DEVELOPMENTAL EFFECTS ON IMMUNITY: HORMONAL AND PROTEINASE CONTROL

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ABSTRACT

Insects are ubiquitous, diverse, and able to combat infections despite their lack of adaptive immunity. Insects have a robust innate immune system that is divided into two branches, cell-mediated and humoral. Activation of cell-mediated immune responses results in phagocytosis, nodule formation, and encapsulation by the insect's immune cells, hemocytes. Activation of humoral immunity results in the production of anti-microbial peptides (AMPs) and phenoloxidase (PO). Insect immune responses can be plastic with development. However, research on how and why insect immunity changes with age as insects develop within a larval developmental stage (instar) is limited and contradictory. In my dissertation research, I answer two main questions: 1) how do immune responses vary within an instar and 2) what drives changes in immunity within an instar? My dissertation research showed that humoral immune responses are more robust at the beginning of the 5th and final instar in *Manduca sexta* (tobacco hornworm) compared to responses from animals later within that instar. Many changes occur within an instar that could affect immunity. For example, I found that protein expression of matrix metalloproteinase (MMP) in immune tissues of *M. sexta* decreases throughout the 5th instar. Though MMPs are involved in immune responses in other insects, MMP was not found to be immunostimulatory in *M. sexta*. Another important factor that changes within an instar is the level of juvenile hormone (JH). JH, a developmental hormone that prevents early molting, peaks early and decreases within an instar until molting. I determined that JH is necessary to survive an infection, control bacterial growth in hemolymph (insect blood), and mount an AMP activity immune response. My dissertation research has established that there is a development-immunity link, and that the naturally fluctuating levels of JH may mediate the effect of development on immunity.

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CHAPTER 1. DEVELOPMENTAL EFFECTS ON IMMUNITY: HORMONAL AND PROTEINASE CONTROL

1.1. Introduction

As juvenile insects grow, they increase in mass and periodically shed their exoskeletons to increase in linear dimensions. Internal development and growth can be significant during an instar, the period between molting. Physiology also changes with development during an instar. For example, insect innate immune responses are developmentally plastic to such a degree that immune responsivity can even change within a single larval instar (Booth et al., 2015; Eleftherianos et al., 2008; Tian et al., 2010). Collectively, studies have shown that both cell-mediated and humoral innate immune responses are more robust in the beginning of the final instar than the end of the final instar of the tobacco hornworm, *Manduca sexta* (Eleftherianos et al., 2008) and the silk worm, *Bombyx mori* (Tian et al., 2010). Though there is an established link between within-instar development and a decline in immune responses, little research has been done to determine what mediates the development-immunity link. In this literature review, I describe the current knowledge of insect immune responses, including a background on the evolution of immune responses across taxa and characterizing how immune responses vary with development. I also describe other aspects of physiology that vary with development, including matrix metalloproteinase (MMP) and juvenile hormone (JH). I specifically describe how MMP and JH are involved in immunity across insect taxa and how they contribute to the development-immunity link.

1.2. Immune responses

1.2.1. Evolution of immune responses

Pathogens have bombarded organisms since the beginning of the diversification of life on earth. The struggle for self-defense against fungal, viral, bacterial, and parasitic pathogens has driven changes in the immune system (Litman and Cooper, 2007). Some aspects of the immune system are highly conserved, while others are specific to only vertebrates. The fast, but non-specific innate immune system was the first type of immunity to evolve 600 million years ago with even metazoans such as porifera (sponges) and cnidarians (jelly fish) demonstrating innate immune responses (Kvell et al., 2007). The innate immune system is conserved and is thus found in both invertebrates and vertebrates alike. The specific, but slow-developing, adaptive immune system was first seen in jawed fishes approximately 500 million years ago (Kvell et al., 2007). The details of the evolution of the innate and adaptive immune systems are further reviewed below.

1.2.1.1. Evolution of the innate immune system

The innate immune system by itself is highly functional, successfully defending invertebrates for hundreds of millions of years. The innate immune system refers to the defense mechanisms used to protect organisms that is considered non-specific and the first response (Kimbrell and Beutler, 2001). The innate immune system does not need previous exposure to a pathogen to confer its broad protection (Kimbrell and Beutler, 2001). The innate immune responses are based on the ability of pathogen recognition receptors (PRRs), specifically pathogen-associated molecular patterns (PAMPs) to distinguish self from non-self. Lipopolysaccharide on gram negative bacteria, peptidoglycan on gram positive bacteria, and β-1,3-glucan on fungi are all examples of PAMPs (Cooper, 2003). However, the innate immune

system is not specific and cannot make distinctions between closely related structures (Cooper, 2003). The first immune system, found in metazoans, was fairly simplistic with basic receptors that could identify self and non-self, made apparent by xenograft tissue rejection (Kvell et al., 2007). It is the presence of these types of receptors that have been conserved from the first immune system to the one found in humans. Although the mechanisms by which the receptor binds to PAMPs are not necessarily the same throughout various taxa, receptor-PAMP binding is the basis by which all immune systems detect invaders (DuPascquier, 2001).

1.2.1.2. Innate immunity across taxa

Since the innate immune system is highly conserved, there are many similarities between the invertebrate and vertebrate innate immune systems. For example, the cuticle in insects and the skin in mammals both function as a primary source of protection against pathogens by serving as a physical barrier (Kvell et al., 2007). Phagocytic cells are seen in all animals from sponges to humans. Antimicrobial peptides, which function to lyse microbes, are conserved between invertebrates and vertebrates. AMPs first evolved in annelids and nematodes; even humans still have AMPs today (Kvell et al., 2007). However, not all innate immune responses are conserved. For example, the phenoloxidase cascade, first seen in annelids, continues to be found in mollusks, arthropods, echinoderms, and is last found evolutionarily in the urochordates (Kvell et al., 2007). Although no immunoglobulin-antibodies are found in the innate immune system of invertebrates and jawless fishes, there are immunoglobulin superfamily proteins that have immune functions (Kvell et al., 2007). For example, in the insect *M. sexta* (tobacco hornworm), the immunoglobulin superfamily protein hemolin may have a function in facilitating phagocytosis and hemocyte aggregation during nodulation and encapsulation (Arala-Chaves and

Sequeira, 2000). The evolutionarily conserved nature of the innate immune system of insects makes them beneficial models to study immune responses in a comparative capacity.

1.2.1.3. Evolution of adaptive immunity

The adaptive immune system evolved more recently, about 500 million years ago. The adaptive immune system was built and scaffolded upon the innate immune system (Kimbrell and Beutler, 2001). The adaptive immune system is specific to each and every type of pathogen. It is based on very specific receptors that can identify very specific epitopes (antigens) to form very specific antibodies and T-cells (Cooper, 2003). The ability of the adaptive immune system to recognize specific pathogens allowed organisms to have immunological memory (Hoffman, 1999). As such an evolutionarily adaptive trait, the adaptive immune system is almost universal throughout all vertebrates (Hoffman, 1999). The adaptive immune system first evolved in jawed fishes (Litman and Cooper, 2007). The immune cell receptors (T-cell receptors and antibodies) that have the ability to recognize and kill a various array of pathogens and have very precise clonal and gene-shuffling (somatic rearrangement) abilities in order to maintain their specific nature (Hoffman, 1999). The specificity is based on the rearrangement of segmental elements encoding the immunoglobulin domains in T and B cell receptors (Litman and Cooper, 2007). The critical genes in regulating the somatic rearrangement are RAGs (recombinase activating genes), which first appeared in the jawed fishes approximately 500 million years ago (Hoffman, 1999). Although jawless fishes (the lowest vertebrate, just before jawed fishes) have been suggested to have adaptive immunity, adaptive immunity experts disagree amongst each other because the specificity of immunity is due to a random shuffle of genes, and jawless fishes do not use RAG for somatic gene shuffling to get specific, intentional receptors on T-cells and antibodies (Kvell et al., 2007).

Interestingly, the formation of the adaptive immune system evolved rather abruptly in jawed fishes. This suggests that all the "genetic equipment" needed for adaptive immunity was present in invertebrates before somatic rearrangement for specificity was introduced in jawed fishes. This "genetic equipment" may have included molecules resembling immunoglobulins (antibodies) or T-cell receptors. Fascinatingly, since somatic rearrangement occurred in the jawed fishes, T-cell receptors and immunoglobulins have not evolved much (DuPascquier, 2001). The adaptive immunity found in jawed fishes is similar to the adaptive immunity found in humans.

1.2.1.4. Immunological memory in invertebrates

What can be considered adaptive immunity is of great debate in the field of immunology. For example, there have been reported cases of immunological memory in invertebrates, but many do not consider it to be adaptive immunity since it is not accompanied by somatic gene shuffling to produce specific T-cell receptors and antibodies (DuPascquier, 2001). Invertebrate immunologists suggest that there may be some sort of "peculiar" adaptive immunity in invertebrates because in many invertebrate species, the hemocyte proliferation rate increases after secondary exposure to pathogens, there are immunoglobulin superfamily proteins with immune functions, and there is increased survival from infection and lower circulating bacterial levels to specific bacteria upon secondary exposure (Arala-Chaves and Sequeira, 2000). In addition, there is some specificity in induced immune responses, with a different variety of genes upregulated depending on the type of infection (Riddell et al., 2009). However, these attempts to claim invertebrate immunological adaptive immunity are refuted by the fact that no immunoglobulin antibodies or T-cell receptors have been found in invertebrates. Immunological memory has yet to be found in many of the major insect immunity models, including *M. sexta*.

1.2.2. Insect immunity

Insects are numerous, diverse, and able to survive a plethora of pathogens despite their lack of adaptive immunity. Insects have a robust innate immune system that is divided into two branches, cell-mediated and humoral (Lavine and Strand, 2002). Measures of immunity are often divided into functional measures of immunity, such as survival from infection and bacterial load in the blood, or specific indices of immunity, such as the specific cell-mediated and humoral immune responses described in fig. 1.

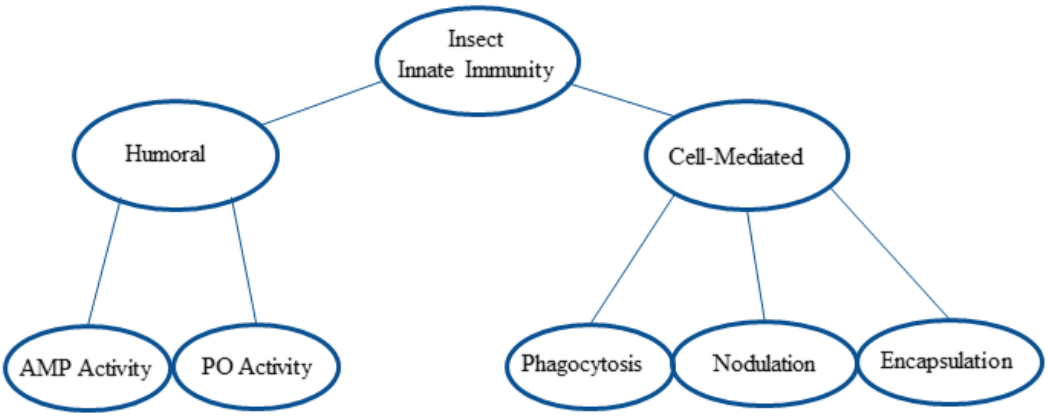

Fig. 1. Insect immunity is considered innate immunity. Innate immunity is divided into humoral immunity, which includes, but is not limited to, AMP and PO activity, and cell-mediated immunity, which includes, but is not limited to, phagocytosis, nodulation, and encapsulation.

1.2.2.1. Cell-mediated immunity

Cell-mediated immune responses are driven by circulating hemocytes, immune cells that perform phagocytosis, nodule formation, and encapsulation (Lavine and Strand, 2002; Strand, 2008). There are varying types of hemocytes across species. The major types of hemocytes in *M. sexta* include prohemocytes, plasmatocytes, granulocytes, oenocytoids, and spherulocytes (Strand, 2008). Phagocytosis, the engulfment and destruction of pathogens, is performed by two types of phagocytic hemocytes, plasmatocytes and granulocytes (Strand, 2008). Nodule

formation and encapsulation are processes in which circulating plasmatocytes and granulocytes bind foreign invaders, spread filopodia, adhere to each other, and form overlapping layers of hemocytes to surround and seal off targets (Lavine and Strand, 2002; Strand, 2008). The formed nodule eventually gets removed from circulation. Nodule formation and encapsulation are essentially the same process with the only difference being the size of the target (Lavine and Strand, 2002; Strand, 2008). Encapsulation is used against larger targets, such as parasites (Rantala and Roff, 2007; Smilanich et al., 2009). Though hemocytes work together to help provide protection against infection, insects also rely on humoral immunity.

1.2.2.2. Humoral immunity

The humoral branch of immunity is driven by the fat body, an organ responsible for metabolic and immune functions. Activation of humoral immunity results in the production of anti-microbial peptides (AMPs) and phenoloxidase (PO). AMPs are small proteins (<25kD) found in all eukaryotic organisms that target a wide variety of pathogens, from bacteria to fungi (Imler and Bulet, 2005; Vilmos and Kurucz, 1998). AMPs are able to enter the cell membrane of pathogens and target intracellular vital transcriptional activity for pathogen reproduction (Matsuzaki, 1999; Yang et al., 2000). AMPs are most often found in the immune tissues of insects, i.e., hemolymph and fat body, but can be isolated in many tissues that face pathogens from the environment, such as the cuticle, gut, and even tracheae (Lemaitre and Hoffmann, 2007). Some specific AMPs have been found to be activated in specific immune challenges in *Drosophila melanogaster* (fruit fly). For example, an AMP called diptericin is produced upon infection with gram-negative bacteria, while drosomycin is produced during gram-positive bacterial infection (Imler and Bulet, 2005; Lemaitre and Hoffmann, 2007). For many insect

species, there is a general up-regulation of several AMPs, such as hemolin, cecropin, and attacin, to fight infections, (Dimarcq et al., 1997).

The other major humoral immune response includes phenoloxidase (PO) activity. PO activity starts with the production of the zymogen proPO from the hemocytes called oenocytoids (Castillo et al., 2006). Serine proteinases called serpins cleave proPO to form the active PO enzyme (Kanost et al., 2004; Zhao et al., 2007). PO activity then results in the production of a variety of cytotoxic molecules, such as 6,6-dihyroxyindole (Kanost et al., 2004). Through a cascade of activation of proteins by proteases, the final result of PO activity is the production of melanin (Cerenius et al., 2008; Cerenius and Soderhall, 2004). One of the main ways PO activity is assessed is by the melanization or darkening of the hemolymph (Kanost et al., 2004). Melanin forms a hardened physical barrier and is the final step in the cell-mediated processes of nodulation and encapsulation (Ling and Yu, 2006). Despite initiation from hemocytes and its involvement as the last step in cell-mediated immunity, PO activity is still considered a humoral immune response because of the involvement of numerous proteins (the Greek origin of "humor" is protein). Interestingly, this supports the argument that the lines between cell-mediated and humoral immunity overlap.

1.2.2.3. Functional immune measures

Overall, regardless of species, immunity encompasses many separate physiological responses that work together to clear an animal of infection. If immune responses are effective, the result is survival of infection and lower pathogen loads. The field of ecoimmunology is moving toward using functional assays, such as survival and bacterial load, to more accurately determine immunocompetency rather than using individual indices of immune responses (Demas and Nelson, 2011). Individual indices of immune responses, like AMP and PO activity, do not

always positively correlate with survival of bacterial infection (Adamo, 2004). However, since the field of ecoimmunology only recently reached consensus on using functional assays, few studies have determined how survival of infection or bacterial load changes with development.

1.3. Insect development

Insect development can be classified as either hemimetabolous (e.g., blattodeans, orthopterans, odonates) or holometabolous (e.g., lepidopterans, dipterans, coleopterans, and hymenopterans). Hemimetabolous insects undergo "simple metamorphosis" with eggs developing via molting into pronymphs, nymphs, and then adults. These stages are considered morphologically and physiologically similar to one another (Champan, 1998). Conversely, holometabolous insects undergo "complete metamorphosis" because eggs develop into larvae, and then into distinct pupae, prior to the molt to adulthood; all stages differ morphologically and physiologically (Champan, 1998). Some aspects of how physiology changes with development in holometabolous insects are well-characterized, such as the hormones controlling development (reviewed below). Other aspects of developmental physiology are largely unstudied, such as how immune responses vary with development. In Chapter 2, I use the holometabolous insect, *M. sexta*, the tobacco hornworm, to determine how immune function varies with development within a single developmental stage.

Manduca sexta is one of the principal model species in insect physiology studies, with the advantage of large amounts of tissue able to be isolated. In fact, a single insect can weigh up to 11 g in the fifth instar, and yield over 1.5 ml of hemolymph. *Manduca sexta* colonies are also relatively easy to maintain in the laboratory.

Manduca sexta hatch from eggs and develop in 5 stages, or instars, throughout the larval life. Each larval stage is punctuated by molting of the exoskeleton, allowing for further feeding

and growth (Reinecke et al. 1980). After developing through 5 larval instars, each lasting about 3-5 days, the final larval molt results in formation of the pupa. The pupal stage lasts approximately 21 days when larvae developed under long days (i.e., summer conditions), before metamorphosing into the adult moth (Reinecke et al. 1980).

1.3.1. Hormonal control of molting

Two developmental hormones, juvenile hormone (JH) and ecdysone, interact to determine the various molts in insects (Truman, 2005). Elevated JH titers throughout larval development prevent holometabolous insects from molting into a pupa (Cole et al., 2002). Within each larval instar, JH levels peak early then decrease until molting (Baker et al., 1987; Nijhout, 1974). JH I, II, and III are the major structural forms of JH produced by *M. sexta*. They are produced by the corpora allatum, an endocrine gland near the insect brain (Baker et al., 1987; Granger et al., 1982). JH I and II are bioactive, while the function of JH III in *M. sexta* is not known (Cole et al., 2002). Circulating levels of JH are controlled by the activity of an enzyme found in the hemolymph called JH esterase, which hydrolyzes JH (Baker et al., 1987). While JH levels and their effects on molting and tissue rearrangement have been characterized in various insects, the precise mechanism(s) by which JH manifests its actions at the molecular level has remained elusive. The search for the JH receptor has been a major area of research in insect endocrinology over the last few decades. The best candidate as a JH receptor found to date is the protein methoprene-tolerant (Met), found in the cuticle of *D. melanogaster* and *Tribolium castaneum* (flour beetle) (Charles et al., 2011; Konopova and Jindra, 2008; Wilson and Fabian, 1986).

