mg optimal diet per day. There were significant differences in Kaplan-Meier survival curves, with less diet resulting in lower survival (χ^2 =16.88; p = 0.0007). (B) comparison of Kaplan-Meier survival curves for larvae reared on 42 mg/day and larvae reared on 25.5 mg/day. Log-Rank test shows no significant differences between these curves (χ^2 = 2.77, p = 0.0961); however, Wilcoxon method did detect significant differences (χ^2 = 4.36, p = 0.0368). This is due to the differences occurring early in the curve, with no differences in survival late. (C) comparison of Kaplan-Meier survival curves for larvae reared on 42 mg/day and larvae reared on 12.6 mg/day (χ^2 = 7.24, p = 0.0071). (D) comparison of Kaplan-Meier survival curves for larvae reared on 42 mg/day and larvae reared on 6.3 mg/day (χ^2 = 23.31, p <0.0001).

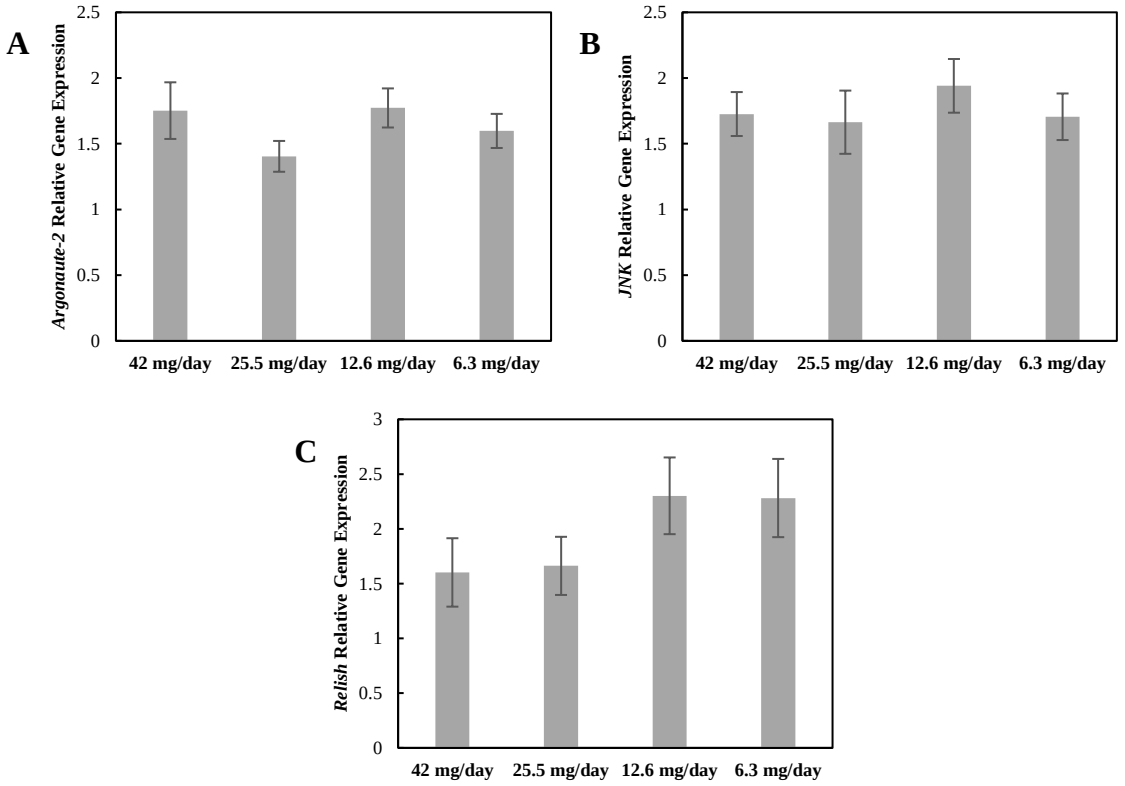

Figure III.13: Comparison of relative expression levels of immune response genes in the caloric restriction experiment. (A) *Argonaute-2* ($F_{1,22} = 0.121$, $p = 0.732$), (B) *JNK* ($F_{1,22} = 0.102$, $p = 0.752$), and (C) *Relish* ($F_{1,22} = 3.30$, $p = 0.083$) when 3rd instar larvae were restricted to feeding on optimal and suboptimal quantities of optimal diet. Larvae feeding on 42 mg/day did not differ in immune response from larvae feeding on 25.5 mg/day, or on either of the heavily restricted diet quantities (12.6 mg/day and 6.3 mg/day).

Discussion

During a pathogenic invasion, many insects have been shown to alter their typical nutritional intake target. This alteration is due to either self-medication, compensatory feeding, or pathogen-induced behavior alteration. Previous studies of lepidopteran insects have shown that they are capable exhibiting all three strategies when challenged by pathogen infections with varying strategies employed by different species. *S. littoralis* larvae that survived an infection by SlMNPV were found to implement a compensatory shift in feeding behavior by ingesting higher protein levels; however, larvae that succumbed to the infection did not exhibit altered feeding behavior (Lee et al. 2006). Povey et al. (2009) found, like *S. littoralis*, *S. exempta* infected with a bacterial pathogen also required an increase in protein consumption to resist the invasion, resulting in an increased physiological immune response. The authors found infected larvae did not alter their carbohydrate intake levels, but did increase their protein intake levels and concluded *S. exempta* exhibited self-medication. Shikano et al. (2016) revealed *Trichoplusia ni* inoculated with a low dose of *Autographa californica* multiple nucleopolyhedrovirus (AcMNPV) survived and developed better on protein-biased diets compared to carbohydrate-biased diets, once again showing the importance of protein in mounting an immune response against viral pathogens. Shikano and Cory (2016) found *T. ni* infected with AcMNPV exhibited compensatory feeding rather than self-medication. Cotter et al. (2011) found both constitutive and induced immune responses were not limited by the quantity of nutrients consumed, but the quality consumed, with different immune responses requiring different ratios of protein and

carbohydrates to be induced. The authors conclude immune-challenged insects modify their allocation of nutrients to improve their immune response. Therefore, no single diet can maximize all components of the immune system simultaneously. Wilson et al. (2018) found *Manduca sexta* immune function varied in response to different p:c diets, but that protein-reduced diets had the largest impact on the immune response, and that it was mostly detrimental. Both *H. zea* and *H. armigera* were less susceptible to a *Bacillus thuringiensis* toxin when developing on diet correlating to their previously selected optimal nutritional ratio (Deans et al. 2017; Tessnow et al. 2018).