As juveniles grow within a larval instar, they reach what is called a critical mass, a mass at which the commitment to molt into the next developmental stage begins (Nijhout and

Williams, 1974). Attainment of a critical body mass triggers the release of prothoracicotropic hormone (PTTH), which stimulates the prothoracic glands to synthesize and release ecdysone (Nijhout and Williams, 1974). Ecdysone levels peak at the end of each larval instar to signal shedding of the exoskeleton. If JH levels are high when ecdysone is secreted, then a larval-to-larval molt will occur. Alternatively, if JH levels are also low during an ecdysone peak, commitment to metamorphosis results (Nijhout, 1974; Truman, 2005; Truman and Riddiford, 2007). The fluctuating levels of development hormones is likely to have more impact than simply triggering molting and the type of molt, specifically it could affect immune responses, which I address in Chapter 4

1.3.2. Insect immunity and development

Insect immune responses can be plastic over development (Eleftherianos et al., 2008; Tian et al., 2010). Many studies have characterized how a single immune responses may vary with development, such as transcription of AMPs within the 5th and final instar of *M. sexta* (Beetz et al., 2008; Eleftherianos et al., 2008; Jiang et al., 2004; Yu and Kanost, 1999), but only a few studies have identified how the overall insect immune responses change within that instar (Eleftherianos et al., 2008). However, there are conflicting results between these studies (Benesova et al., 2009; Eleftherianos et al., 2008; Lu et al., 2006; Tian et al., 2010). Some results indicated that cell-mediated and humoral immune responses were more robust in the beginning of the final instar of *M. sexta* (Eleftherianos et al., 2008) and *B. mori* (Tian et al., 2010). For example, *M. sexta* at the end of the 5th instar has less than half of the phagocytosis, nodulation, bacterial killing capacity, AMP and PO activity against *Photorhabdus luminescens* than *M. sexta* at the beginning of the 5th instar (Eleftherianos et al., 2008). In *B. mori*, AMP activity and

transcription reduces 50% against *Staphylococcus aureus* and *Escherichia coli* as they age within their 4th and final instar (Tian et al., 2010).

In direct contrast, others have shown immune responses to be more robust at the end of the final instar of a variety of insect species (Beetz et al., 2008; Benesova et al., 2009; Lu et al., 2006). In *Galleria mellonella* (wax moth), PO activity peaks at the end of the 7th and final instar (Benesova et al., 2009), and in *Ostrinia furnacalis* (Asian corn borer), both encapsulation and PO activity peak at the end of the 5th and final instar (Lu et al., 2006). In *M. sexta*, lysozyme activity and hemocyte concentration are 5 times higher in pre-pupating caterpillars than in freshly molted 5th instar caterpillars (Beetz et al., 2008). Because of these contradictions in the literature regarding how immune function changes with development, I found it important to determine how and precisely when immune responses start to change during development, which I address in Chapter 2.

1.4. Mediators of the development-immunity link

Chapter 2 of this dissertation provides evidence that insect innate immune responses are developmentally plastic to such a degree that immune responsivity can change within a single larval instar (Eleftherianos et al., 2008; Tian et al., 2010). Though there is an established link between within-instar development and declines in immune responses, little research has been carried out to determine what mediates the development-immunity link. In Chapter 3, I show a link between development and a matrix metalloproteinase (MMP) and then determine how MMP is involved in immunity. In Chapter 4, I hypothesize how known variations in JH with development affect immunity. My dissertation research is summarized in fig. 2.

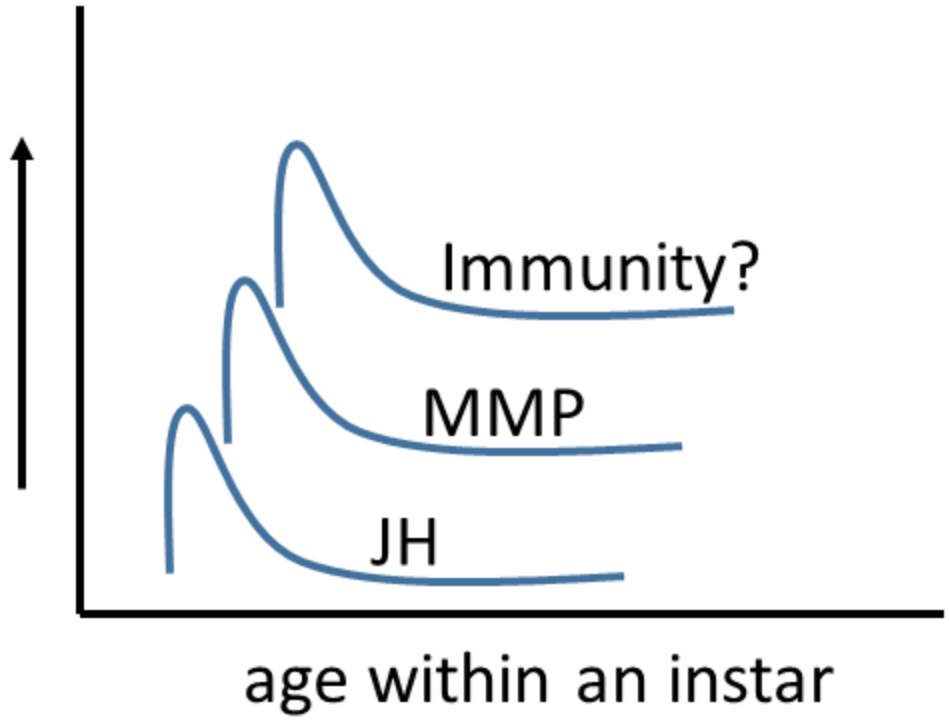

Fig. 2. As insects age within an instar, JH levels and MMP expression decrease, possibly driving a decrease in the capacity for immune responses.

1.4.1. Matrix metalloproteinases

Though there is an established link between within-instar development and a decline in immune responses, little research has been carried out to determine what mediates the development-immunity link. One of the many changes that occur within an instar that could potentially affect immunity includes expression of matrix metalloproteinase (MMP). MMPs are a family of evolutionarily-conserved zinc-dependent endoproteinases that play a role in many physiological and developmental processes, including immunity. There are about 25 types of MMPs found in vertebrates (Brinckerhoff and Matrisian, 2002; Mannello et al., 2005; Mott and Werb, 2004), whereas there are typically only 1-3 MMPs found in a given insect species (Altincicek and Vilcinskas, 2006; Knorr et al., 2009; Vishnuvardhan et al., 2013).

Since MMPs were first discovered to be involved in tadpole metamorphosis in 1962 by Gross and Lapiere, many more functions of these enzymes have been revealed (Gross and Lapiere, 1962). A few of the physiological processes MMPs are involved in include tissue remodeling, tissue repair, wound healing, immune cell migration, and innate immunity (Dubois et al., 2002; Greenlee et al., 2006; Gross and Lapiere, 1962; Hong et al., 2011; Page-McCaw et al., 2007; Vanlaere and Libert, 2009; Vu and Werb, 2000). They are known to be involved in extracellular matrix remodeling and repair as they have the ability to break down extracellular matrix molecules such as collagen (Vu and Werb, 2000). MMPs are involved in in tissue repair in response to wound-forming injuries, infection, or inflammation (Page-McCaw et al., 2007). MMPs play a role in immune cell migration by cleaving cell adhesion molecules, cytokines, and chemokines known to be crucial in immune cell migration (Greenlee et al., 2006; Zhang et al., 2006).

Because many different types of MMPs are found in vertebrates and because many of them have overlapping and redundant roles (Lambert et al., 2003; Stickens et al., 2004), it has been difficult to identify the exact function of MMPs in many physiological processes. Insects lend themselves well to studying the role of MMP in physiology, because they often have one or very few MMPs, though this doesn't overcome the problem of multifunctionality. The first MMPs in insects were discovered in *D. melanogaster* and *G. mellonella*, and were implicated in metamorphosis and tissue remodeling (Altincicek and Vilcinskas, 2006; Llano et al., 2000). More recently, an MMP (Ms-MMP) has been sequenced in *M. sexta* (Vishnuvardhan et al., 2013). Ms-MMP was initially found in the tracheae of *M. sexta* and also in the immune tissues of hemocytes and fat body, further supporting a role for MMPs in immune function (Vishnuvardhan et al., 2013).

1.4.2. MMPs and immunity

MMPs are thought to be important for immune function based on the evidence that MMPs prevent infection and mortality in mice (Dubois et al., 2002; Hong et al., 2011; Vanlaere and Libert, 2009). In insects, MMPs are though to play a role in innate immunity and wound healing. For example, in *T. castaneum* without MMP have high rates of mortality in response to *Beauveria bassiana* fungal infection (Knorr et al., 2009). MMPs play a role in innate immunity and wound healing in the flour beetle, *T. castaneum* (Knorr et al., 2009) and the wax moth, *G. melonella* (Altincicek and Vilcinskas, 2008). The role of MMP in wound healing starts with MMP secretion from the epithelium at the site of damage (Nagase and Woessner, 1999). Upon release at the wound site, MMP cleaves extracellular matrix proteins and alters cell signaling molecules involved in recruiting immune cells (Nagase and Woessner, 1999). In *G. melonella*, inhibition of MMP activity by the MMP inhibitor, GM6001 significantly suppressed lipopolysaccharide-induced antibacterial activity of hemolymph (Altincicek and Vilcinskas, 2008) while injection of purified MMP protein stimulated AMP mRNA expression (Altincicek and Vilcinskas, 2006). Additionally, MMP has been shown to cleave and activate the AMP α-defensin in mice (Wilson et al., 1999), providing a rationale for studying the role of MMP in humoral immune responses such as AMP activity. Further support for a role of MMPs in immune function is seen in *G. melonella*, whereby MMP mRNA expression in hemocytes and fat body is up-regulated after LPS-activation of the immune system (Altincicek and Vilcinskas, 2008). Additionally, LPS-injection also led to increased MMP mRNA expression and activity in hemocytes in *T. castaneum* (Knorr et al., 2009).

In addition to the link between MMP and immunity, MMP has also been shown, in insects, to have differential expression during development. In *G. melonella*, MMP mRNA

expression is up-regulated during pupation and associated with enhanced AMP synthesis at the onset of metamorphosis (Altincicek and Vilcinskas, 2006). More specifically, in *M. sexta*, gene and protein expression of MMP in tracheal tissue of *M. sexta* decrease during the 4th and 5th instars (Vishnuvardhan et al., 2013). Though it is also established that MMP is involved in immunity in other insects, it is unclear whether MMP is directly involved in the decline in immune responses throughout the 5th instar of *M. sexta*. I address this point in Chapter 3.

1.4.3. JH and immunity

Another of the developmental changes that occur within an instar that could potentially affect immunity is JH titer. JH is known for regulating development and reproduction in insects, but recently has been shown to have other roles, including mediating the immune system. For example, JH analog applied topically to prepupal 4th instar silkworms, *B. mori*, resulted in a dramatic increase in gene expression of several AMPs, including cecropin, morincin, lebocin, and neucin, and the gloverin-like proteins 1 and 2 (Tian et al., 2010). In addition, JH analog application increased AMP activity against both *E. coli* and *S. aureus* in a traditional zone of inhibition assay (Tian et al., 2010). In contrast, other studies using JH analogs and inhibitors, have determined that JH suppresses AMP production, PO activity, and hemocyte concentration and spreading in insects such as *D. melanogaster*, *Plutella xylostella* (diamond back moth), and *Tenebrio molitor* (mealworm beetle) (Flatt et al., 2008; Kwon and Kim, 2007; Rolff and Siva-Jothy, 2002). For example, application of JH analog resulted in decreased gene expression of many anti-microbial peptides including diptericin, attacin, and cecropin in *D. melanogaster* (Flatt et al., 2008). Application of JH analog also resulted in decreased PO activity in *Calopteryx virgo* (beautiful demoiselle) (Contreras-Garduño et al., 2009). JH analog application also reduced hemocyte spreading in *P. xylostella* (Kwon and Kim, 2007) and abundance in *G. melonella*

(Sezar and Ozlap, 2015). Whether JH is immunosuppressive or immunostimulatory remains controversial. Thus, to resolve this conflict, more studies are needed to understand how developmental changes in JH contribute to changes in immunity, which I address in Chapter 4.

All of the studies investigating the role of JH in immunity have only used a single age of insect, with JH inhibitor applied when natural levels of JH are high or JH analog applied when JH levels are low. This method of assessing the role of JH in immunity may lead to flawed results, because it may not take into account variables such as saturation of JH receptors or varying levels of JH receptor expression at different ages (Charles et al., 2011). Further, no studies have investigated the role of JH using functional immunity assays, such as survival and hemolymph bacterial load. Functional immunity assays have been shown to be a more accurate depiction of immunocompetency compared to individual indices of immune responses, like PO activity and hemocyte spreading. Individual indices of immune responses do not always positively correlate with survival of bacterial infection and lower bacterial loads in hemolymph (Adamo, 2004; Booth et al., 2015). I study how age-related changes in JH affect immune responses and underlie the previously observed decline in immune responses during 5^{th} instar development in Chapter 4.

1.5. Significance of research

While humans need insects for pollination and removal of detritus, many insects are crop or animal (including human) health pests. Caterpillars (larvae of Lepidoptera) as a group are, perhaps, the most significant pests of food crops, and more sustainable methods for their control are needed. Identification of vulnerable stages of larval immunity could allow for development of more sustainable control methods. For insect scientists, my studies emphasize the need to control for age in experimental design. *Manduca sexta* is one of the most important model

species in insect physiology. Solely comparing studies using the same age of *M. sexta* may not be enough to provide an accurate portrayal of *M. sexta*'s immunity across the insect's lifetime.

Furthermore, insects are relevant models for studying mammalian innate immunity because many insect immune responses (phagocytosis and anti-microbial peptides) are evolutionarily conserved across a wide range of species (Lavine and Strand, 2002). MMP is also an evolutionary conserved proteinase and MMPs in mammals are important for proper immune function (Greenlee et al., 2007). If it is determined that MMP is involved in evolutionarily conserved immune responses, this research may better inform the roles of MMPs in human immunity. In addition, understanding the developmental physiology of *M. sexta* and how JH regulates immunity throughout development will allow for better understanding of how hormones may contribute to immune function. For society, determining the factors causing development-dependent changes in immunity could help in the production of new, more efficient pest control methods and reduce impact on the planet.

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CHAPTER 2. IMMUNE DEFENSE VARIES WITHIN AN INSTAR IN THE TOBACCO HORNWORM, *MANDUCA SEXTA*[1]

2.1. Introduction

Insects are numerous, diverse, and able to survive a plethora of pathogens despite their lack of antibody-mediated immunity. Insects have a robust innate immune system that is divided into two branches, cell-mediated and humoral (Tanaka and Yamakawa 2011; Satyavathi et al. 2014). Cell-mediated immune responses are driven by circulating hemocytes, immune cells that perform phagocytosis, nodule formation, and encapsulation (Strand 2008; Tanaka and Yamakawa 2011; Satyavathi et al. 2014). The humoral branch is driven by both hemocytes and the fat body, an organ responsible for metabolic and immune functions. Activation of humoral immunity results in anti-microbial peptide (AMP) production and phenoloxidase activation (PO) (Jiang et al. 2010; Demas and Nelson 2011). AMPs kill pathogens by altering their transcriptional activity or cell membrane structures, whereas PO results in the production of cytotoxic molecules and melanin (Kanost et al. 2004; Tanaka and Yamakawa 2011).

Insect immune responses can be plastic with development (Eleftherianos et al. 2008; Tian et al. 2010). One of the most common model organisms for studying insect immunity is *Manduca sexta* (tobacco hornworm), which develops in 5 stages, or instars, throughout their

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larval life cycle (Reinecke et al. 1980). Many studies have characterized transcription of AMPs within the 5th instar of *M. sexta* (Yu and Kanost 1999; Jiang et al. 2004; Beetz et al. 2008; Eleftherianos et al. 2008; Tanaka and Yamakawa 2011), but only a few studies have identified how the overall insect immune responses change within that instar (Eleftherianos et al. 2008). However, there are conflicting results between these studies. Some results indicated that cell-mediated and humoral immune responses were more robust in the beginning of the final instar of *M. sexta* (Eleftherianos et al. 2008) and *Bombyx mori* (silk worm) (Tian et al. 2010). In direct contrast, others have shown humoral immune responses were more robust at the end of the final instar of *Galleria mellonella* (wax moth) (Benesova et al. 2009) and *Ostrinia furnacalis* (Asian corn borer) (Lu et al. 2006). Although these contradictory results could be species differences, the timing of the development of immune systems remains unclear. In addition, none of these studies identifies precisely when immune responses start to change during development.

Insect immunity encompasses many separate physiological responses that work together to clear an animal of infection. When faced with an infection, animals may respond with resistance, tolerance, or a combination of both. Tolerance is characterized by the survival of animals despite a heavy pathogen load, while resistance is characterized by survival and a low pathogen load (Moreno-García et al. 2014). Resistance is mediated by effective immune responses to lower the pathogen load (Tanaka and Yamakawa 2011; Satyavathi et al. 2014). The field of ecoimmunology is moving toward using functional assays, such as survival and pathogen load, to more accurately determine immunocompetency rather than using individual indices of immune responses (Demas and Nelson 2011). Individual indices of immune responses, like AMP and PO activity, do not always positively correlate with survival of bacterial infection (Adamo 2004). However, since the consensus in ecoimmunology to use functional assays has

recently been reached, few studies have yet determined how survival of infection or bacterial load changes with development. Although Eleftherianos *et al*. (2008) observed increases in susceptibility to bacterial infection in *M. sexta* later in the 5th instar, more studies are needed to test how functional immunity varies with development.

Immune responses are a metabolically costly physiological function (Freitak et al. 2003; Ardia et al. 2012; Catalan et al. 2012). If an animal is perturbed with an immune challenge such as a bacterial infection, normal allocation of energy toward processes like growth, development, metabolism, and reproduction may be disrupted since resources are limited. (Stearns 1992). Delayed growth and development are often the energetic costs of an immune challenge in many insects, including *M. sexta* and *Acheta domesticus* (house cricket) (Adamo et al. 2007; Bascunan-Garcia et al. 2010; Diamond and Kingsolver 2011). Based on life history theory, predictions can be made about how animals will respond to perturbations like an immune challenge, but no studies have investigated how energy allocations may vary with age upon an immune challenge in insect larvae.

In this study, we used several assays to test three main hypotheses. 1) To test the hypothesis that humoral immune responses vary with development, we measured AMP and PO activity in *M. sexta* at each day of the 5th instar (days 0-4). We predicted that AMP and PO activity would decrease with each day of development within the 5th instar. 2) To test the hypothesis that functional immunity varies with development, we assessed bacterial load and survival of infection throughout 5th instar development in *M. sexta*. Based on the predicted outcomes for hypothesis 1, we also predicted that bacterial load would increase and survival would decrease during 5th instar development. 3) To test the hypothesis that changes in energy allocation upon infection vary with development, we measured growth rate and development

time of infected *M. sexta* at each day of the 5th instar (days 0-4). We predicted that growth rate would decrease and development time would increase in infected early 5th instar caterpillars.

2.2. Material and methods

2.2.1. Animal care

M. sexta larvae (Carolina Biological, Burlington, NC) were reared at 25°C under a 16L:8D photoperiod with *ad libitum* access to an artificial, wheat germ-based diet as previously described (Vishnuvardhan et al. 2013). *M. sexta* were observed daily to determine the age within an instar, indicated by the presence or absence of head capsules. Head capsule formation indicates the last day of each instar, while head capsule slippage marks the first day of each instar (day 0). The end of the 5th instar is indicated by cessation of feeding and visualization of the dorsal heart, typically day 4 of the 5th instar (Reinecke et al. 1980). The following life stage is prepupal formation, in which caterpillars have a lighter pigmentation, a moist cuticle, and increased activity (Reinecke et al. 1980).

2.2.2. Micrococcus lysodeikticus preparation

Lyophilized *M. lysodeikticus* (Sigma-Aldrich, St. Louis, MO) used in the AMP activity assay were prepared at a concentration of 10 mg/ml in *Manduca* saline buffer (MSB; 4mM NaCl, 40mM KCl, 18mM $MgCl_2 \bullet 6H_2O$, and 3mM $CaCl_2$).

2.2.3. Bacterial cultures

Escherischia coli and *Stenotrophomonas maltophilia* were grown to stationary phase in Luria broth (LB) and the optical densities of the bacterial suspensions were measured using a spectrophotometer at 600nm (Beckman Coulter, Indianapolis, MN). The bacterial suspensions were centrifuged at 4000 x g for 15 min. Resulting pellets were washed with PBS (137mM NaCl, 3mM KCl, 10mM Na_2HPO_4, 2mM KH_2PO_4, pH 7.4) and centrifuged again at 4000 x g for

15 min. The final *E. coli* bacterial pellet was re-suspended in PBS at a concentration of 1.00×10^6 colony forming units (CFU) per 10µl.The final *S. maltophilia* bacterial pellet was re-suspended in 1.66 ml of PBS. Then 10µl of bacterial dilutions (10^{-1}-10^{-5}) in PBS were plated on LB agar, and bacterial colonies were counted 24 h later to determine the concentration of bacteria injected into each caterpillar.

2.2.4. Injections

Regardless of injected treatment and age of *M. sexta,* the injection method was the same. Larvae were cooled on ice for 5 min and surface sterilized with 75% ethanol. A 25 µl syringe (Hamilton, Reno, NV) with a 26 gauge needle was filled with 10 µl of control buffer or bacterial suspension. The needle was inserted subcutaneously into the hemocoel of the caterpillar between the first and second pro-legs. Once an injection was completed, the injection site was monitored for potential leaking of hemolymph, but no caterpillars exhibited leaking. Caterpillars were then transferred to individual containers with *ad libitum* access to food and monitored daily.

According to standards in developmental insect immune studies, caterpillars were given the same amount of bacteria, regardless of body mass (Hung and Boucias 1996; Zerofsky et al. 2005; Beetz et al. 2008; Eleftherianos et al. 2008). To validate this approach for our study, we conducted a preliminary experiment comparing the immune responses of caterpillars of similar ages and body mass that were given either the standard dose or a mass-corrected dose of bacteria. Younger (end of fourth instar) and older (fifth instar day 2) caterpillars were injected with either 10 µl of heat-killed *Micrococcus lysodeikticus* (10mg/ml in MSB) or 10 µl of a mass-corrected dose. We corrected the dose based on the average mass of a day 0, fifth instar caterpillar, by using the following correction factor, (caterpillar mass mg x 10 mg)/1100 mg.

Twenty-four hours post- injection, hemolymph was collected and prepared for hemocyte count by diluting 10 µl into 90 µl of PBS for a 1:10 dilution. The hemolymph solution was vortexed, and 10 µl placed on a hemocytometer. Hemocytes were counted at 40x magnification, and hemocyte concentration (hemocytes/ml of HL) and total hemocyte counts (hemocytes/insect), were calculated. Because we found no significant difference between young and old caterpillars in their responses to standard or mass-corrected bacterial doses, we used the standard dose for all of our experiments (Fig. 1; effect of dose, $F_{1,8} = 0.6$, $p = 0.46$).

Caterpillars for AMP assays were injected with 10 mg of *M. lysodeikticus* in 10 µl of *Manduca* saline. Caterpillars for the PO activity assay were injected with 10 µl of *Manduca* saline. Caterpillars for bacterial load, growth rate, and development time assays were injected with 1.00×10^6CFU of *E. coli* in 10 µl of PBS. Caterpillars for the mortality rate from *S. maltophilia* assay were injected with varying amounts of bacteria (see mortality assay for details).

2.2.5. Hemolymph collection

Hemolymph was collected 12 h post-*E. coli* injection in the bacterial load assay and 24 h post-*M. lysodeikticus* injection in the AMP and PO activity assays. *M. sexta* larvae were cooled on ice for 5 min and surface sterilized with 75% ethanol before sample collection. To collect hemolymph, the first right pro-leg was cut with a sterilized razor blade and hemolymph was expelled into a 1.5 ml Eppendorf tube on ice. The hemolymph for the bacterial load assay was used immediately while the hemolymph for the AMP and PO activity assays was stored at -20°C.

2.2.6. Anti-microbial peptide (AMP) activity assay

To determine the activity of the AMPs produced as a part of the humoral immune response, a traditional zone-of-inhibition assay was performed (Hoffmann et al. 1981). Caterpillars on each day of the 5th instar (day 0-4) were injected with 10 µl of lyophilized *M. lysodeikticus* at a concentration of 10 mg/ml to induce the production of AMPs. Hemolymph was collected 24 h later. AMPs were isolated by centrifugation at 7000 x g for 5 min to separate hemocytes from hemolymph. The plasma layer was collected and heated at 95°C for 5 min and spun again at 13,400 x g for 10 min. AMPs remain in the supernatant. Wells (3 mm diameter) were carved into LB agar plates embedded with DH5α *E. coli.* Five µl of AMPs from each caterpillar (n=6) were loaded into the wells in duplicate. The plates were incubated at 37°C for 1 h, then at 25°C overnight. The next morning, the plates were photographed with a ruler for calibration, to measure the zone of inhibition, the diameter around the well where the AMPs inhibited bacterial growth. Using Image J (1.45K version) (Rasband 1997-2011), the diameter of the area lacking bacteria was measured vertically, horizontally, and diagonally across the cleared area. Then using the oval selection tool, the circumference of the zone of inhibition was measured and from that a diameter calculated. These four measures of diameter were averaged for each well to yield one zone of inhibition per well. The zone of inhibition for each caterpillar was divided by the average hemolymph volume collected from the appropriate caterpillar age to account for increases in hemolymph volume during 5th instar development.

2.2.7. Phenoloxidase activity assay

Caterpillars of each day of the 5th instar (day 0-4) were injected with 10 µl of *Manduca* saline to induce the production of PO. Hemolymph was collected 24 h later and centrifuged at 7000 x g at 4°C for 10 min to separate the hemocytes from the hemolymph plasma. A 10 µl

fraction of the hemolymph plasma was mixed for 1 h with *E. coli* LPS (Sigma-Aldrich, St. Louis, MO), an effective pro-PO to PO activator (Laughton and Siva-Jothy 2010). The reaction was started by adding 20mM 4-methylcatechol (Sigma-Aldrich, St. Louis, MO). The conversion of 4-methylcatechol to colorimetric quinone by phenoloxidase was marked by a change in absorbance (at 490 nm), which was measured every 15 min for 1 h at room temperature by a microplate reader (BioRad, Hercules, CA). The change in absorbency over time (mOD/min) is directly related to phenoloxidase activity (Eleftherianos et al. 2008). Slopes from each hemolymph sample were calculated and compared. PO activity for each caterpillar was divided by the average hemolymph volume collected from the appropriate caterpillar age to account for increases in hemolymph volume during 5^{th} instar development.

2.2.8. Bacterial load assay

Caterpillars of day 0, day 2, and day 4 of the 5^{th} instar were injected with 1.00×10^6CFU of *E. coli* or 10 µl of PBS. Hemolymph was collected 12 h later and the concentration of bacteria was determined via standard colony counts after serial dilution. We assumed that the proportion of bacteria adhering to tissues was similar among days. Thus, this measurement of load indicates the amount of circulating bacteria.

2.2.9. Mortality assay

Caterpillars on day 0, 2, and 4 of the 5^{th} instar were injected with between 6.85×10^7 and 1.72×10^8 CFU of *S. maltophilia*. Caterpillars were assessed for mortality from the time of injection until 48 h post-injection. Caterpillars unable to right themselves after being placed on their dorsum were considered dead. Observations of mortality were made hourly for 8 h post-injection and 10, 14, 18, 22, 24, 26, 30, 42, 46, and 48 h post-injection.

2.2.10. Growth rate assay

Caterpillars from each day of the 5^{th} instar (day 0-4) were injected with 1.00×10^6CFU of *E. coli* or 10 µl PBS. Caterpillars were weighed 0, 4, and 8 h post-injection. The rate of change in growth over 8 h post-injection was calculated to represent growth rate. The growth rate over the first 8 h post-injection was chosen because the 5^{th} instar day 4 animals develop into prepupae 16 h post-injection. Prepupae normally lose weight and were thus not included in the growth rate calculations.

2.2.11. Development time assay

Caterpillars from each day of the 5^{th} instar (day 0-4) were injected with 1.00×10^6CFU of *E. coli* or 10 µl PBS. Caterpillars were observed twice daily for signs of reaching the prepupal life stage. The number of days it took for caterpillars to reach prepupal formation from the day of 5^{th} instar ecdysis was recorded to yield development time.

2.2.12. Data analysis

Statistical analyses were performed using IBM SPSS version 21. To analyze hemocyte counts, we used two-way Analysis of Variance (ANOVA). For the PO activity assay, bacterial load assay, growth rate assay, and development time assay, an ANOVAtest was used to obtain F-values and Bonferroni post-hoc tests were used to detect differences among treatment groups. . Because the AMP activity data were not normally distributed, we used a Kruskal-Wallis test to obtain a χ^2-value, and Tamhane's post-hoc tests were used to detect differences between treatment groups. For the mortality assays, the Cox-Regression test was used to obtain a χ^2-value and Kaplan-Meier post-hoc tests were used to detect differences between treatment groups. A probability value < 0.05 indicates statistical significance. Data are expressed as mean ± SEM throughout.

2.3. Results

2.3.1. AMP and PO activity vary with development

AMP activity peaked at the beginning of the 5th instar (day 0) and significantly decreased on subsequent days during the 5th instar (days 1, 2, 3, and 4), approximately halving AMP activity with each consecutive day (Fig. 3, χ^2=41.22, P<0.05; Kruskal-Wallis; n=6 for all treatment groups). PO activity also peaked at the beginning of the 5th instar (day 0). PO activity in hemolymph from 5th instar day 0 larvae was 2-fold higher than from hemolymph extracted from day 1 or 2 larvae, 5-fold higher than day 3, and 8-fold higher than day 4 (Fig. 4, $F_{6,14}$=17.823, P<0.05; ANOVA; n=3 for all treatment groups).

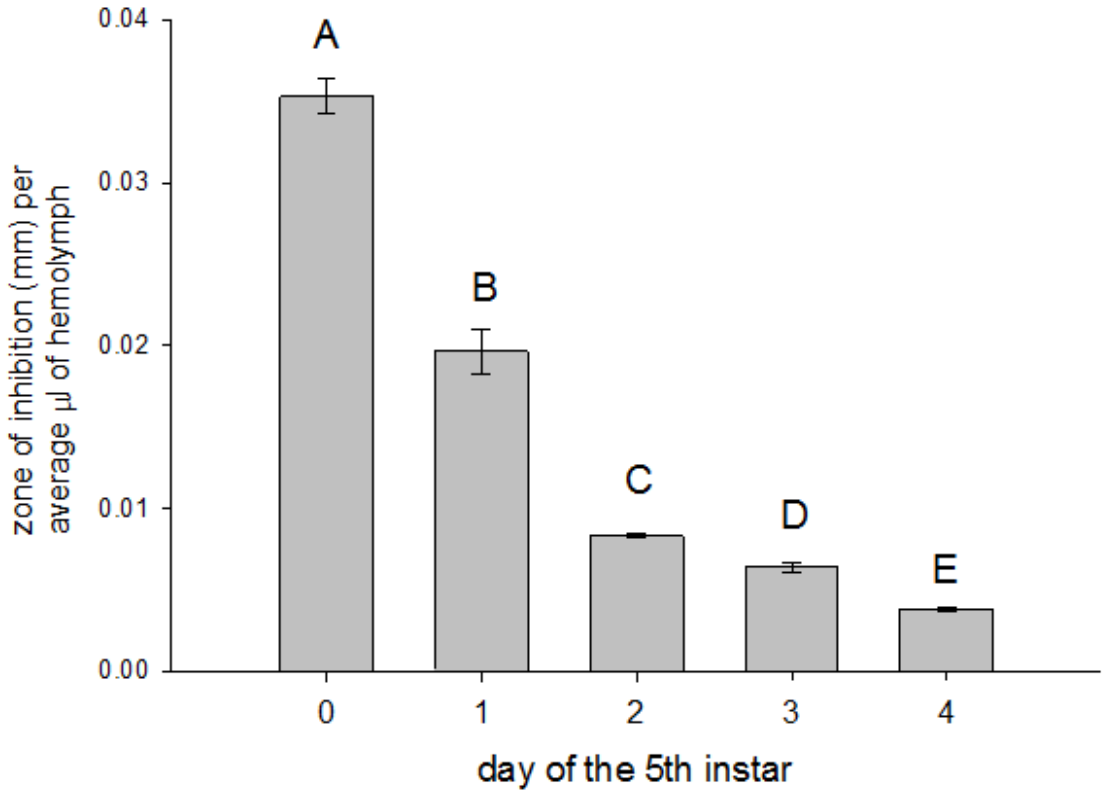

Fig. 3. Anti-microbial peptide (AMP) activity throughout the 5th instar in *M. sexta*. *M. sexta* of each day of the 5th instar (day 0-4) were injected with dried *M. lysodeikticus*, and AMP activity was assessed 24 h later. Bars with different letters are significantly different from one another (χ^2=41.22, P<0.05; Kruskal-Wallis). Error bars indicate +/- SEM.

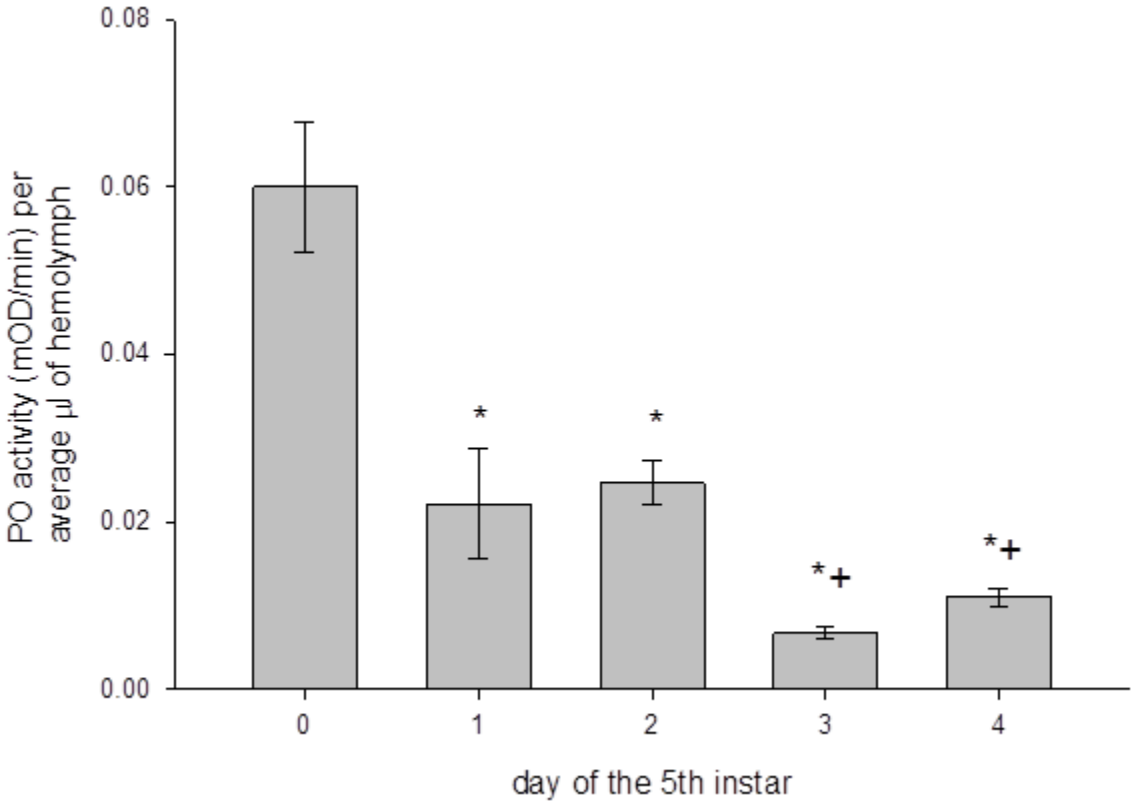

Fig. 4. Phenoloxidase (PO) activity throughout the 5th instar in *M. sexta*. *M. sexta* on each day of the 5th instar (day 0-4) were injected with MSB, and PO activity was assessed 24 h later. Bars with * indicate values significantly lower than day 0 and bars with + indicate values significantly lower than day 1 ($F_{6,14}$=17.823, P<0.05; ANOVA). Error bars indicate +/- SEM.

2.3.2. Bacterial load and survival vary with development

Hemolymph from 5th instar day 0 caterpillars had about 25-fold higher *E. coli* bacterial loads than hemolymph from 5th instar day 4 caterpillars (Fig. 5, $F_{2, 53}$= 4.107, P<0.05; ANOVA; n=10 for all treatment groups). Hemolymph from *M. sexta* injected with PBS did not grow any bacteria (data not shown). *M. sexta* were also challenged with a lethal dose of *S. maltophilia* on varying days within the 5th instar to examine differences in survival kinetics. *M. sexta* injected with *S. maltophilia* as 5th instar day 0 died twice as quickly as caterpillars injected as 5th instar day 4 (Fig. 6 , 5th instar day 0 vs. day 2: χ^2=7.442, P<0.05, Kaplan Meier; 5th instar day 0 vs. 4:

χ^2=9.751, *P*<0.005, Kaplan Meier; 5th instar day 2 vs. 4: χ^2=8.310, *P*<0.005; n=10 for all treatment groups). Regardless of age upon injection, 100% of *M. sexta* survived PBS injections.