We tested for differences in nutritional regulation between healthy and HearNPV-infected *H. zea* larvae, and determined which factor was contributing to the altered behavior. Our results suggest that *H. zea* larvae infected with HearNPV are no less susceptible when reared on nutritionally optimal diets, unlike previous studies where ingestion of protein-rich diets decreased susceptibility to viral pathogens (Lee et al. 2006; Povey et al. 2009; Povey et al. 2013). Our results indicate no differences in survival for larvae restricted to carbohydrate-biased or protein-biased diets, except larvae that fed on p35:c7 survived significantly longer than larvae that fed on p14:c28 diets. However, even when restricted to a single nutritional diet, *H. zea* larvae did reduce their overall intake when infected compared to control larvae, potentially pointing to illness-induced anorexia. A similar trend was observed by Povey et al. (2009), where a reduction in ingestion of a carbohydrate-rich diet bolstered the immune response of *S. exempta* to SeMNPV. Furthermore, in the intake target deviation choice test, infected larvae did not significantly alter their intake target from healthy larvae, but again did

significantly reduce ingestion of both protein and carbohydrates, once more implying illness induced anorexia.

Illness-induced anorexia is a specific type of self-medication in which the host reduces consumption of protein and/or carbohydrates in order to stimulate a specific immune response that increases the survival of the host, which was not observed in our experiments. Adamo et al. (2010) determined that *Gryllus texensis* crickets exhibited illness-induced anorexia when exposed to a pathogen. They concluded this was to reduce lipid transport occurring during digestion, thereby reducing metabolic activity to maximize immune activity. Povey et al. (2013) determined that *S. exempta*, while choosing diets higher in protein, were significantly reducing their overall intake by decreasing their carbohydrate ingestion. This reduction in feeding was hypothesized to reduce the potential of ingesting more pathogen or restricting a key nutrient from the pathogen. Our results show *H. zea* larvae infected with HearNPV ingested significantly less than uninfected larvae without altering the relative amounts of specific macronutrients consumed. To test if this was due to illness-induced anorexia or pathogen-mediated host manipulation, we restricted access to a nutritionally optimal diet based on empirically-determined daily ingestion amounts of healthy and infected larvae. A healthy *H. zea* larvae ingested 42 mg/day, an infected larva ingested 25.5 mg/day. Both infected and healthy larvae regulated to an intake target of 1.2 p:c. From this caloric restriction test, we determined survival was not significantly increased by decreasing ingestion, and therefore is not consistent with being a strategy that improves host fitness. We were unable to quantify viral loads to determine if the pathogen directly

benefited; however, development time was delayed due to the decreased consumption and it can be hypothesized that this delay would benefit HearNPV reproduction. Furthermore, qPCR was used to determine if certain immune response genes were upregulated during caloric restriction. Our results indicate no effects of starvation on the transcriptional levels of *Argonaute-2*, *JNK*, or *Relish*. *Argonaute-2* is important in the antiviral small-interfering RNA pathway, *JNK* is important in apoptotic immune responses, and *Relish* is important in antimicrobial peptide production. This lack of transcriptional response to starvation further supports our conclusion that reduced overall consumption by infected *H. zea* does not improve host defenses, but is more likely pathogen-mediated host manipulation that benefits the pathogen.

In conclusion, we have demonstrated *H. zea* caterpillars infected with HearNPV do not survive better on protein-biased diets compared to carbohydrate-biased diets as has previously been observed for both *S. littoralis* and *S. exempta* (Lee et al. 2006; Povey et al. 2009). When allowed the choice, infected larvae do not regulate their macronutrient intake target to a higher protein-biased ratio, nor do they alter their intake target at all. However, infected larvae do tend to reduce overall diet consumption, possibly implicating illness-induced anorexia (Adamo et al. 2010; Povey et al. 2013). When infected *H. zea* larvae were restricted in their access to a nutritionally optimal diet, survival did not increase relative to larvae that were not restricted, and no differences in transcriptional immune responses were observed. Therefore, *H. zea* larvae do not seem to respond to HearNPV via self-medication, nor do they seem to

compensate for lost resources via compensatory feeding. Our data supports the conclusion that HearNPV alters *H. zea* behavior which could benefit the virus.

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CHAPTER IV

TRI-TROPHIC INTERACTIONS BETWEEN COTTON, CATERPILLARS, AND A VIRAL PATHOGEN

Introduction

Insects are exposed to entomopathogens regularly, and rely on physical barriers to keep infectious particles from invading their bodies. Once a pathogen has successfully surmounted the insect cuticular layer or hostile midgut environment and invaded the host epithelial cells, the insect host's innate immune system is induced to combat the infection (Reviewed in Black et al. unpublished). While the survival of an infected host insect has been shown to be correlated with nutritional intake in some species (Lee et al. 2006; Povey et al. 2009), nutritional state does not appear to be equally important for all species (Black et al. unpublished). Nevertheless, an insect's host plant has the potential to greatly affect the virulence of entomopathogens, enhancing or reducing the ability of a pathogen to overcome its host (Monobrullah et al. 2007; Sarfraz et al. 2011; Gasmi et al. 2019; Forschler et al. 1992).