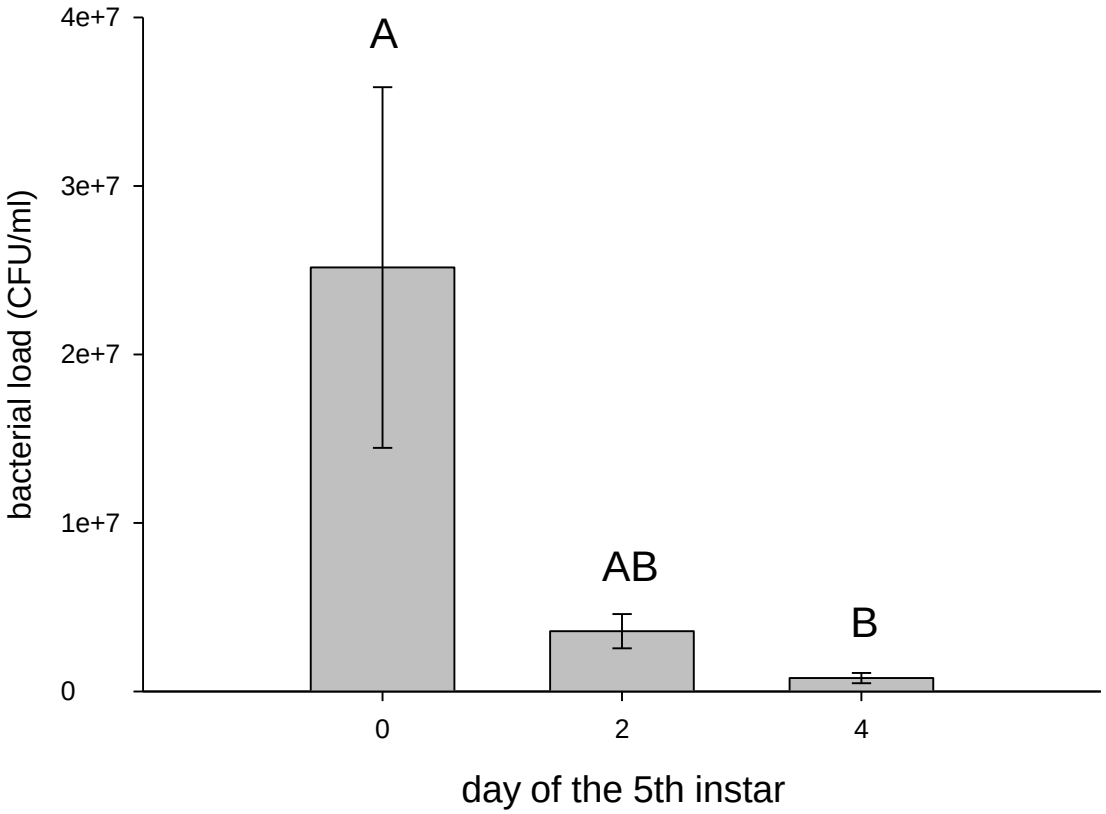

Fig. 5. Bacterial load of *M. sexta* injected with *E. coli* on days 0, 2, and 4 of the 5th instar. Hemolymph was collected 12 h post-injection, and bacterial growth assessed. Bars with the same letter are not significantly different from one another ($F_{2, 53}$= 4.107, *P*<0.05; ANOVA). Error bars indicate +/- SEM.

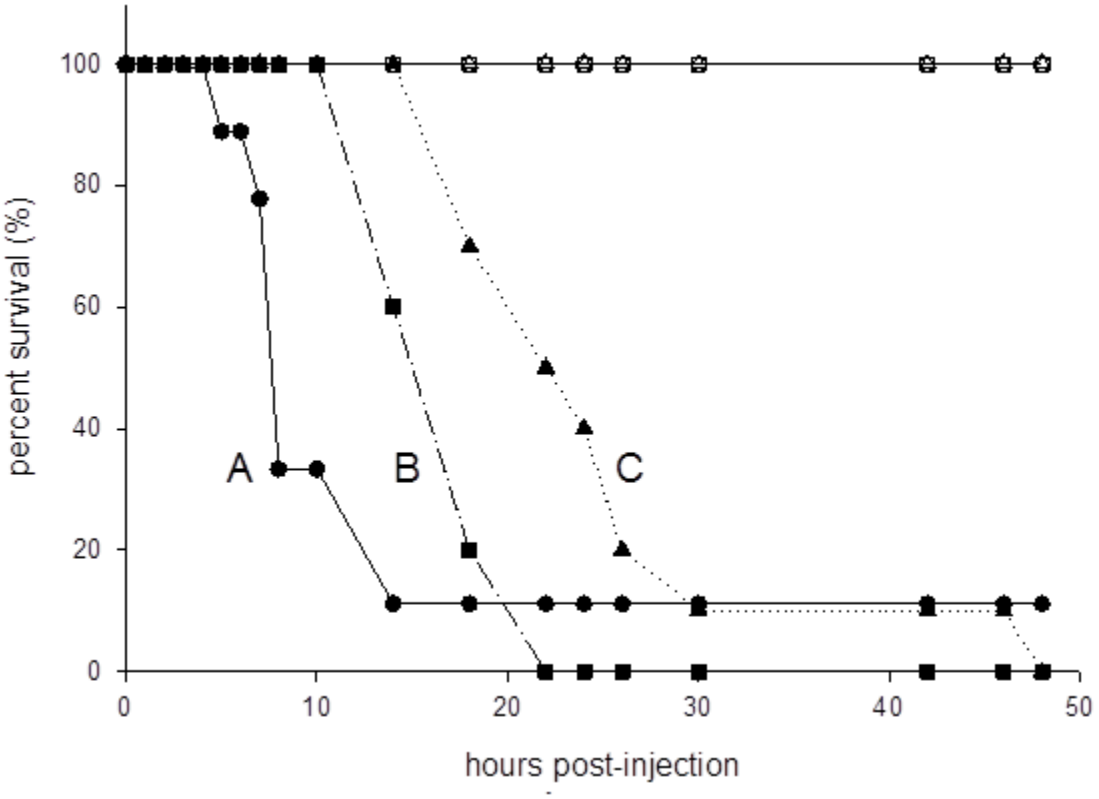

Fig. 6. Survival of *S. maltophilia* infection by *M. sexta* injected on day 0, day 2, and day 4 of the 5th instar. Caterpillars were checked hourly for 8 h and then every 4 h for signs of life. PBS-injected caterpillars are represented with open symbols and *S. maltophilia*-injected caterpillars are represented with closed symbols. All 5th instar day 0 caterpillars are represented by circles, 5th instar day 2 caterpillars by squares, and 5th instar day 4 caterpillars with triangles. Lines with different letters are significantly different from one another (χ^2=57.691, P<0.05; Cox-Regression).

2.3.3. Growth and development vary with infection

Injection with *E. coli* significantly delayed growth in caterpillars that were early in the instar (Fig. 7, $F_{1,87}$ = 32.328, P<0.05; ANOVA; n=10 for all treatment groups). Differences in growth rates depended on treatment injection (PBS or *E. coli*) and only existed in *M. sexta* injected as 5th instar day 0, 1, and 2. *M. sexta* injected with PBS as 5th instar day 0 or day 1 caterpillars had 4-fold higher growth rates over 8 h post-injection than caterpillars of the same age injected with *E. coli*. *M. sexta* injected with PBS as 5th instar day 2 had 2-fold higher growth rates than caterpillars of the same age injected with *E. coli*. The delayed development also

affected the time to reach the pre-pupal stage, but the effect differed depending on the age of the caterpillar at injection (Fig. 8, $F_{1,85} = 74.420$, $P<0.05$; ANOVA; n=10 for all treatment groups). *M. sexta* injected with *E. coli* as 5th instar day 0 caterpillars took twice as long to reach the pre-pupal stage as caterpillars injected with PBS. Regardless of the day on which the 5th instar caterpillars are injected, those injected with PBS reached the prepupal stage in about 5 +/- 0.1 days.

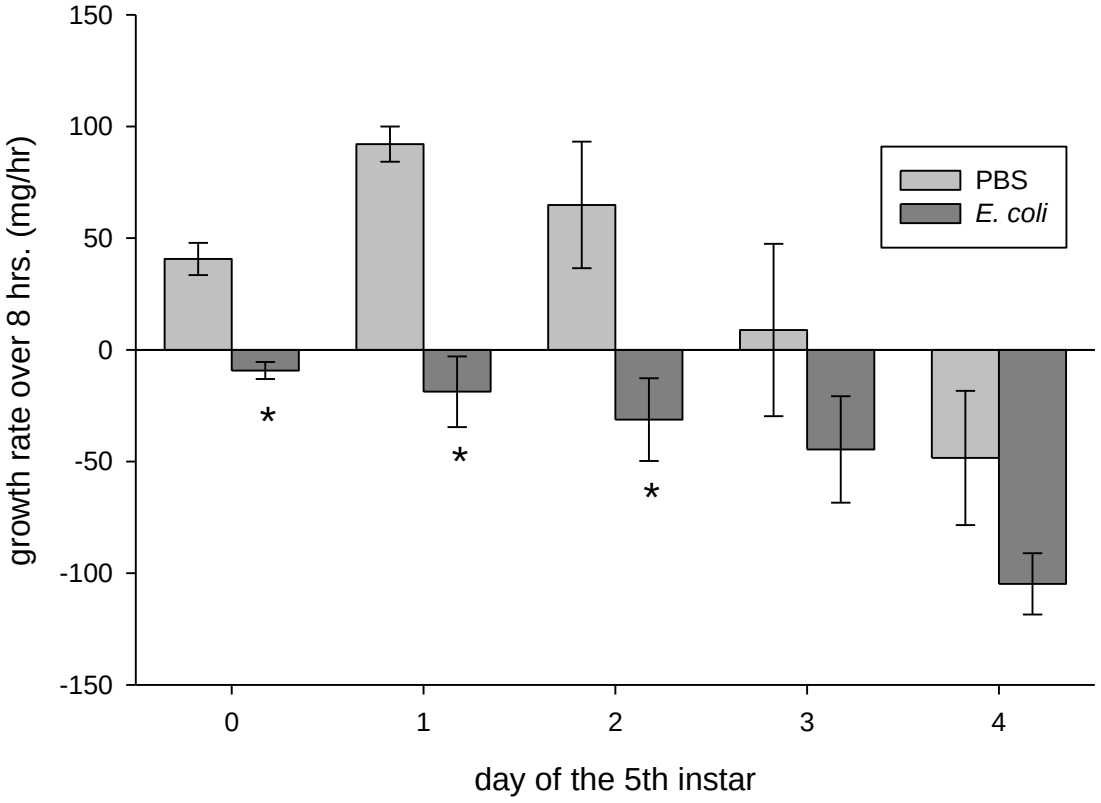

Fig. 7. Growth rates of *M. sexta* injected with either PBS or *E. coli* on each day of the 5th instar (day 0-4). Caterpillars were weighed immediately, 4 h and 8 h post-infection. Bars with* indicate values significantly lower than the PBS control for the given age ($F_{1,87} = 32.328$, $P<0.05$; ANOVA). Error bars indicate +/- SEM.

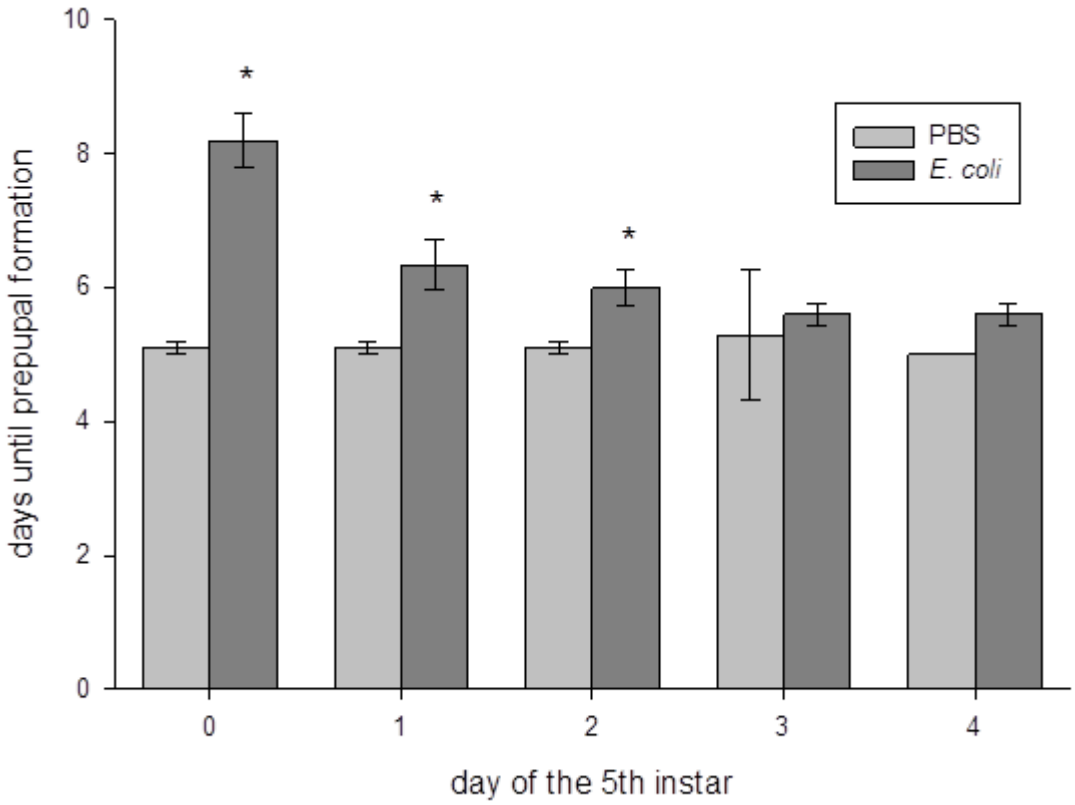

Fig. 8. Development times of *M. sexta* larvae injected with either PBS or *E. coli* on each day of the 5th instar (day 0-4). Larvae were observed for signs of prepupal formation twice daily. Bars with* indicate values significantly higher than the PBS control for the given age ($F_{1,85}$ = 74.420, P<0.05; ANOVA). Error bars indicate +/- SEM.

2.4. Discussion

2.4.1. AMP and PO activity vary with development

Our results concur with previous findings that humoral immune responses, AMP and PO activity, peak at the beginning of the 5th instar in insects (Figs. 3 and 4) (Eleftherianos et al. 2008; Tian et al. 2010). Supporting our results, Eleftherianos et al. determined *M. sexta* larvae early in the 5th instar had more robust cell-mediated (phagocytosis and nodule formation) and humoral immune responses (AMP gene expression and PO activity) to *Photorhabdus luminescens* infection when compared to animals later within the 5th instar (Eleftherianos et al. 2008). In another study, Tian et al. determined *B. mori* had decreased AMP activity against

Staphylococcus aureus and *E. coli* as they age within the 4th and final instar (Tian et al. 2010). Our data provide a unique perspective on this topic because it is the first study to track every day of the 5th instar *M. sexta*, specifically determining humoral immune responses decline after just a single day in the 5th instar. Although many physiological changes occur in the 5th and final instar, it is striking that such drastic changes in humoral immunity occur in as short of a timespan as 1 day. This emphasizes the need to control for age in experimental design. Scientists should also use caution when interpreting and integrating information from various physiological studies using different ages of insects. *M. sexta* in the 5th instar are used most often in insect physiology studies because of the large amount of tissue that can be isolated from them. Even comparing studies using the same *M. sexta* instar may not be enough to provide an accurate portrayal of the field of *M. sexta* immunity as a whole.

Although the body of research is limited, our results clarify how humoral immunity varies with development in insects. Our results provide evidence for development-related declines in humoral immunity in direct contrast to what others have observed. PO activity peaked at the end of the 7th and final instar of *G. mellonella* (Benesova et al. 2009) and the 5th and final instar of *O. furnacalis* (Lu et al. 2006). Immune function against *E.coli* increased until the last developmental stage before adult onset of *Tachycineta bicolor* (Stambaugh 2009). As larval Lepidoptera are voracious crop pests, identification of the particularly vulnerable stages of larval immunity could allow for better biological control methods. Farmers would be able to apply pesticides at a targeted time when larval Lepidoptera are most vulnerable. The opposite could be true for other animals, especially endangered populations. Because infectious diseases could be a contributing factor to causing a fluctuation in population (MacPhee and Greenwood

2013), finding a point in development where treatments would be most effective would be beneficial.

From an ecological perspective, it is still unclear why there would be such drastic declines in immunity during the 5th instar. It could be beneficial for caterpillars to have robust immune responses immediately after molting into the 5th instar, before the cuticle has fully sclerotized. Our alternative hypothesis relies on the life history theory changes in energy allocation during immune challenge. We hypothesize that energy is already allocated toward storage for pupation at the end of the 5th instar and is not available for immune responses. From a mechanistic perspective, the driving force behind development-related changes in immunity within the 5th instar remains unclear. Many changes occur within an instar that could affect immunity, including the level of juvenile hormone (JH). JH, a developmental hormone that prevents early molting, peaks early and decreases within an instar until molting (Nijhout 1974; Baker 1987). JH analog applied topically to early wandering 4th instar silkworms, *B. mori,* resulted in a dramatic increase in gene expression of several AMPs, including cecropin, morincin, lebocin, and neucin, and the gloverin-like proteins 1 and 2 (Tian et al. 2010). In addition, JH analog application increased AMP activity against both *E. coli* and *S. aureus* in a traditional zone of inhibition assay (Tian et al. 2010). Although JH is immunostimulatory in larval *B.mori*, it is unclear how JH might be involved in immunity in *M. sexta*.

Alternatively, the changes in growth may be an adaptive response from the host, mediated by the insect cytokine growth-blocking peptide (GBP). Hayakawa (1995) and Noguchi *et al*. (Noguchi et al. 2003) showed that armyworms, *Pseudaletis separata,* display elevated levels of GBP when parasitized by the wasp, *Cotesia kariyai*. The release of GBP upon infection has multiple benefits for the host, such as creating a higher JH concentrated environment, which

could possibly increase AMP activity (Tian et al. 2010), and delaying growth, which would be able to provide more time for healing before molting. GBP causes increased gene expression of dopa decarboxylase (DDC), which produces the enzyme necessary to convert dopa into dopamine (Noguchi et al. 2003). Elevated levels of dopamine in the hemolymph and nerve cord delay both growth and metamorphosis (Hayakawa 1995; Noguchi et al. 2003) and repress juvenile hormone esterase (JHE) synthesis (Hayakawa 1995; Noguchi et al. 2003), keeping JH levels high, perhaps delaying molting (Nijhout 1974; Baker 1987). The elevated levels of dopamine also cause a paralysis effect, resulting in reduced feeding and delayed growth (Hayakawa 1995). A better understanding of the roles of JH and GBP in modulating development-related changes in humoral immunity is clearly necessary.

2.4.2. Bacterial load and survival vary with development

Interestingly, our results show that although *M. sexta* have more robust AMP and PO activity earlier in 5th instar, they also have 25-fold higher *E. coli* bacterial loads and die twice as quickly by *S. maltophilia* infection, indicating ineffective immune responses (Figs. 5 and 6). The disparity between our results of the individual insect immune responses (AMP and PO activity) and the functional immune assays (bacterial load and survival) is puzzling because of the assumption that active immune responses will clear an infection. A possible explanation for this disparity is varying AMP production across ages, resulting in different responses to infection. Although the body of research is limited, there are some reports of this phenomenon in the literature. For example, total PO activity and baseline lysozyme-like activity did not predict survival to *Serratia marcescens*, *Serratia liquefaciens*, or *Bacillus cereus* in male crickets *Gryllus texensis* (Adamo 2004).

The ability to survive an infection depends on a vast number of immune responses working together, which does not translate into all immune responses with peak activity upon an immune challenge. There could be trade-offs even within an individual's immune response. For example, increased encapsulation and PO activity was associated with lower lysozyme activity in *Tenebrio molitor* (mealworm beetle) and *A. domesticus* (Ardia et al. 2012). Lysozyme-like activity was also negatively correlated with hemocyte density and PO activity in *Spodoptera littoralis* (African cotton leafworm) (Cotter et al. 2004). Animals assessed with only certain immune assays may appear to have greater immune capacity. However, unassessed immune responses may be equally important in clearing an animal of infection. For example, perhaps the unmeasured cell-mediated immune responses at the end of the 5th instar are more active and effective than humoral immune responses at clearing *E. coli* and *S. maltophilia* infections. In support of this hypothesis, mass-corrected total circulating hemocytes were significantly higher at the end of the 5th instar in *M. sexta*, possibly indicating more inducible cell-mediated responses than earlier in the 5th instar (Beetz et al. 2008). Our results emphasize the importance of shifting ecoimmunology research toward functional immune assays. Because the immune response to infection is so complex, measuring all aspects of the immune response is difficult. Performing bacterial load and survival assays in conjunction with specific immune responses assays can improve our interpretation of results.

2.4.3. Growth and development vary with infection

Our results also indicate *M. sexta* early in the 5th instar injected with *E. coli* exhibit lower growth rates and longer development times than caterpillars of the same age injected with sham (Figs. 7 and 8). This could indicate a shift in energy allocation from growth and development to metabolically costly immune responses. As indicated with sham-injected caterpillars, growth

rates in *M. sexta* normally decline with 5th instar development and represent a change in energy allocation from juvenile growth to storage for pupation (Sears et al. 2012). Our results are consistent with previous studies in other insect-pathogen systems. For example, *M. sexta* injected with *S. marcescens* had lower growth rates than caterpillars injected with sham (Adamo et al. 2007), and *M. sexta* with higher levels of encapsulation of Sephadex beads had slower growth rates (Diamond and Kingsolver 2011). *A. domesticus* had lower body masses upon immune challenge with nylon compared to control crickets (Bascunan-Garcia et al. 2010). Immune responses are indeed metabolically costly. At times if the cost is considered too high, energy could be allocated to other responses that are more beneficial (Krams et al. 2014; Moreno-García et al. 2014). Encapsulation of thread was positively correlated with increased metabolic rates measured as CO_2 emission rates in T. *molitor, A. domesticus, Cotinis nitida* (June bug) and *Periplaneta Americana* (American cockroach) (Ardia et al. 2012). *Pieris brassicae* pupae (white cabbage butterfly) challenged with nylon also showed increases in metabolic rate (Freitak et al. 2003). To provide further support, even *M. sexta* with high quality food had higher levels of encapsulation than those fed a regular diet (Diamond and Kingsolver 2011). Although there is plenty of evidence for changes in energy allocation during an immune challenge, other mechanisms (e.g., sickness-induced anorexia) may better explain infection-induced declines in growth rates and development times.

Sickness-induced anorexia is a puzzling paradox because it seems counterintuitive for animals to cease feeding upon infection, when they need energy most to combat an infection. However, the observation of sickness-induced anorexia is pervasive among diverse taxa and has been observed in *M. sexta* in response to infection with *S. marcescens* (Adamo et al. 2007). The cessation of feeding during the response to bacterial infection is associated with lower growth

rates (Adamo et al. 2007), and may be the cause of our observed decreases in growth rate during *E. coli* infection in early 5th instar *M. sexta*. In addition, we anecdotally observed that *M. sexta* injected with *E. coli* do not consume as much food as caterpillars injected with sham. However, it is unclear whether this is an age-dependent response, since *M. sexta* infected later in the 5th instar did not experience changes in growth rate and development time compared to sham-injected caterpillars. It would be interesting to investigate if *M. sexta* injected later in the 5th instar show any long-term effects of infection. For example, even though *M. sexta* infected later in the 5th instar do not show changes in growth rate or development time, perhaps their survival through pupation, longevity, or fecundity may be affected.

2.4.4. Conclusions

When reviewing the data as a whole, it is important to note that as animals age, they may undergo a reconfiguration of the immune response from resistance, fighting off an infection, to tolerance, surviving despite a high bacterial load. According to Moreno-García *et al*. (2014) insects could decide between resisting or tolerating a pathogen by sensing the levels of possible damage. If the damage were sensed to be minimal or below the damage threshold, it would be tolerated. If it were above the threshold, an immune response would activate for resistance. However, if the resistance was predicted to be ineffective, all energy would be put towards reproduction (Krams et al. 2014; Moreno-García et al. 2014). In *T. molitor* this type of terminal investment towards reproduction causes an increase in attractiveness, ensuring reproduction while costing its survival (Krams et al. 2014). Perhaps if *M. sexta* injected later in the 5th instar sensed the damage to be minimal, a reconfiguration to tolerance would be possible. The opposite is also plausible; if damage was sensed to be too high energy could have been allocated towards pupation and reproduction.

We are the first to identify insect immune responses decline even within a single day of development in the 5th instar, which is crucial information for scientists and farmers alike. Scientists should recognize that they should not overlook age as a confounding factor in experimental design. Lepidopteran larvae are notorious crop pests, particularly during the 5th instar when they are getting ready for pupation. Our results will help to understand their physiology and possibly identify vulnerable life stages of insects. We also encourage the use of functional immune assays to help interpret immune response data because immune response data may not always be an accurate depiction of an animal's defensive state. Although we did not measure energy variations or reserves, we speculate that the vulnerable life stage of insects could be caused by changes in how energy is allocated throughout development.

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CHAPTER 3. MATRIX METALLOPROTEINASE IS UP-REGULATED IN RESPONSE TO IMMUNE CHALLENGE DURING LARVAL DEVELOPMENT OF THE TOBACCO HORNWORM, *MANDUCA SEXTA*

3.1. Introduction

Insect innate immune responses are developmentally plastic to such a degree that immune responsivity can even change within a single larval instar, a juvenile developmental stage (Booth et al., 2015; Eleftherianos et al., 2008; Tian et al., 2010). Collectively, studies have shown that both cell-mediated and humoral innate immune responses are more robust in the beginning than the end of the final instar of the tobacco hornworm, *Manduca sexta* (Eleftherianos et al., 2008) and the related silk worm, *Bombyx mori* (Tian et al., 2010). Though there is an established link between within-instar development and a decline in immune responses, little research has been done to determine what mediates the development-immunity link.

One of the many changes that occur within an instar that could potentially affect immunity includes expression of matrix metalloproteinase (MMP). MMPs are a family of evolutionarily-conserved endoproteinases that play a role in many physiological and developmental processes, such as tissue remodeling, tissue repair, wound healing, immune cell migration, and innate immunity (Dubois et al., 2002; Greenlee et al., 2006; Gross and Lapiere, 1962; Hong et al., 2011; Page-McCaw et al., 2007; Vanlaere and Libert, 2009; Vu and Werb, 2000). It is thought MMPs are important for immune function because they prevent infection and mortality in mice (Dubois et al., 2002; Hong et al., 2011; Vanlaere and Libert, 2009). MMPs play a role in innate immunity and wound healing in the flour beetle, *Tribolium castaneum* (Knorr et al., 2009) and the wax moth, *Galleria melonella* (Altincicek and Vilcinskas, 2008). Also, MMP has been shown to cleave and activate the anti-microbial peptide (AMP) α-defensin

in mice (Wilson et al., 1999) and provides rationale for studying the role of MMP in humoral immune responses such as AMP activity. In addition, recent research has shown that gene and protein expression of MMP in tracheal tissue of *M. sexta* decrease within the 4th and 5th instars (Vishnuvardhan et al., 2013). Though it is established that MMP is involved in immunity in other insects without considering development as a factor, it is unclear if MMP may mediate the link between development and the decline in immune responses in the 5th instar of *M. sexta*.

In this study, we hypothesized that MMP mediates immune responses in *M. sexta* and that its expression is responsible for the observed decline in immune responses during 5th instar development. To test this hypothesis, we first observed how MMP protein expression varied with development in immune tissues. We also observed how MMP mRNA and protein expression vary in response to bacterial infection in immune tissues. Finally, we knocked down MMP using RNA inhibition and measured the subsequent immune response of these insects, including survival from infection, anti-microbial peptide activity, and phenoloxidase activity.

3.2. Materials and methods

3.2.1. Study organism and rearing conditions

Manduca sexta colonies were reared as described in Booth et al., 2015. Briefly, *M. sexta* larvae (Carolina Biological, Burlington, NC) were reared at 25°C under a 16L:8D photoperiod with *ad libitum* access to an artificial, wheat germ-based diet. *M. sexta* were observed daily to determine the stage.

3.2.2. Experimental design

To quantify MMP mRNA and protein expression in immune tissues following an infection, freshly molted 5th instar *M. sexta* larvae were injected with *Escherichia coli* and hemocyte and fat body samples were collected 6 and 24 h after injection; these were also

collected from non-injected 5th instar *M. sexta* for controls. Quantitative PCR and western blotting were performed in these samples to assess relative mRNA abundance and protein expression. To determine MMP protein expression in immune tissues during 5th instar development, hemocyte, hemolymph, and fat body samples were collected from naïve *M. sexta* on each day (days 0-4) of the 5th instar. Western blotting was performed to assess protein expression.

We used RNA inhibition (RNAi) to determine the role of MMP in surviving an infection. Freshly molted 5th instar *M. sexta* larvae were treated with either GFP control double-stranded RNA (dsRNA) or dsRNA to inhibit MMP mRNA expression. Immediately after RNAi treatment, treatment groups were split into two, with *M. sexta* injected with either phosphate buffer saline (PBS) vehicle or *E. coli*. *Manduca sexta* were checked for mortality at various time points up to 48 h post-infection.

To determine the role of MMP in mediating AMP and PO activity, freshly molted 5th instar *M. sexta* were treated with either GFP control dsRNA or zincin dsRNA. Immediately after RNAi treatment, treatment groups were divided, and *M. sexta* were injected with either *Manduca* saline buffer (MSB) vehicle or lyophilized *Micrococcus lysodeikticus*. Hemolymph was collected 24 h after injection, and AMPs were isolated. Isolated AMPs were used in the AMP activity assay, and hemolymph was used in the PO activity assay.

To test if RNAi reduced protein expression, freshly molted 5th instar *M. sexta* were treated with either GFP control dsRNA or zincin dsRNA. Hemolymph and hemocyte samples were collected 24 h after injection and western blotting was performed to assess MMP protein expression.

We used an alternative MMP inhibitor to RNAi, GM6001 to test the role of MMP in various immune indices (hemocyte concentration, hemocyte spreading, phagocytosis, and bacterial load).

3.2.3. *E. coli* cultures

DH5-α *E.coli* used in the qPCR, western blotting, and mortality assays were grown to stationary phase in Luria broth (LB) and the optical densities of the bacterial suspensions were measured using a spectrophotometer (Beckman Coulter, Brea, CA). The bacterial suspensions were centrifuged at 4000 x g for 15 min. Resulting pellets were washed with PBS (137mM NaCl, 3mM KCl, 10mM Na_2HPO_4, 2mM KH_2PO_4, pH 7.4) and centrifuged again at 4000 x g for 15 min. The final *E. coli* bacterial pellet was re-suspended in PBS at a concentration of 1 million CFU per 10 μl.

3.2.4. *Micrococcus lysodeikticus* preparation

Lyophilized *M. lysodeikticus* (Sigma-Aldrich, St. Louis, MO), used in the AMP and PO activity assays, were prepared at a concentration of 10 mg/ml in *Manduca* saline buffer (MSB; 4mM NaCl, 40mM KCl, 18mM $MgCl_2•6H_2O$, and 3mM $CaCl_2$).

3.2.5. RNA interference

Double-stranded RNA (dsRNA) used in the mortality, AMP, and PO activity assays was synthesized using a DNA-derived template from *M. sexta* MMP (GenBank: JN415760.1). MEGAscript® RNAi kit (Life Technologies Corporation, USA) was used to prepare dsRNA according to the manufacturer's protocol. Appropriate templates for *Ms*-MMP were generated by PCR using gene-specific primers containing a T7 polymerase promoter sequence at the 5' end (forward primer: TAATACGACTCACTATAGGG, reverse primer: TAATACGACTCACTATAGGG) and purified with MEGAscript® RNAi kit prior to use.

Within 24 h of molting into the 5th instar, *M. sexta* were injected with 5 ng of dsRNA/mg of larval body mass.

3.2.6. Injections

Regardless of treatment and age of *M. sexta*, the injection method was the same as previously described (Booth et al., 2015). Briefly, larvae were cooled on ice for 5 min and surface sterilized with 75% ethanol. A 25 µl Hamilton syringe (Hamilton, Reno, NV) with a 26 gauge needle was filled with 10 µl of control buffer or bacterial suspension. The needle was inserted subcutaneously into the hemocoel of the caterpillar between the first and second pro-legs. Once injections were complete, caterpillars were transferred to individual containers with *ad libitum* access to food and monitored daily.

Caterpillars for the qPCR, western blotting, and mortality assays were injected with 1 million CFU of *E. coli* in 10 µl of PBS. Caterpillars for the AMP and PO activity assays were injected with 100 µg of *M. lysodeikticus* in 10 µl of MSB or 10 µl of MSB. Caterpillars for the mortality, AMP, and PO activity assays were injected with purified dsRNA at a concentration of 5ng dsRNA diluted in nuclease free water per milligram of body weight in a total volume of 10-15 µl.

3.2.7. Sample collection

The samples collected in the experiments included hemolymph, hemocytes, and fat body. *Manduca sexta* larvae were cooled on ice for 5 min, and surface sterilized with 75% ethanol before sample collection. To collect hemolymph, the first, right pro-leg was cut with a sterilized razor blade, and hemolymph was collected in a 1.5ml Eppendorf tube on ice. Hemocytes were isolated from the hemolymph by centrifugation at 7000 x g for 10 min. at 4°C. After centrifuging, the pellet consisted of hemocytes, while the supernatant consisted of hemolymph

plasma. The hemocytes and plasma were kept at -20°C. The fat body was collected by making a dorsal incision with a razor and using the razor to scrape the cream-colored fat body out of the hemocoel, while avoiding major tracheae, into a 1.5 ml Eppendorf tube. The fat body was diluted in 200 µl of MSB and kept at -20°C.

3.2.8. Quantitative PCR

Briefly, total RNA was extracted from hemocyte and fat body samples from freshly molted 5th instar *M. sexta* larvae 6 and 24 h after *E. coli* injection. Non-injected freshly molted 5th instar *M. sexta* were used as controls. Quantitative PCR was performed to assess relative mRNA abundance according to Vishnuvardhan et al. (2013).

3.2.9. Western blotting

Western blotting was used to determine protein expression of MMP, using a method modified from (Vishnuvardhan et al., 2013). Briefly, protein concentration of each sample was measured, and then each sample was dissolved in loading buffer (5% β-mercaptoethanol in Lamelli sample buffer, BioRad, Hercules, CA). Ten micrograms of each sample in buffer suspension were loaded into each well of a 12% acrylamide gel (BioRad, Hercules, CA), and each separate gel was run with a Western C ladder (BioRad, Hercules, CA). Proteins were separated by molecular weight using electrophoresis for 30 min at 300 V. Separated proteins were transferred to a nitrocellulose membrane at 25 V for 7 min using the manufacturer's protocol (TransBlot Turbo, BioRad, Hercules, CA). The membrane was blocked with 5% milk in TBST (tris-buffered saline with tween, 100mM Tris, 300 mM NaCl, 0.0005% tween) overnight at 4°C on a shaker. The membrane was then incubated with a primary antibody (1:500 rabbit anti-*M. sexta* MMP from Vishnuvardhan et al., 2013) for 1 h at room temperature while shaking. The membrane was washed with TBST 3 times for 10 min, and then incubated with a

1:100,000 dilution of goat anti-rabbit secondary antibody (ThermoScientific, Waltham, MA) for 1 h at room temperature while shaking. The membrane was then washed with TBST 6 times for 5 min. Protein expression was detected using West Femto Chemiluminescence (ThermoScientific, Waltham, MA) and a chemi-imager (Alpha-Innotec, Kasendorf, Germany) with 2 min of exposure. MMP protein expression was quantified using densitometry on specific bands.

3.2.10. Mortality assay

After RNAi treatment and bacterial/vehicle injection of newly molted 5th instars caterpillars, mortality from infection was assessed up to 48 h post-injection. Observations of mortality were made 16, 20, 24, 40, 44, and 48 h post-injection. Caterpillars unable to right themselves after being placed on their dorsum were considered dead.

3.2.11. Anti-microbial peptide activity assay

To determine the activity of the AMPs produced as a part of the humoral immune response, a traditional zone-of-inhibition assay was performed (Hoffmann et al., 1981). Fifth instar day 0 *M. sexta* were injected with 10 µl of lyophilized *M. lysodeikticus* at 10 mg/ml to induce the production of AMPs. Hemolymph was collected 24 h later. AMPs were isolated by centrifugation at 7000 x g for 5 min to separate hemocytes from hemolymph. The plasma layer was collected and heated at 95°C for 5 min and spun again at 13,400 x g for 10 min. AMPs were isolated in the supernatant. Wells were carved into LB agar plates embedded with DH5α *E. coli*. Five microliters of AMPs from each caterpillar (n=9) were loaded into the wells in duplicate. The plates were incubated at 37°C for 1 h, then at 25°C overnight. The next morning, plates were photographed and the zone of inhibition, the diameter around the well where the AMPs inhibited bacterial growth, was measured using Image J (1.45K version; Rasband, 1997-2011).

3.2.12. Phenoloxidase activity assay

Fifth instar day 0 *M. sexta* were injected with 10 µl of lyophilized *M. lysodeikticus* at a 10 mg/ml to induce the production of PO. Hemolymph was collected 24 h post-injection and centrifuged at 7000 x g at 4°C for 10 min to separate hemocytes from hemolymph plasma. A 10 µl fraction of the hemolymph plasma was mixed with *E. coli* lipopolysaccharide (Sigma-Aldrich, St. Louis, MO) for 1 h to allow conversion of pro-PO to PO. The reaction was started by adding 20 mM 4-methylcatechol (Sigma-Aldrich, St. Louis, MO). The conversion of 4-methylcatechol to colorimetric quinone by phenoloxidase was marked by a change in absorbance (at 490 nm), which was measured every 15 min for 1 h at room temperature by a microplate reader (BioRad, Hercules, CA). The change in absorbance over time (mOD/min) is directly related to PO activity (Eleftherianos et al., 2008). Slopes from each hemolymph sample were calculated and compared.

3.2.13. Hemocyte concentration assay

Freshly molted 5th instar *M. sexta* were treated with either PBS, 0.01% DMSO vehicle, the MMP inhibitor GM6001 (Millipore, Billerica, CA) at 100 µM in 0.01% DMSO. Immediately after treatment, DMSO and GM6001 treatment groups were split into two. *Manduca sexta* were injected with either phosphate buffer saline (PBS) vehicle or 1 million CFU of *E. coli*. Hemolymph was collected 18 h post-injection, diluted 1:10 in PBS before 10 µl of the diluted hemolymph was placed on a hemocytometer (Hausser Scientific, Horsham, PA). Hemocytes were counted in 4 grids using a light microscope (Leica, Wetzlar, Germany) at 20x magnification. An average hemocyte concentration was calculated using the following equation: average number of hemocytes per grid x 10^4 x dilution factor. The dilution factor was $1x10^{-1}$ for each animal.