Herbivorous insects are constantly exposed to a variety of stressors that can reduce fitness and sometimes result in mortality such as unsuitable environmental conditions, plant exudates or structures, and other micro- or macro-organisms (Clissold et al. 2009; Shikano et al. 2010; Shikano et al. 2018a; Nix et al. 2017). Insects developing on different host plants can be exposed to different stressors such as differing nutritional composition and various suites of constitutive or induced plant defensive metabolites, collectively termed host plant quality (Shikano et al. 2018a;

Shikano et al. 2017b). The quality of the host plant is a key determinant of an herbivore's overall fitness, including developmental rates and fecundity (Clissold et al. 2009; Sarfraz et al. 2011; Forschler et al. 1992). Plant structural attributes such as trichomes and leaf texture can deter or inhibit herbivory damage, or accelerate the deterioration of an herbivore's mandibles (Sarfraz et al. 2011; Shikano et al. 2017a). Plants are also capable of inducing defenses against herbivores or pathogens, with defensive responses known to be mediated by three main phytohormones, jasmonic acid (JA), salicylic acid (SA), and ethylene (ET) (Bostock 2005). The induced systemic resistance (ISR) defense pathway is mediated by JA and ET, and has been shown to be important in defending against chewing herbivory, while the inducible systemic acquired resistance (SAR) defense pathway is mediated by SA, and is important in defending against plant pathogens or piercing-sucking insects (Bostock 2005). Both pathways result in the production and release of different suites of plant secondary compounds that can help defend the plant from attack (Bruinsma et al. 2007; Stotz et al. 2000; Nix et al. 2017; Walling 2001; War et al. 2018; Bruinsma et al. 2009; Thaler 1999a). These compounds can have a direct effect on the fitness of an herbivore, or an indirect affect by recruiting natural enemies such as predators and parasitoids (Thaler 1999a; Thaler 1999b; Bruinsma et al. 2009). While this tri-trophic interaction between plants, herbivore, and natural enemy has been intensively studied (Ali and Agrawal 2012; Carrasco et al. 2015; Walling 2001; Qi et al. 2016; Bruisma et al. 2007; War et al. 2018; Howe and Schaller 2008; Bruinsma et al. 2009; Onkokesung et al. 2010; Thaler 1999a; Thaler 1999b), plant-herbivore-entomopathogen interactions have been studied

infrequently at best, with most focusing on changes to infectivity, rather than virulence of entomopathogens (Franco et al. 2017; Gasmi et al. 2019; Shikano et al. 2017a,b; Shikano et al. 2018a,b; Monobrullah et al. 2007; Hoover et al. 2000). To our knowledge, the effects of these stressors on insects with pre-established early infections have not been examined to date. Therefore, our objective in this study was to understand the effects of the induction of both the plant ISR and SAR defensive pathways on the virulence of a viral pathogen in its host.

We utilized *Helicoverpa zea* larvae as an herbivore, upland cotton *Gossypoum hirsutum* as a host plant, and the viral pathogen *Helicoverpa armigera* nucleopolyhedrovirus (HearNPV) as an agriculturally-important model system for this study. *H. zea* is highly polyphagous and one of the most important crop pests in the New World feeding on soybeans, sorghum, cotton, corn and a variety of other host plants (Quaintance and Brues 1905; Fitt 1989; Musser et al. 2018; Cook 2018). *H. zea* infestations in crops are typically controlled by the application of an insecticide; however, they have become resistant to many insecticide classes (Abd-Elghafar et al. 1993; Kanga et al. 1996; Musser et al. 2015). Currently, the main viable control options are to utilize *Bt*-technology seeds at planting, apply an expensive insecticide such as Diamides, or utilize a natural entomopathogenic virus that is specific to Heliothines, *Helicoverpa armigera* nucleopolyhedrovirus (HearNPV) (Adams et al. 2016; Black 2017). Although much more cost effective, efficacy of HearNPV in the field can be affected by several environmental factors that must be considered before use. Developmental resistance usually occurs by the 4th instar, resulting in an unsuitable

environment for the virus within the host (Ignoffo et al. 1978; Luttrell et al. 1982; Alam et al. 1987). Viral occlusion bodies are degraded by ultraviolet light and quickly lose efficacy after foliar applications, especially if suitable host populations are not present (Ignoffo et al. 1972; Young and Yearian 1974; McLeod et al. 1977). In addition, temperature and humidity can both affect the ability of HearNPV to infect a suitable host (Ignoffo et al. 1976; Black et al. 2019). Due to these factors, it is common for HearNPV applications to result in highly variable levels of *H. zea* mortality, especially in cotton. Variability of HearNPV efficacy in cotton relative to other crops such as soybeans has been well documented, and is hypothesized to be caused by high pH levels of around 9.3 of evaporated dew on cotton leaves that are high enough to inactivate the viral particles (McLeod et al. 1977; Young et al. 1977). Further studies have shown the importance of plant phenolic levels which are capable of inducing excess midgut cell sloughing as observed in Shikano et al. (2017a), and as plant phenolic production is inducible, the effect on insects and entomopathogens could be affected by plant defenses. Notably, these studies demonstrate the importance of environmental conditions and plant defenses in altering an insect's susceptibility to a viral entomopathogen, but fail to explore the effects of this tri-trophic interaction on an entomopathogen's virulence once established in the host insect. Therefore, this study was conducted to demonstrate the importance of inducible plant defense pathways (JA-induced and SA-induced) on HearNPV virulence in infected *H. zea* larvae.

Materials and Methods

Insects, Viruses, and Cotton Varieties

Helicoverpa zea caterpillars were purchased from Benzon Research Inc. (Carlisle, PA) as eggs and were reared on artificial diet purchased from Southland Products Inc. (Lake Village, AR) until reaching the targeted instar. The strain of *Helicoverpa armigera* nucleopolyhedrovirus (HearNPV) was provided by AgBiTech LLC (Fort Worth, TX), and is listed under the trade name Heligen®. To get to the desired concentration for experiments, the highly concentrated viral solution was serially diluted from 7.5×10^9 occlusion bodies/mL to 7.5×10^5 occlusion bodies/mL using deionized water. The cotton seeds used for all experiments were variety NG 4050 XF (Americot, Inc. Lubbock, TX). Seeds were planted in individual 3.5in square pots containing unsterilized Pro-Line C/25 growing soil mix consisting of 55% aged pine bark, Canadian sphagnum peat moss, perlite, and vermiculite (Jolly Gardener Products, Inc.). All plants were grown in a greenhouse at ~25°C with natural photoperiod for the duration of the experiments. In an attempt to limit horizontal transmission of HearNPV, pots were placed in a block design, watered as needed, with no fertilizer applied throughout the experiments.

Experiment 1: Inducible Plant Defenses and Virulence of HearNPV in H. zea

This experiment was conducted to determine the effects inducible plant defenses had on the virulence of HearNPV in *Helicoverpa zea* using a 2 × 3 factorial design, with virus (Infected and Control) as one factor and plant induction (Control, ISR, and SAR) as the other factor. Each treatment group had 30 biological replicates evenly distributed

across two identical trials. Larvae were reared on artificial diet until molting to the 3rd instar, at which point they were given a small piece of diet inoculated with 10µL of deionized water or diluted HearNPV solution containing 750 occlusion bodies (OBs). Larvae were given 24 hours to ingest the entire diet or were excluded from the experiment. Simultaneously, cotton plants at the 4-5 leaf stage were sprayed with foliar treatments of either deionized water, 78mg Actigard 50 WG per liter of deionized water (Syngenta; Basel, Switzerland) to stimulate SAR (Inbar et al. 2001), or 42.5µL methyl-jasmonate per liter deionized water (TCI Chemicals; Portland, OR) to stimulate ISR (Rodriguez-Saona et al. 2001), until the leaves were dripping. After larvae ingested the entire diet cube provided, they were individually caged on the sprayed cotton plants after they had dried. Larvae were checked daily for mortality or pupation. Survival data were analyzed in JMP 15 using Kaplan-Meier survival curves and Log-Rank tests to determine differences between curves.

Experiment 2: Inducible Plant Defenses and Virulence of HearNPV Dosages in H. zea

This experiment was conducted to determine the effects of plant inducible defensive pathways on HearNPV virulence in *H. zea* when various dosages were utilized. There were twelve treatments with *H. zea* larvae either being left uninfected (Control), or infected with one of three possible dosages (7.5 OBs, 75 OBs, or 750 OBs). Cotton plants were either sprayed with deionized water, Actigard 50 WG, or methyl-jasmonate. As in the previous experiment, inoculation of 3rd instar larvae and stimulation of plant defense pathways occurred 24 hours prior to experiment initiation; however, larvae were infected 24 hours after molting to 3rd instars, which meant they

molted to 4th instars quickly upon being placed in the cage. Larvae were caged onto individual cotton plants and monitored daily for mortality or pupation. Survival data was analyzed in JMP 15 using Kaplan-Meier survival curves and Log-Rank tests to determine differences between curves.

Results

Experiment 1: Inducible Plant Defenses and Virulence of HearNPV in H. zea

A significant difference in survival was observed in Kaplan-Meier survival curves for *Helicoverpa zea* larvae infected with *Helicoverpa armigera* nucleopolyhedrovirus compared to uninfected Control larvae, regardless of cotton plant defenses (Figure IV.1). Uninfected larvae caged on cotton plants sprayed with methyl-jasmonate to stimulate the ISR defense pathway had significantly reduced survival compared to the Control plants or plants sprayed with Actigard 50 WG which stimulated the SAR pathway (Figure IV.2). Furthermore, there were no significant differences in survival of larvae infected with HearNPV across cotton plant treatments (Figure IV.3). Infected larvae did not survive better on Control plants compared to ISR-induced or SAR-induced plants, indicating minimal to no effect of inducible plant defenses on HearNPV virulence in *H. zea*. The lack of an observed plant defense effect could have been due to the viral dosage used, so a follow-up experiment was initiated to determine if the dosage used was over-powering.