3.2.14. Hemocyte spreading assay

Hemolymph was collected from 5th instar day 2 *M. sexta* and centrifuged at 100 x g at 4ºC for 10 minutes to separate the hemocytes in the pellet from the hemolymph plasma in the supernatant. The hemolymph plasma was discarded, 500 µl of Grace's Insect Medium (Sigma-Aldrich, St. Louis, MO) was added to the hemocyte pellet and mixed. The hemocyte solution was mixed with either 0.4 µl of PBS, 0.01% DMSO vehicle, or the MMP inhibitor GM6001 (Millipore, Billerica, CA) at 10 µM in 0.01% DMSO. Ten µl of the hemocyte solution was placed on a glass slide and incubated for 1 h. The hemocytes were viewed on a phase contrast microscope (Leica, Wetzlar, Germany) at 40x magnification. The average percent of hemocytes that spread onto the glass slide was calculated.

3.2.15. Phagocytosis assay

Freshly molted 5th instar *M. sexta* were injected with 10 µl of lyophilized *M. lysodeikticus* at 10 mg/ml. Hemolymph was collected 18 h post-injection and hemocyte concentration was calculated using method described above. Hemolymph was centrifuged at 400xg for 10 minutes at 4 ºC and hemocytes in the pellet were separated from hemolymph plasma in the supernatant. The hemolymph plasma was discarded, 170 µl of Grace's Insect Medium (Sigma-Aldrich, St. Louis, MO) was added to the hemocyte pellet and mixed. The hemocyte solution was mixed with either 20 µl of PBS, 0.01% DMSO vehicle, or the MMP inhibitor GM6001 (Millipore, Billerica, CA) at 10 µM in 0.01% DMSO and incubated for 1 h. FITC-labeled latex beads (Sigma-Aldrich, St. Louis, MO) were added in a 1 bead: 2 hemocyte ratio. Immediately, 10 minutes, and 30 minutes after beads were added, the hemocyte solution was washed by centrifuging at 400xg for 10 minutes at 4 ºC. Immediately after washing, hemocytes were fixed in 1% paraformaldehyde. The fluorescence emitted by the hemocytes was then analyzed using an Accuri C6 Flow

Cytometer and Flow Jo software (Beckman Coulter, Indianapolis, IN). The percentage of hemocytes phagocytosing as evidenced by higher fluorescence was calculated.

3.2.16. Bacterial load assay

Freshly molted 5th instar *M. sexta* were treated with either 0.01% DMSO vehicle or the MMP inhibitor GM6001 (Millipore, Billerica, CA) at 100 µM in 0.01% DMSO and injected with 1 million CFU of *E. coli*. Hemolymph was collected 18 h post-injection, serially diluted in PBS (10^{-2}, 10^{-3}, 10^{-4}), and spread on LB agar plates. The concentration of bacteria was determined via standard colony counts 24 h later.

3.2.17. Data analysis

Statistical analyses were performed using IBM SPSS version 21. Analysis of Variance (ANOVA) was used to determine significant differences among treatments, and Bonferroni post-hoc tests were used to distinguish differences for all pair-wise comparisons for western blots, qPCR, AMP activity, PO activity, hemocyte concentration and spreading, phagocytosis, and bacterial load assays. Differences in mortality among treatment groups were assessed by Kaplan-Meier time-to-event analysis. A p-value < 0.05 indicated statistical significance. Data are expressed as mean ± SEM throughout.

3.3. Results

3.3.1. MMP protein expression with 5th instar development

MMP protein expression in hemolymph extracted from *M. sexta* was highest at the beginning of the 5th instar and decreased gradually through to the end of the 5th instar (Fig. 9B, $F_{4,10}=14.56$, $P<0.005$; ANOVA; n=3 for each treatment group). Hemolymph extracted from day 1 5th instars had 33% less MMP protein expression compared to hemolymph extracted from day 0 5th instars. Furthermore, hemolymph extracted from day 2 5th instars showed about ¼ of MMP

protein expression compared to hemolymph extracted from day 0 5^{th} instars. MMP protein expression in hemocytes did not change significantly within 5^{th} instars (Fig. 9D, $F_{4,10}$=0.76, *P*=0.573; ANOVA; n=3 for each treatment group). MMP expression in fat body extracted from uninjected *M. sexta* was greatest at the beginning (day 0) of the 5^{th} instar and decreased by about 13-fold by the end (day 3) of the 5^{th} instar, while increasing slightly on day 4 (Fig. 9E, $F_{4,10}$ =5.43, *P*<0.05 on ANOVA; *P*<0.05 on Bonferonni post-hoc test; n=3 for each treatment group).

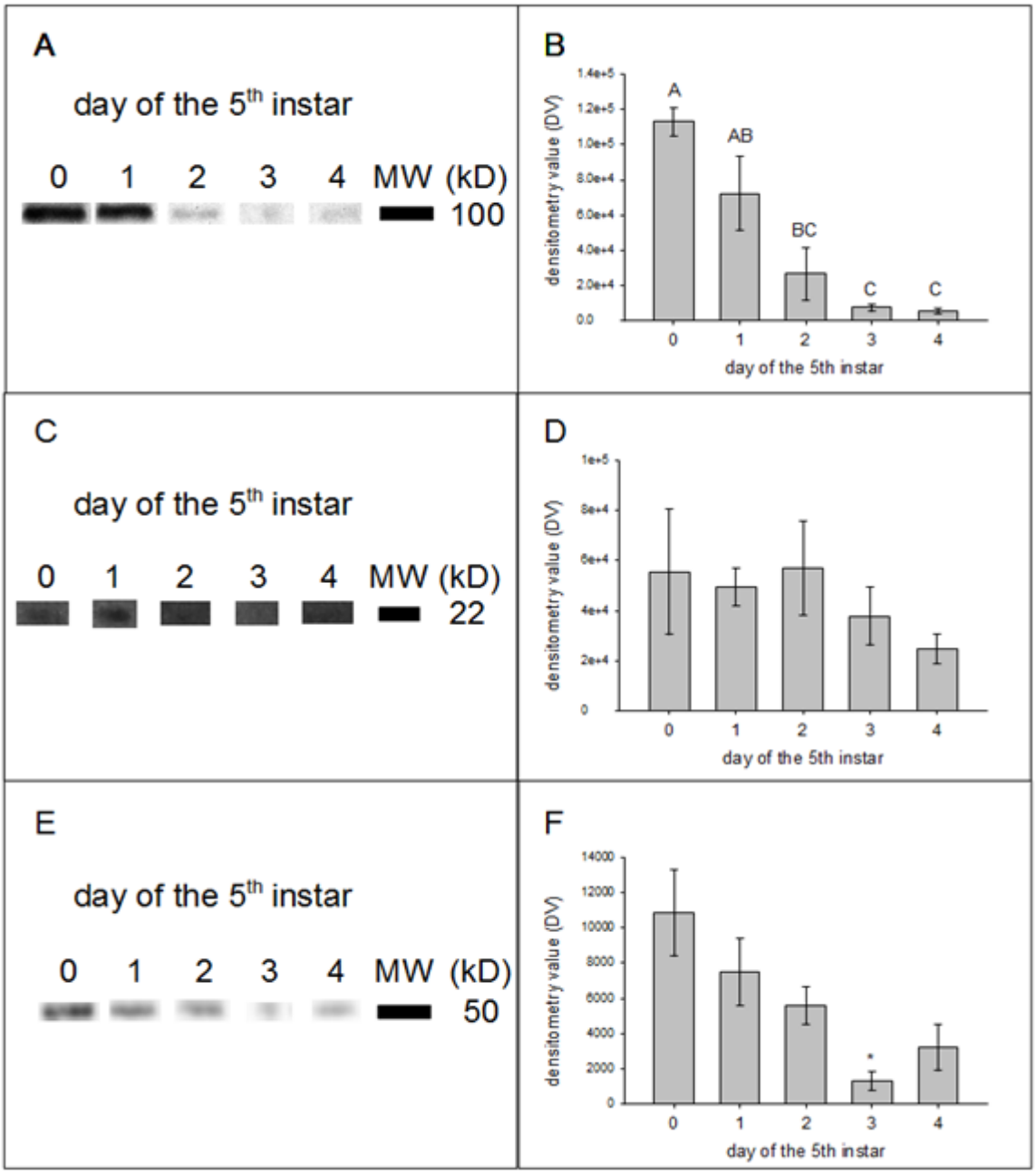

Fig. 9. MMP protein expression throughout 5th instar development in hemolymph (A,B), hemocytes (C,D), and fat body (E, F) (western blots on left and densitometry graphs on right). Tissues were extracted from naïve *M. sexta* on each day of the 5th instar (0-4), and MMP protein expression was assessed using Western blotting. For panel B, bars with different letters are significantly different from one another ($F_{4,10}$=14.56, P<0.005; ANOVA). For panel D, there were no statistically significant differences ($F_{4,10}$=0.76, p=0.573; ANOVA). For panel F, bars with* indicate values significantly lower than at day 0 ($F_{4,10}$ =5.43, P<0.05; ANOVA). Error bars indicate +/- SEM.

3.3.2. MMP mRNA and protein expression with infection

MMP mRNA expression in hemocytes extracted from 5th instar day 0 *M. sexta* was up-regulated 6 and 24 h post-*E. coli* infection (Fig. 10, $F_{2, 26}$=3.67, P<0.05; ANOVA; n=10, 10 and 7 for 0, 6, and 24 h, respectively). MMP mRNA expression was about 5-fold higher in hemocytes extracted from *M. sexta* 6 and 24 hrs post-*E. coli* infection than in uninfected caterpillars. MMP mRNA expression in fat body extracted from 5th instar day 0 *M. sexta* was up-regulated 6 and 24 h post-*E. coli* infection (Fig. 11, $F_{2, 25}$=3.46, P<0.05; ANOVA; n=10 for the naïve treatment group and n=8 for each infected treatment group). MMP mRNA expression was about 4-fold higher in fat body extracted from *M. sexta* 6 h post-*E. coli* infection and about 9-fold higher 24 h post-infection than in uninfected *M. sexta*. MMP protein expression did not vary in any of the immune tissues in response to infection. (Hemolymph: Fig. 12B, $F_{2,12}$=2.28, P =0.145; ANOVA; n=5 for each treatment group. Hemocytes: Fig. 12D, $F_{2,9}$ = 0.06, P=0.947; ANOVA; n=4 for each treatment group. Fat body: Fig. 12E=F, $F_{2,12}$ = 0.57, P =0.581; ANOVA; n=5 for each treatment group.)

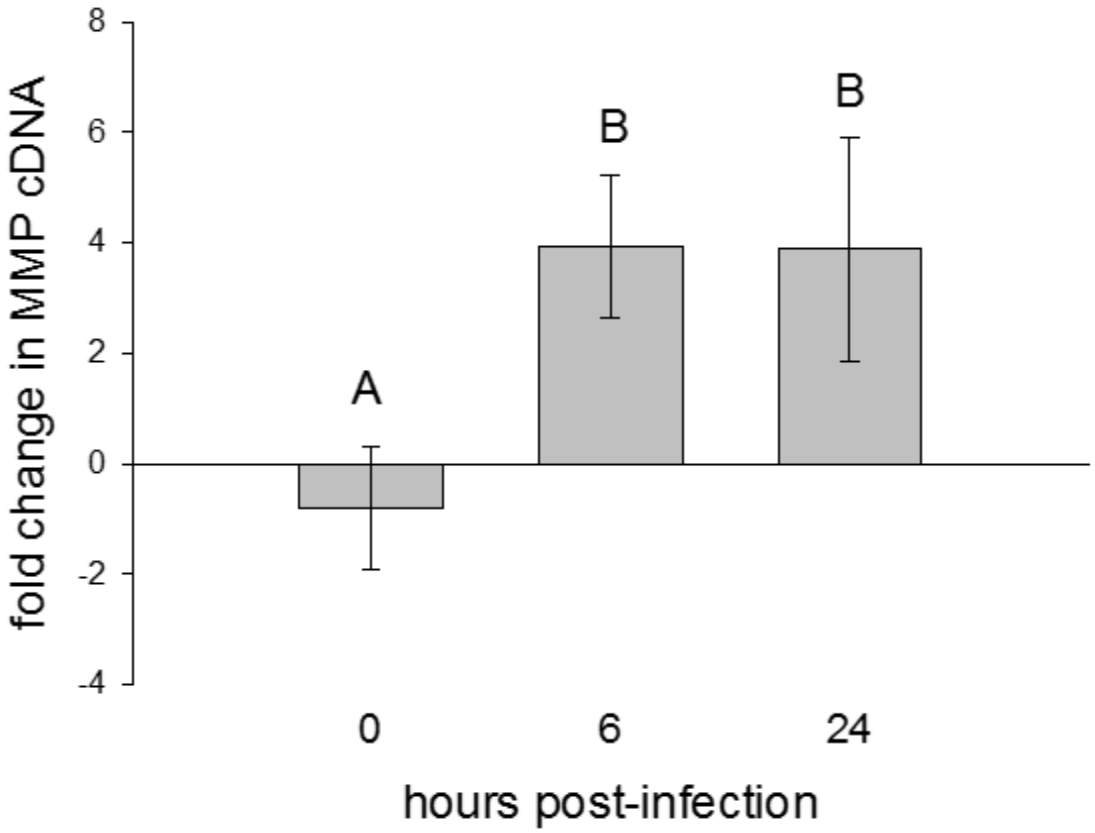

Fig. 10. MMP mRNA expression in hemocytes 0, 6, and 24 h post-*E. coli* infection. Fifth instar day 0 *M. sexta* were injected with *E. coli*, hemocytes were extracted immediately, 6, or 24 h post-*E coli* infection, with MMP mRNA expression measured using qPCR. Bars with different letters are significantly different from one another ($F_{2, 26}$=3.67, P<0.05; ANOVA; Fischer's LSD post hoc tests, 0 h vs. 6 h, P<0.05, 0 h vs. 24 h, P<0.05). Error bars indicate +/- SEM.

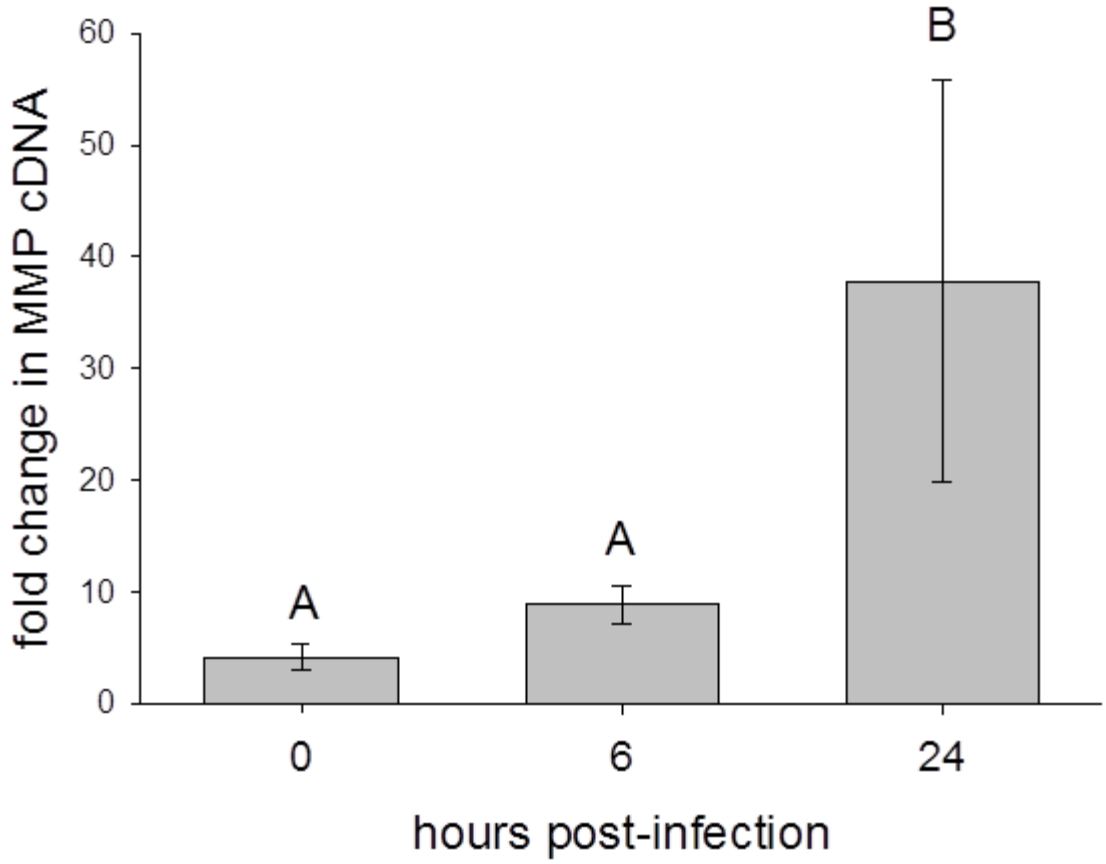

Fig. 11. MMP mRNA expression in fat body 0, 6, and 24 h post-*E. coli* infection. Fifth instar day 0 *M. sexta* were injected with *E. coli*, fat body was extracted immediately, 6, or 24 h post-*E coli* infection, with MMP mRNA expression measured using qPCR. Bars with different letters are significantly different from one another ($F_{2,27} = 6.96$, $P<0.01$, ANOVA; Bonferonni post-hoc test, 0 h vs. 24 h, $P<0.005$). Error bars indicate +/- SEM.