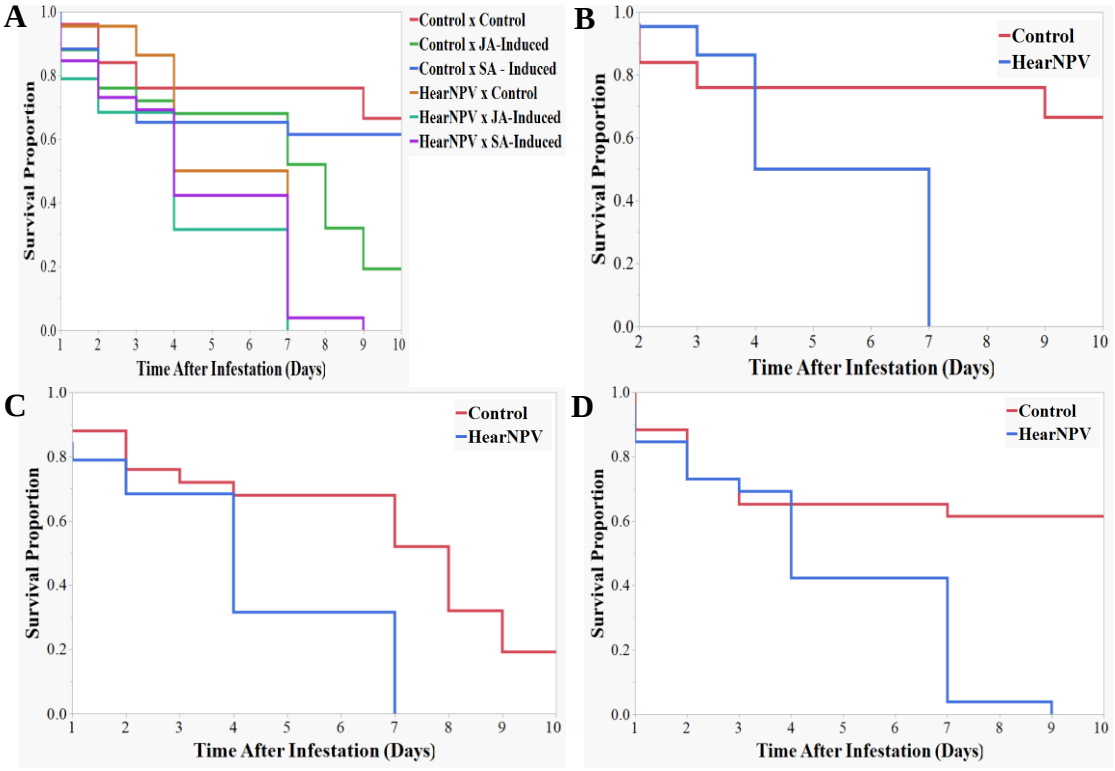

Figure IV.1: Kaplan-Meier survival curves for Control and *Helicoverpa armigera* nucleopolyhedrovirus-infected *Helicoverpa zea* larvae caged on treated and untreated cotton plants. (A) Kaplan-Meier survival curves for all treatment combinations (χ^2 = 45.3025, *P-value* < 0.0001). (B) Kaplan-Meier survival curves for Control and HearNPV-infected larvae caged on untreated cotton plants (χ^2 = 17.1058, *P-value* < 0.0001). (C) Kaplan-Meier survival curves for Control and HearNPV-infected larvae caged on cotton plants treated with methyl-jasmonate to stimulate the jasmonic acid defense pathway, systemic acquired resistance (SAR) (χ^2 = 10.4965, *P-value* = 0.0012). (D) Kaplan-Meier survival curves for Control and HearNPV-infected larvae caged on cotton plants treated with Actigard 50 WG to stimulate the salicylic acid defense pathway, induced systemic resistance (ISR) (χ^2 = 11.8577, *P-value* = 0.0006).

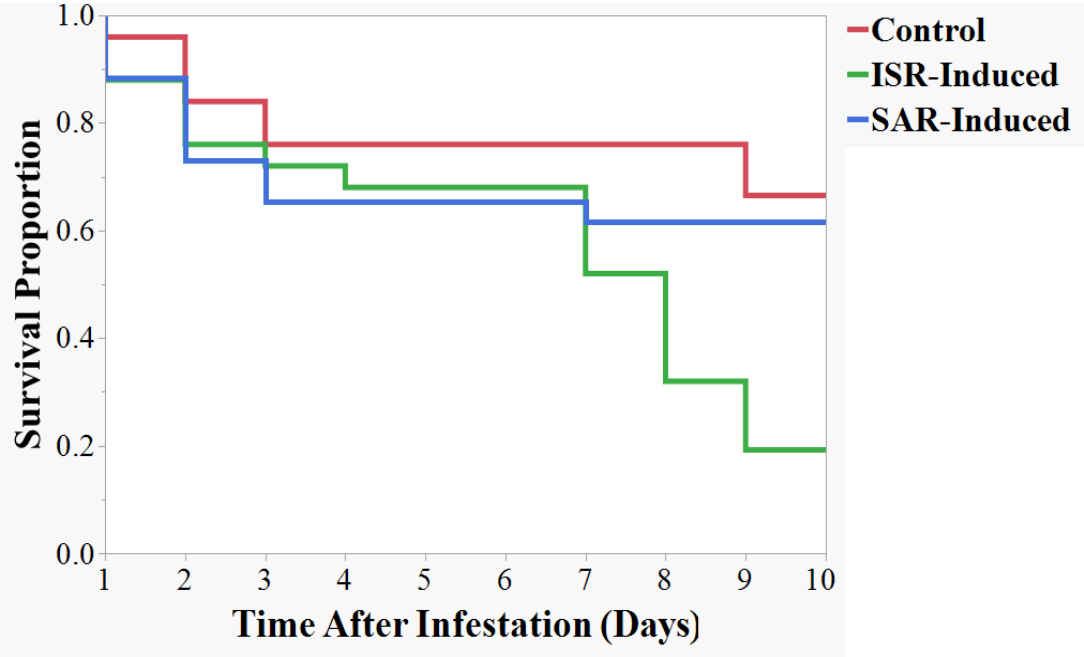

Figure IV.2: Kaplan-Meier survival curves for Control *Helicoverpa zea* larvae caged on cotton plants. (Red) untreated cotton plants, (Green) cotton plants treated with methyl-jasmonate to stimulate the jasmonic acid defense pathway, and (Blue) cotton plants treated with Actigard 50 WG to stimulate the salicylic acid defense pathway. There was a significant reduction in survival of Control larvae caged on cotton plants treated with methyl-jasmonate (Green) compared to the other treatments (χ^2 = 8.2895, *P-value* = 0.0158).

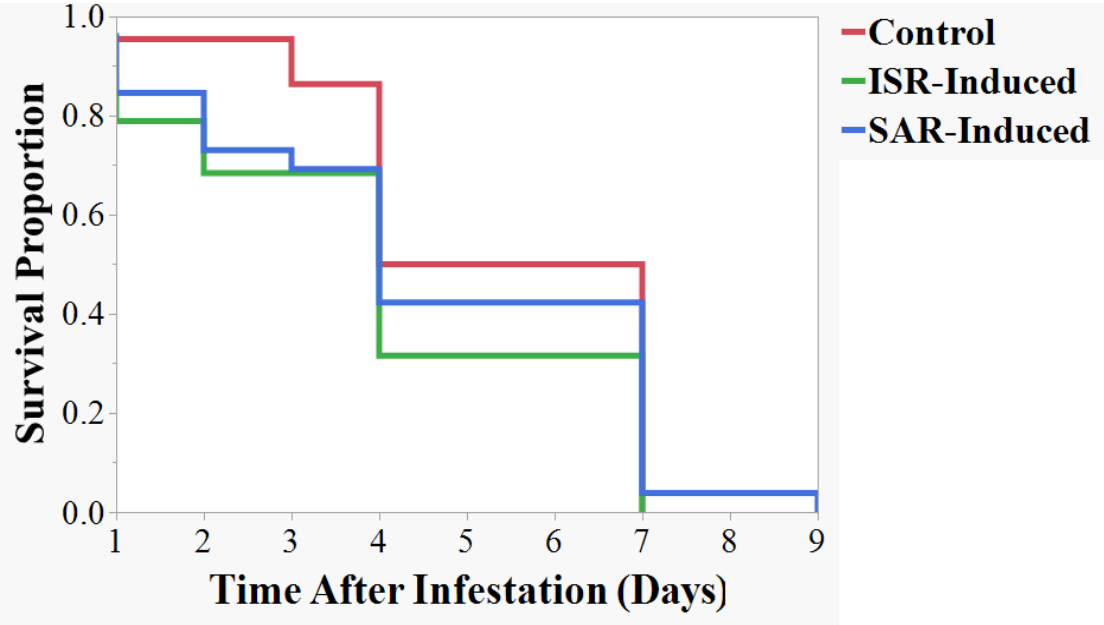

Figure IV.3: Kaplan-Meier survival curves for *Helicoverpa armigera* nucleopolyhedrovirus-infected *Helicoverpa zea* larvae caged on treated and untreated cotton plants. (Red) untreated, (Green) methyl-jasmonate to stimulate the jasmonic acid defense pathway, and (Blue) Actigard 50 WG to stimulate the salicylic acid defense pathway. There were no significant differences in survival for any of the HearNPV-infected larval treatments (χ^2 = 1.9941, *P-value* = 0.369).

Experiment 2: Inducible Plant Defenses and Virulence of HearNPV Dosages in H. zea

This experiment resulted in very low levels of mortality for all treatment combinations, probably due to developmental resistance occurring during the 24-hour delay between molting to 3rd instars and inoculation (Figure IV.4). However, there were still significant differences between treatments (Figure IV.5). No larvae died in the uninfected larval group regardless of plant defense treatment. When comparing plant defense effects within a specific viral dosage, no significant differences in survival were observed (7.5 OBs: $\chi^2 = 4.1723$, *P-value* = 0.1242; 75 OBs: $\chi^2 = 2.1435$, *P-value* = 0.3424; 750 OBs: $\chi^2 = 0.9352$, *P-value* = 0.6265).

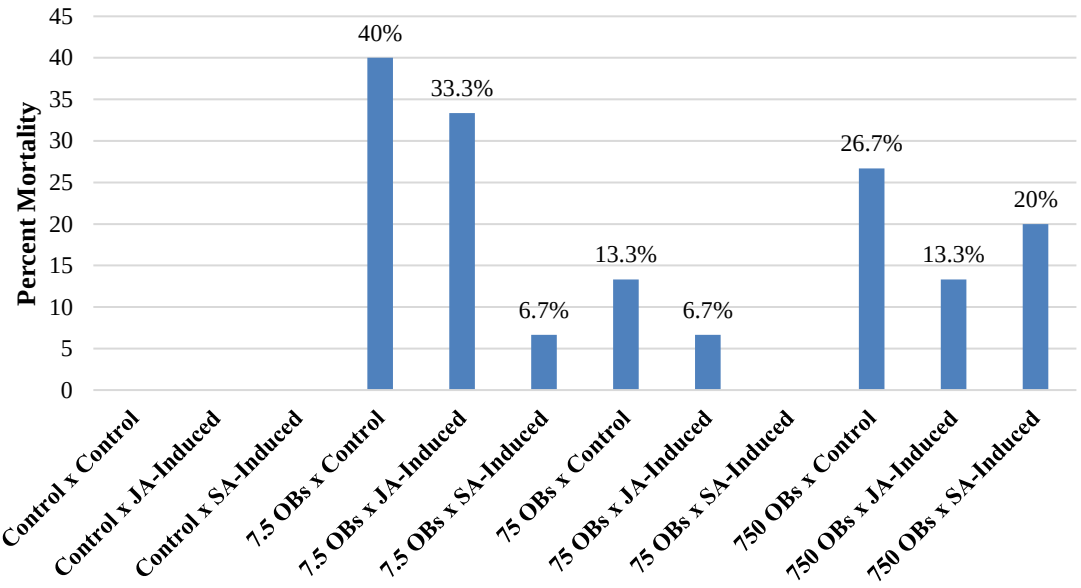

Figure IV.4: Mortality rates for each combination of *Helicoverpa armigera* nucleopolyhedrovirus dosage and cotton plant foliar treatment. No treatment combination had mortality rates above 50%, which was probably due to developmental resistance as these larvae were 24 hours older than larvae used in all other experiments. Mortality rates for uninfected larvae (Control) were all 0%. Mortality rates for 7.5 OBs are 40%, 33.3%, and 6.7% respective to the figure above. Mortality rates for 75 OBs are 13.3%, 6.7%, and 0% respective to the figure above. Mortality rates for 750 OBs are 26.7%, 13.3%, and 20% respective to the figure above.