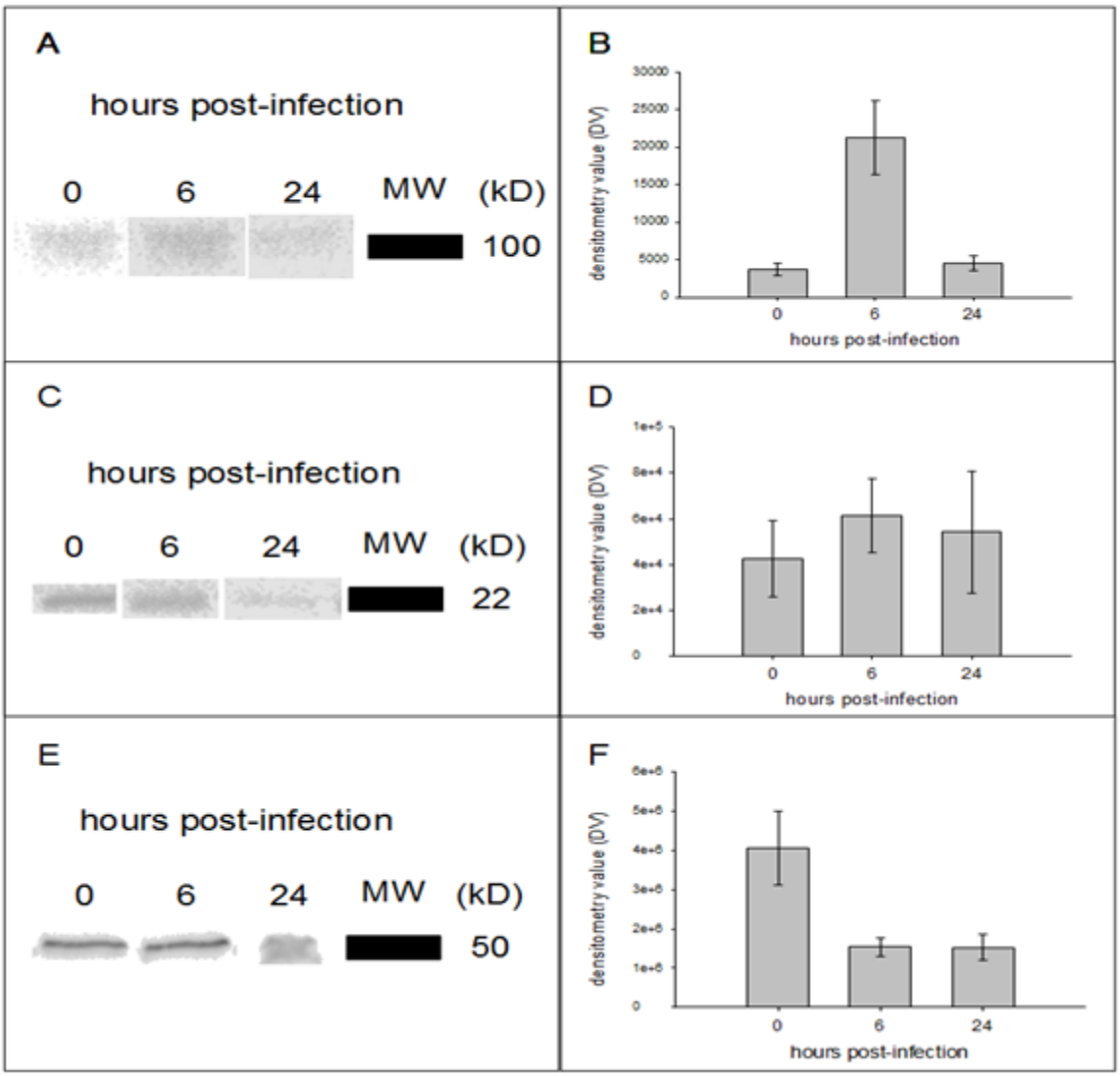

Fig. 12. MMP protein expression 0, 6, and 24 h post-*E. coli* infection in hemolymph (A,B), hemocytes (C,D), and fat body (E,F) (western blots on left and densitometry graphs on right). Fifth instar day 0 *M. sexta* were injected with *E. coli*, hemolymph, hemocytes, and fat body were extracted immediately, 6, and 24 h post-*E coli* infection, and MMP protein expression was measured using Western blotting. There were no statistically significant differences in protein expression in all three tissues extracted. Error bars indicate +/- SEM.

3.3.3. Role of MMP in immune function

When MMP mRNA expression was inhibited by RNAi, 5th instar day 0 *M. sexta* show improved survival to *E. coli* infection (Fig. 13: Kaplan-Meier time to event analysis, χ^2=7.38, P<0.05; n=10 for each treatment group). About twice as many *M. sexta* with MMP inhibited by

RNAi survived than *M. sexta* injected with control dsRNA against GFP. Regardless of dsRNA injection, 100% of *M. sexta* survived PBS injections.

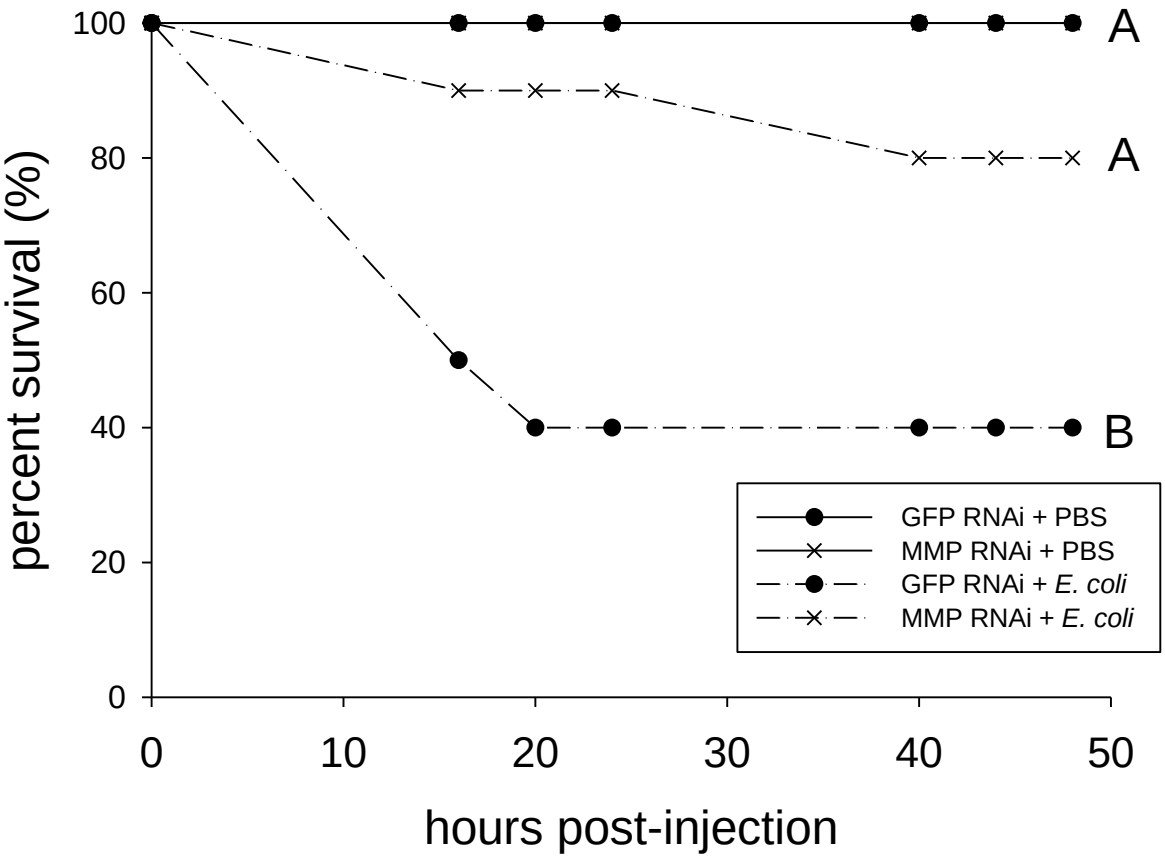

Fig. 13. Survival of *E. coli* infection by *M. sexta* injected with dsRNA to inhibit MMP mRNA expression. *M. sexta* were injected with PBS (solid line) or *E. coli* (dashed line), and control dsRNA (circle) or dsRNA (X) to inhibit MMP mRNA expression, and were checked at varying times up to 48 h later for signs of life. Lines with different letters are significantly different from one another (χ^2=7.38, P<0.05; Kaplan-Meier).

MMP mRNA inhibition did not affect AMP activity of hemolymph extracted from 5th instar day 0 *M. sexta* (Fig. 14, ANOVA, $F_{1,16}$=3.05, P= 0.100; n=9 for each treatment group). In addition, after injection of *M. lysodeikticus*, MMP mRNA inhibition did not affect PO activity of hemolymph extracted from 5th instar day 0 *M. sexta*. However, without *M. lysodekticus* injection, *M. sexta* with MMP mRNA inhibition significantly increased PO activity (Fig. 15, ANOVA, $F_{3,}$

$_{24}$=72.31, *P*<0.005; n=7 for each treatment group). PO activity of hemolymph extracted from *M. sexta* with MMP mRNA inhibition was about 1.5-fold higher than controls.

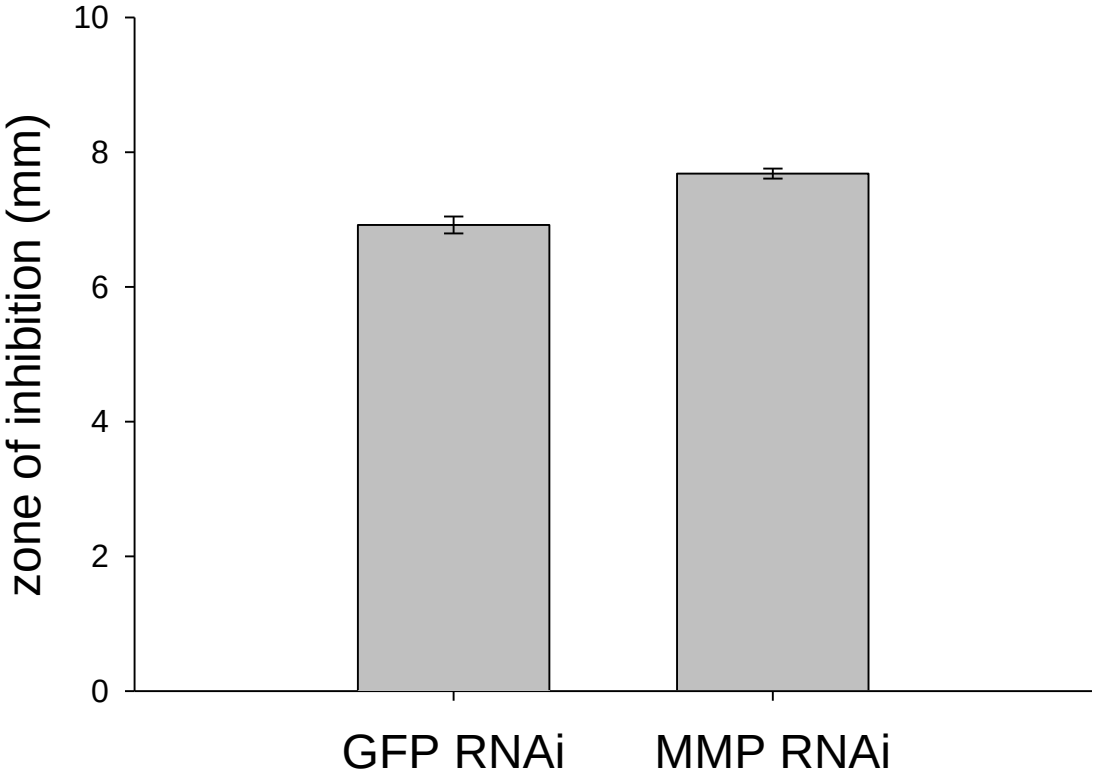

Fig. 14. Anti-microbial peptide (AMP) activity in hemolymph extracted from *M. sexta* injected with control dsRNA or dsRNA to inhibit MMP mRNA expression. Fifth instar day 0 *M. sexta* were injected with dried *M. lysodeikticus,* and either control dsRNA or dsRNA to inhibit MMP mRNA expression, and AMP activity was assessed 24 h later. No statistical significance was detected ($F_{1,16}$=3.05, *P*=.100; ANOVA). Error bars indicate +/- SEM.

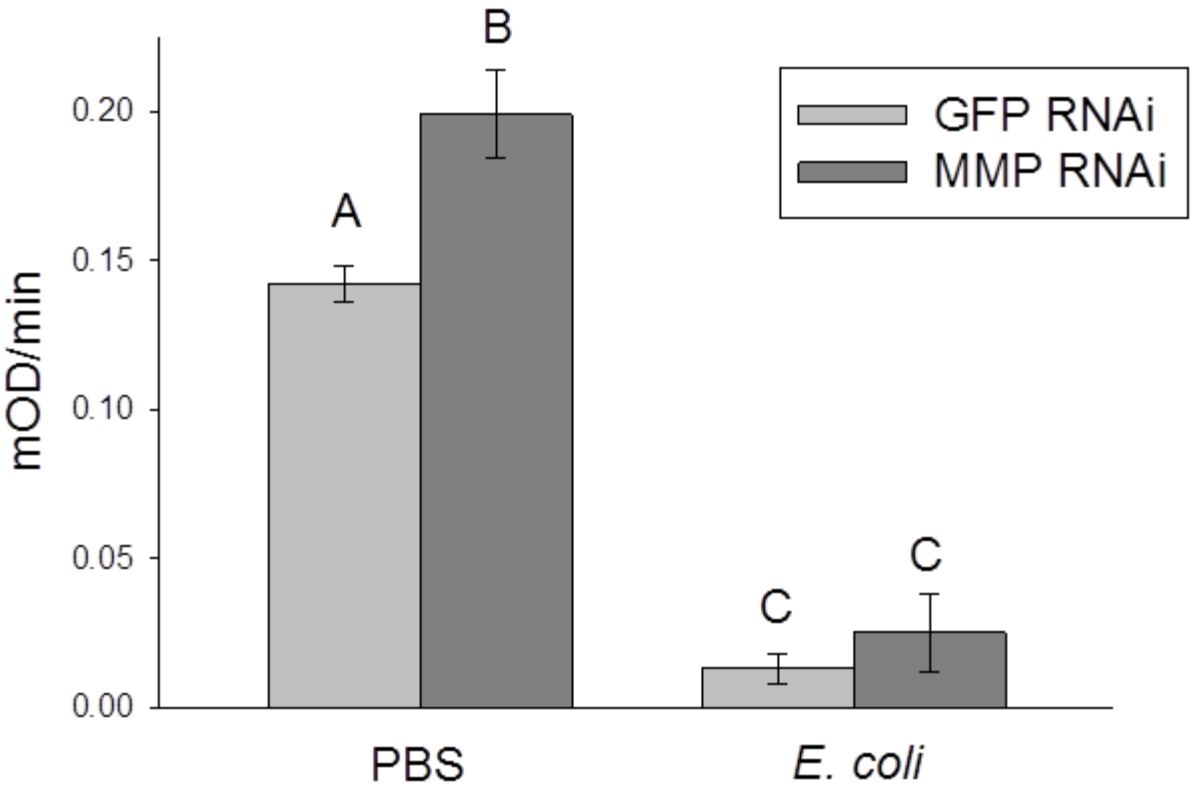

Fig. 15. Phenoloxidase (PO) activity in hemolymph extracted from *M. sexta* injected with dsRNA to inhibit MMP mRNA expression. Fifth instar day 0 *M. sexta* were injected with either MSB or *E. coli*, and either control dsRNA or dsRNA to inhibit MMP mRNA expression, and PO activity was assessed 24 h later. Bars with different letters are significantly different from one another ($F_{3, 24}$=72.31, P<0.005, ANOVA). Error bars indicate +/- SEM.

3.3.4. MMP protein expression with RNA inhibition

MMP protein expression did not vary in hemocytes or hemolymph extracted from 5th instar day 0 *M. sexta* treated with RNA inhibition. (Hemolymph: Fig. 16, $F_{1,4}$= 0.479, P=0.527; ANOVA; n=3 for both treatment groups. Hemocytes: Fig. 17, $F_{1,4}$=0.069, P =0.806; ANOVA; n=3 for each treatment group.).

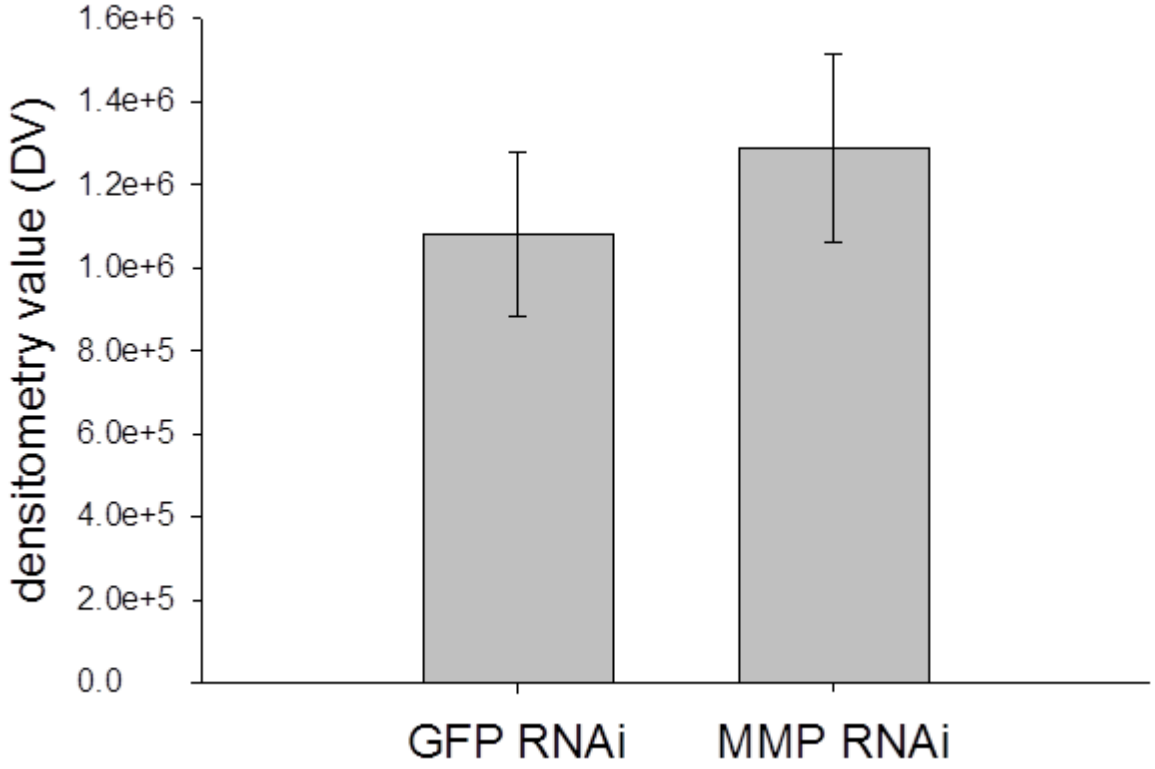

Fig. 16. MMP protein expression in hemolymph extracted from 5th instar day 0 *M. sexta* injected with dsRNA to inhibit MMP mRNA expression. MMP protein expression was measured using Western blotting and densitometry values were assessed. There were no statistically significant differences in protein expression. ($F_{1,4}$= 0.479, *P*=0.527; ANOVA). Error bars indicate +/- SEM.