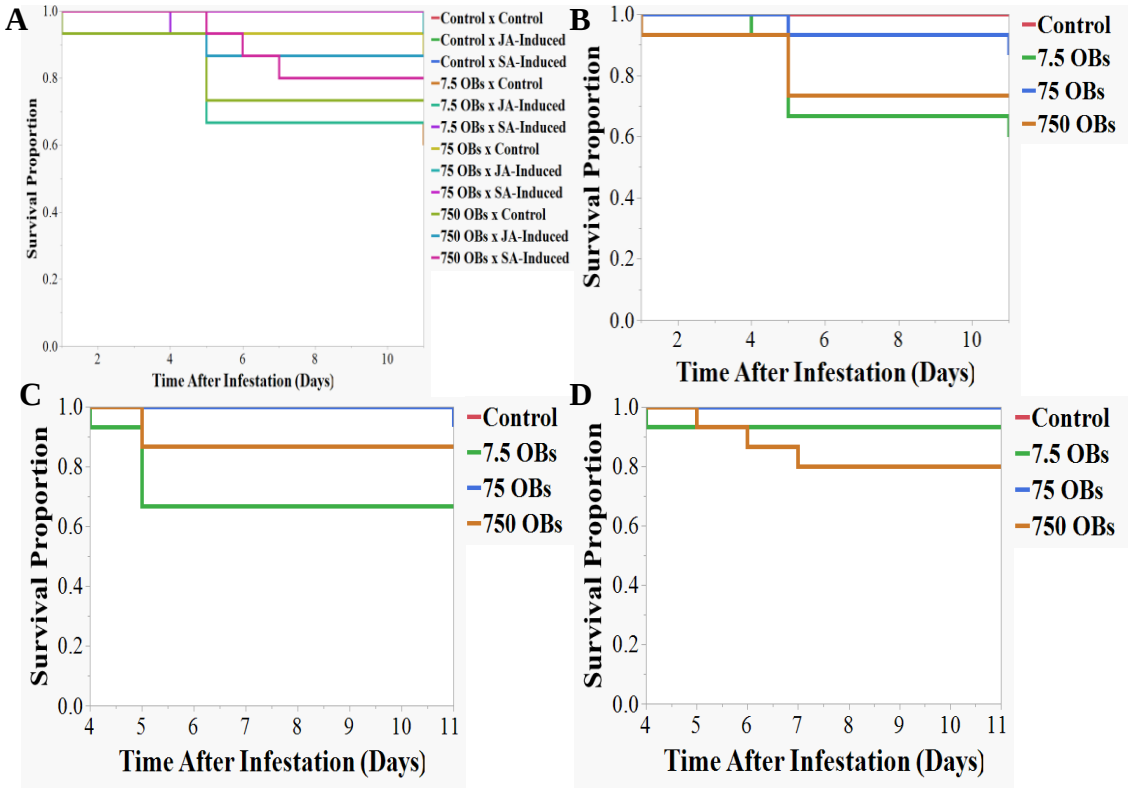

Figure IV.5: Kaplan-Meier survival curves for *Helicoverpa zea* larvae infected with different dosages of *Helicoverpa armigera* nucleopolyhedrovirus caged on treated and untreated cotton plants. Viral Dosages: Control, 7.5 OBs, 75 OBs, and 750 OBs. Cotton treatment: deionized water (Control), methyl-jasmonate (JA-Induced), or Actigard 50 WG (SA-Induced). (A) Kaplan-Meier survival curves for all treatment combinations revealing a significant difference between treatments (χ^2 = 28.771, *P-value* = 0.0025). (B) Kaplan-Meier survival curves for all HearNPV dosages caged on Control cotton plants, revealing a significant difference between viral dosages (χ^2 = 8.3014, *P-value* = 0.0402). (C) Kaplan-Meier survival curves for all HearNPV dosages caged on JA-Induced cotton plants, revealing a significant difference between viral dosages (χ^2 = 8.4568, *P-value* = 0.0375). (D) Kaplan-Meier survival curves for all HearNPV dosages caged on SA-Induced cotton plants, revealing no significant difference between viral dosages (χ^2 = 6.2732, *P-value* = 0.0991).

Discussion

JA-mediated induced plant defenses have been shown to negatively impact development or survival of herbivorous insects (Walling 2001). Our results confirm this effect with uninfected *H. zea* larvae reared on cotton having significantly lower survival on JA-induced plants relative to those reared on either untreated or SA-induced plants. However, an effect of inducible plant defenses on caterpillar survival was not observed in *H. zea* larvae infected with HearNPV. Therefore, our data indicate that the virulence of HearNPV to *H. zea* larvae and its resulting mortality were unaffected by induced plant defense pathways. However, if larvae had been uninfected prior to caging, the effect of plant defenses might have been stronger as seen in other studies (Shikano et al. 2017a; Shikano et al. 2018a; Monobrullah et al. 2007; Hoover et al. 2000).

Multiple studies have shown an effect of host plant on insect susceptibility to entomopathogens and identified several plant-associated stressors capable of altering an insect host's susceptibility to certain viral pathogens (Gasmi et al. 2019; Shikano et al. 2017a,b; Shikano et al. 2018a,b; Pan et al. 2019; Shikano et al 2010; Shikano 2017; Monobrullah et al. 2007; Cory and Hoover 2006; Franco et al. 2017; Hoover et al. 2000). Gasmi et al. (2019) found *Spodoptera exigua* inoculated with *Spodoptera exigua* multiple nucleopolyhedrovirus (SeMNPV) were more susceptible to SeMNPV when exposed to indole or linalool, two herbivore induced plant volatiles (HIPVs), but not when exposed to (Z)-3-hexenyl acetate. Their conclusion being that certain HIPVs can strongly enhance susceptibility through effects on insect gut microbiota, while others were not so effective. Shikano et al. (2017a) found *S. frugiperda* inoculated with

Spodoptera frugiperda multiple nucleopolyhedrovirus (SfMNPV) differed in survival on various soybean genotypes, which varied in their phenolic content. Plants with higher phenolic content induced *S. frugiperda* larvae to slough midgut epithelial cells, thus reducing infectivity of SfMNPV. They found soybean genotypes that utilized plant structural attributes, rather than phytochemicals, as defense mechanisms did not affect SfMNPV pathogenicity, potentially pointing to the ability of entomopathogens to exhort selective pressure on plants. Most studies concluded that the effects of host plant on pathogen susceptibility, whatever the stressor, were due to an increase in midgut cell sloughing, brought on by induction of the JA-mediated ISR plant defense pathway (Shikano et al. 2018a; Gasmi et al. 2019; Shikano et al. 2018b; Pan et al. 2019; Shikano et al 2017b; Hoover et al. 2000). While these studies illustrate how the host plant can affect an insect-pathogen interaction, they all examined the effect of the host plant on establishing an infection in the insect host. Our study examined the effects of inducible plant defenses on entomopathogen virulence after an infection was successfully established. Unlike these studies, our experiments found no effect on host susceptibility or pathogen virulence once an infection was established. These results further define the effects of host plant choice on insect-pathogen interactions, possibly constraining the effects to the site of the infection, rather than having a lasting effect on a pathogen's virulence.

Our study also revealed a high likelihood of developmental resistance in *H. zea* during the 3rd instar, as larvae inoculated directly following a molt were much more susceptible to HearNPV compared to larvae inoculated 24 hours post-molt. While

developmental resistance has been observed in other insect-pathogen interactions (McNeil et al. 2010; Hoover et al. 2002), to our knowledge this is the first time intra-stadial developmental resistance has been observed for *H. zea* infected with HearNPV. Taken together, these data suggest that activation of ISR by application of me-JA can reduce survival of early 3rd instar uninfected *H. zea* larvae, but there is no added benefit to activating plant defenses in terms of increasing HearNPV virulence after the larvae have been infected. However, further studies should be conducted to determine if induction of plant defenses is able to enhance the initial susceptibility of *H. zea* to HearNPV infection in cotton, and explore the possibility of intra-stadial developmental resistance in *H. zea* and HearNPV interactions.

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CHAPTER V

SUMMARY AND CONCLUSIONS

The first objective of this study revealed how *Helicoverpa zea* larvae respond to immune challenge by four different entomopathogens. *Helicoverpa zea* challenged with *Helicoverpa armigera* nucleopolyhedrovirus, a co-evolved viral pathogen, did not exhibit much ability to mount an effective immune response, with all immune response characteristics measured, physiological or transcriptional, either being significantly reduced compared to the Control, or remaining at normal levels, except for hemocyte counts at 4 hours post-inoculation (hpi), which was the only immune response variable measured that was significantly higher in HearNPV-infected larvae compared to Controls. *Bacillus thuringiensis*-infected larvae exhibited the greatest level of response among the different pathogens used. Larvae infected with *B. thuringiensis* exhibited upregulation of prophenoloxidase and phenoloxidase 24- and 48-hpi. A decrease in hemocyte counts was coupled with an increase in encapsulation ability at 48 hpi. Transcriptionally, *Argonaute-2*, *PPO2*, *JNK*, and *Dorsal* were all down-regulated or consistant with Control transcription levels, but *Relish* was up-regulated 24 and 72 hpi. This points to *H. zea* inducing antimicrobial peptides specific to gram negative bacteria and *Bacillus* species, implying a mounted immune response by *H. zea*. *Beauveria bassiana*-infected larvae upregulated both *JNK* and *Relish* at 72 hpi, and *Steinernema carpocapsae*-infected larvae upregulated both *JNK* and *Dorsal* at 72 hpi, revealing that *H. zea* utilize the IMD pathway to combat *B. bassiana* infections and the Toll pathway to combat *S. carpocapsae* infections. Ultimately, this reveals how *H. zea* is capable of

mounting a different immune response to different pathogen types, and how some pathogens appear to be able to inhibit *H. zea* from mounting a successful response.

The second objective of this study revealed the overall lack of importance of nutritional protein and carbohydrate ingestion when *H. zea* were infected with HearNPV. While Control larvae restricted to individual diet cubes of various nutritional content did have different levels of survival, implying an ideal intake target, infected larvae did not exhibit a difference in survival based on diet. Infected larvae died significantly faster than Control larvae on all diets except the most extreme protein-deficient diet (p7:c35), which showed no differences in survival. Among infected larvae, no differences were observed in survival except larvae reared on p35:c7 lived significantly longer than larvae reared on p14:c28. Also, infected larvae ingested significantly less diet than their uninfected conspecifics. When given the option of feeding on a pair of diets, larvae were found to feed non-randomly, indicating nutritional selection; however, p:c ratios were not different between infected and Control larvae. The main difference was Control larvae consumed significantly more diet than infected larvae, pointing to a possible case of self-medication (illness-induced anorexia). However, this was found to not be the case when infected larvae given a daily diet equal in quantity to an infected larval ingestion rate did not enhance survival compared to other diet restrictions, and furthermore failed to increase any immune responses measured. In conclusion, nutrition is a key element in uninfected larval development; however, larvae infected with HearNPV appear to be affected by the viral pathogen to reduce consumption, possibly enhancing the virulence of the pathogen.

The third objective of this study revealed the importance of plant defenses in combating herbivory from uninfected *H. zea* larvae, and the lack of importance in combating herbivory from HearNPV-infected larvae. Cotton plants sprayed with methyl-jasmonate were less suitable hosts for Control *H. zea* larvae, resulting in significantly reduced survival compared to Actigard 50 WG sprayed plants and Control plants. When larvae were previously infected with HearNPV, plant defenses did not affect survival. Further research should be conducted to explore the effects plant defenses have in altering initial susceptibility to a pathogenic infection, rather than a pathogen's virulence.

In conclusion, this study shows the intricate immune response of an important crop pest to entomopathogenic invasions. These data show, for the first time, how *H. zea* responds to four different entomopathogens as the infection cycle progresses. These data also reveal the importance of nutrition on larval survival and the role nutrition plays in immunity. Finally, these data show the importance of plant-defenses in combating herbivory from *H. zea*, and the lack of increased virulence in HearNPV infections. Further studies should be conducted to determine if *H. zea* respond differently to host-specific viruses like HearNPV compared to generalist viruses like *Autographa californica* multiple nucleopolyhedrovirus, and delve more into plant-insect-pathogen interactions.