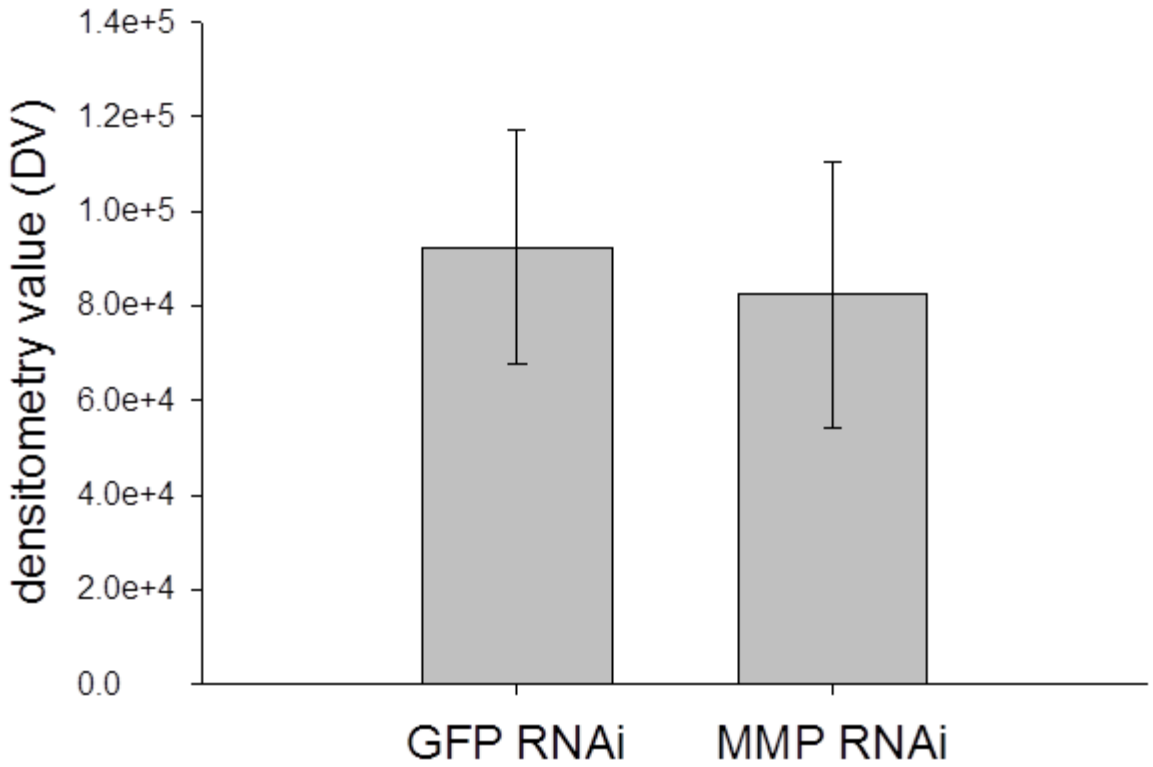

Fig. 17. MMP protein expression in hemocytes extracted from 5th instar day 0 *M. sexta* injected with dsRNA to inhibit MMP mRNA expression. MMP protein expression was measured using Western blotting and densitometry values were assessed. There were no statistically significant differences in protein expression. ($F_{1,4}$=0.069, P =0.806; ANOVA). Error bars indicate +/- SEM.

3.3.4. The effect of GM6001 on immune indices

MMP inhibition by the inhibitor GM6001, regardless of *E. coli* infection, doubled hemocyte concentration in the hemolymph of 5th instar day 0 *M. sexta* (Fig. 18; ANOVA, $F_{4, 44}$=0.351, P<0.001; Bonferonni post-hoc test DMSO vs. GM6001, P<0.05; DMSO+*E. coli* vs. GM6001+*E. coli*, P<0.01; n=10 for each treatment group).

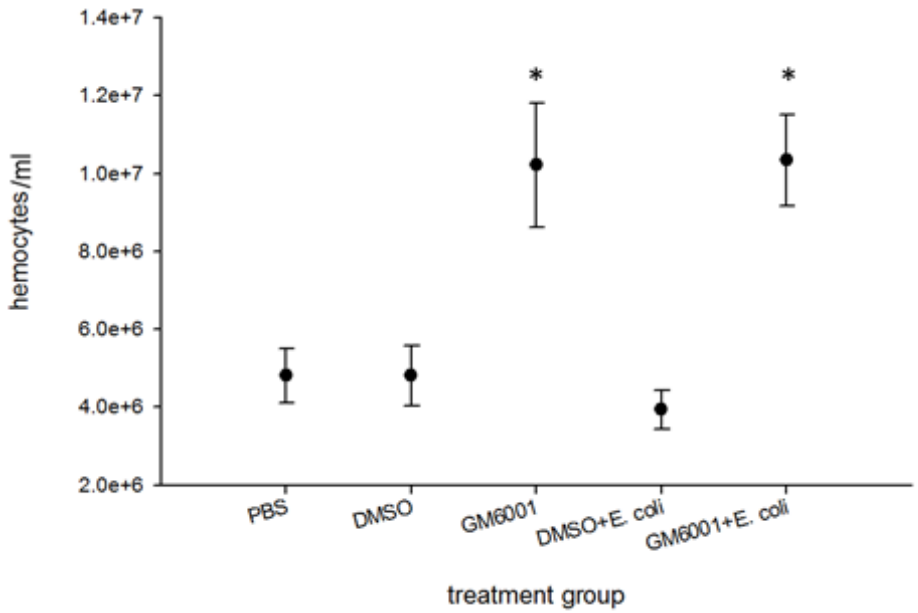

Fig. 18. Hemocyte concentration in hemolymph extracted from 5th instar day 0 *M. sexta* injected with GM6001 to inhibit MMP activity. *M. sexta* were injected with either PBS, DMSO vehicle, GM6001, DMSO + *E. coli*, or GM6001 + *E. coli* and hemocytes were counted 18 h later. * indicates significantly different from DMSO ($F_{4, 44}$=0.351, P<0.001, ANOVA; Bonferonni post-hoc test DMSO vs. GM6001, P<0.05; DMSO+*E. coli* vs. GM6001+*E. coli*, P<0.01). Error bars indicate +/- SEM.

MMP inhibition by the inhibitor GM6001, did not have an effect on average percent of hemocyte spreading in *M. sexta* (Fig. 19; ANOVA, $F_{2,1}$=0.902, P>0.05, n=9 for each treatment group). MMP inhibition by the inhibitor GM6001, did not have an effect on average percent of hemocytes that phagocytose FITC-labeled beads (Fig. 20; ANOVA, $F_{2,126}$=0.151, P>0.05, n=10 for each treatment group). MMP inhibition by the inhibitor GM6001, did not have an effect on the bacterial load of *M. sexta* (Fig. 21; ANOVA, $F_{1,6}$=0.683, P>0.05, n=4 for each treatment group).

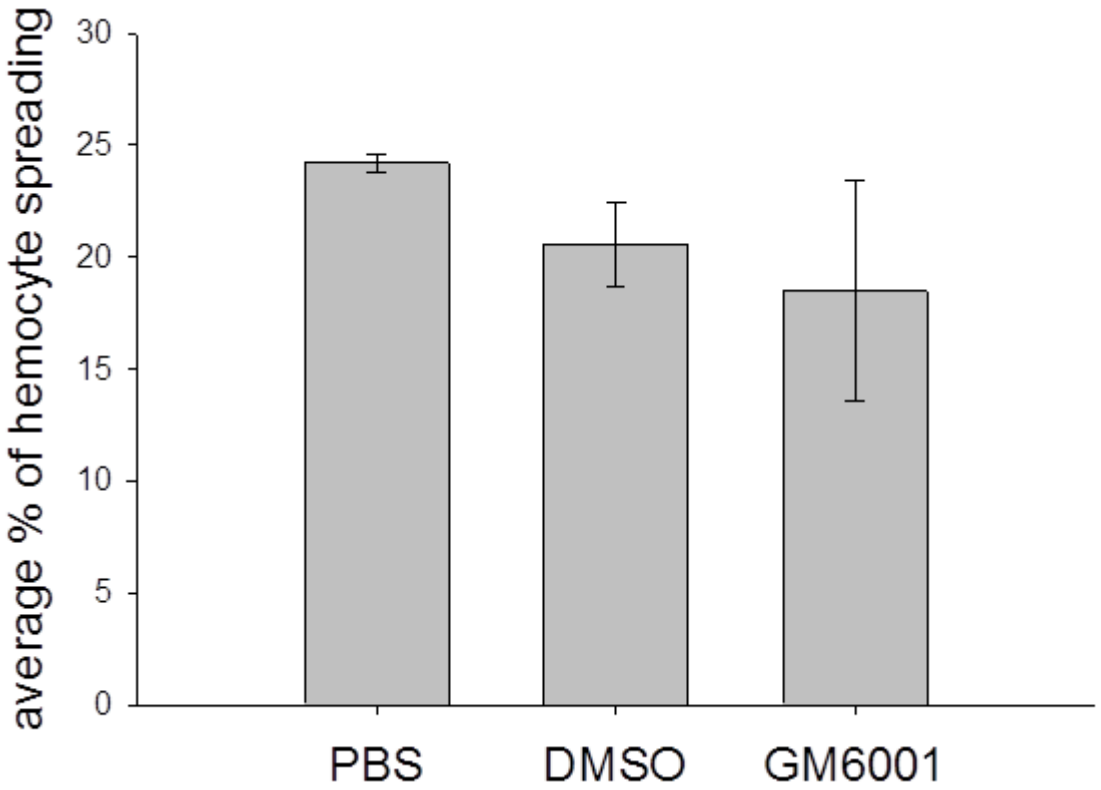

Fig. 19. Average percent of hemocyte spreading in hemolymph incubated with either PBS, DMSO vehicle, or GM6001 to inhibit MMP activity. There were no statistically significant differences. ($F_{2,1}$=0.902, P>0.05; ANOVA). Error bars indicate +/- SEM.

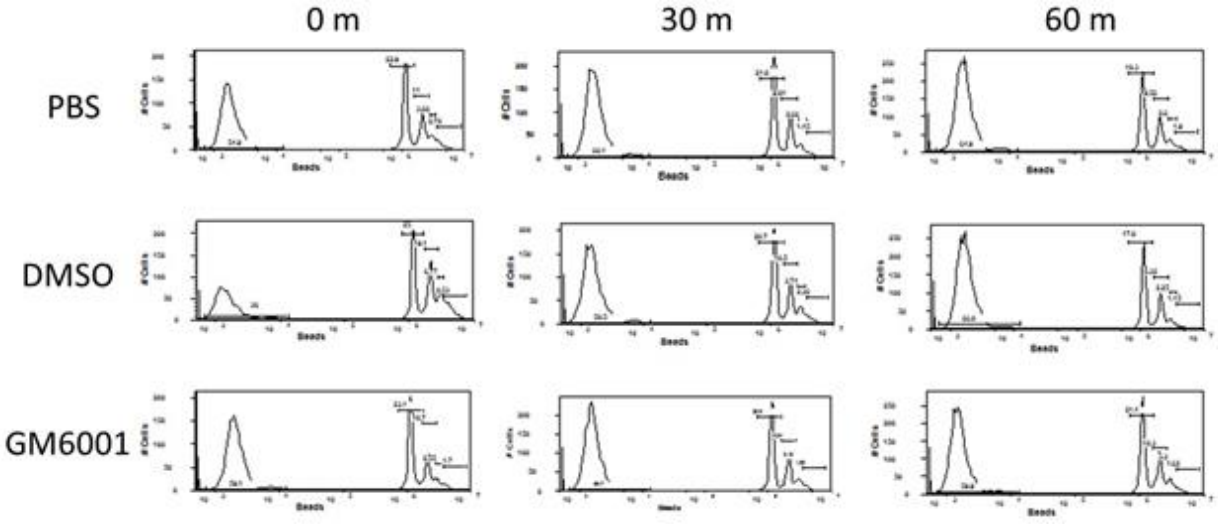

Fig. 20. Phagocytosis of hemocytes incubated with either PBS, DMSO vehicle, or GM6001 to inhibit MMP activity. There were no statistically significant differences. ($F_{2,126}$=0.151, P>0.05; ANOVA).

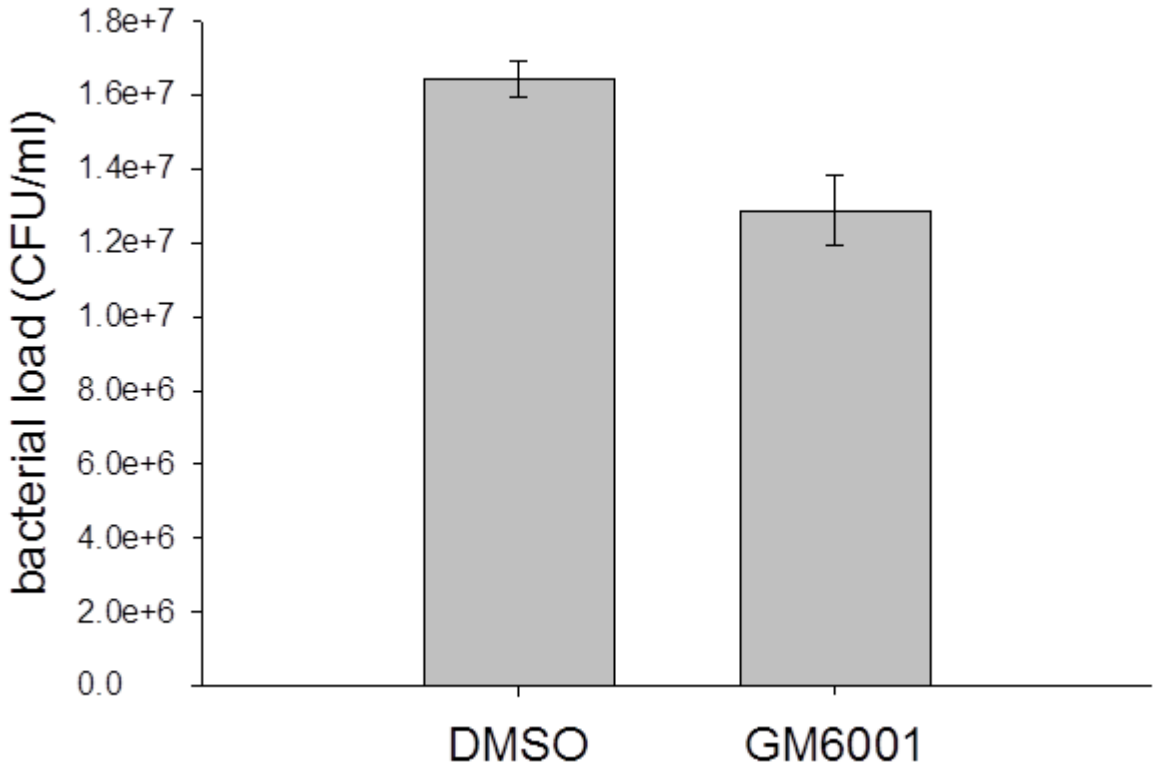

Fig. 21. Bacterial load of *M. sexta* injected *E. coli* and with DMSO vehicle or GM6001 to inhibit MMP activity. Hemolymph was collected 18 h post-*E. coli* injection, and bacterial load assessed. There were no statistically significant differences. ($F_{1,6}$=0.683, P>0.05; ANOVA). Error bars indicate +/- SEM

3.4. Discussion

In this study, we tested the hypothesis that MMP is involved in immunity in the tobacco hornworm. Our results are the first to show that MMP protein expression declines in immune tissues during 5th instar development and that MMP mRNA expression increases after *E. coli* infection. These results are exciting, because they provided a rationale for investigating the role of MMP in mediating immunity throughout development. Therefore, we tested the hypothesis that MMP was involved in survival from infection and AMP and PO activity. Interestingly, we found that MMP was deleterious to survival from infection, mounting a response with PO activity and hemocyte counts. These results are important because they provide an alternative hypothesis to explain the role of MMP in insect immunity.

3.4.1. MMP protein expression varies with 5th instar development in immune tissues

To determine if MMP mediates the established link between 5th instar development and decline in immune responses, we first observed how MMP protein expression varies with development in immune tissues. MMP protein expression peaks at the beginning and decreases by the end of the 5th instar in hemolymph and fat body, but remains unchanged in hemocytes (Fig. 9). Because hemolymph and fat body are major immune tissues, these data indicate that MMP plays a role in immunity. The decline in MMP protein expression throughout 5th instar development correlates with the observed decline in immune responses during 5th instar development (Booth et al., 2015; Eleftherianos et al., 2008). Our results parallel studies that show MMP expression varies with development and immune responses (Altincicek and Vilcinskas, 2008). For example, Altincicek and Vilinskas (2008) found MMP mRNA expression is up-regulated during pupation and is associated with enhanced AMP synthesis at the onset of metamorphosis in *G. melonella*. Moreover, it is interesting that all immune tissues do not show the same MMP protein expression pattern throughout 5th instar development. Perhaps hemocytes produce and secrete MMP into the hemolymph and fat body, but reduce production and secretion throughout 5th instar development. MMP is often secreted from granular cells. For example, in infected mice, MMP-9 is released from granules of leukocytes (Dubois et al., 2002), and MMP-2 and MMP-9 are produced in the granules of eosinophils (Greenlee et al., 2006). Perhaps, granulocytes, the granular cells in *M. sexta* store and secrete MMP upon infection. More experiments, such as in-situ hybridization, are required to further explore the kinetics of MMP expression and secretion in insects both throughout development and among immune tissues.

3.4.2. MMP mRNA expression varies in response to bacterial infection in immune tissues

Because both MMP expression and immune responses vary with development in the 5th instar of *M. sexta*, we observed how MMP mRNA and protein expression varies in response to bacterial infection in immune tissues. We found MMP mRNA expression, but not protein expression, increases 6 and 24 h after *E. coli* injection in both hemocytes and fat body in freshly molted 5th instar *M. sexta* (Figs. 10, 11, and 12). It is peculiar that an increase in MMP mRNA expression does not lead to an increase in MMP protein expression in immune tissues after bacterial injection. Perhaps changes in mRNA transcription occur more quickly than changes in protein expression so that 6 and 24 h after *E. coli* injection was not enough time to detect changes in MMP protein expression. Alternatively, perhaps the MMP protein is produced, secreted, and used so quickly that changes in MMP protein expression could not be detected at 6 or 24 h after *E. coli* injection. Because of the quick increases in MMP mRNA expression, MMP may be involved in the immune response to *E. coli* as early as 6 h post-infection. This hypothesis is also supported by a study in *G. mellonella* that found MMP mRNA expression in hemocytes and fat body is up-regulated after 8 h LPS- activation of the immune system (Altincicek and Vilcinskas, 2008). Further, LPS-injection also led to increased MMP mRNA expression and activity in hemocytes in *T. castaneum* (Knorr et al., 2009).

3.4.3. MMP inhibition improves survival

Because MMP mRNA expression in immune tissues increases in response to *E. coli* infection, we hypothesized that MMP plays an important role in immunity. We also hypothesized that the decline in MMP throughout an instar (Fig. 9) (Vishnuvardhan et al., 2013) may mediate the previously observed decline in immune responses during 5th instar development (Booth et al., 2015). We first observed the effect of MMP inhibition on survival to bacterial

infection as an index of collective immune responses. In freshly molted 5th instar *M. sexta*, survival to *E. coli* injection is improved when MMP protein expression is inhibited by RNA interference (Fig. 13). This was a particularly surprising finding, because mice without MMP have high rates of mortality in response to endotoxin (Dubois et al., 2002; Hong et al., 2011; Vanlaere and Libert, 2009) and *Streptococcus pneumonia* infection (Hong et al., 2011). Additionally, *T. castaneum* without MMP have high rates of mortality in response to *Beauveria bassiana* fungal infection (Knorr et al., 2009). The observation that MMP inhibition improves survival in *M. sexta* suggests that MMP may have harmful effects on the survival of the organism during infection. In fact, there is literature to suggest that MMP may not always have a positive role in physiological function and may need to be regulated. MMP is tightly regulated by endogenous MMP inhibitors to control the negative effects of MMP over-activation (Vilcinskas and Wedde, 2002). Moreover, MMP over-activity has been shown to have negative outcomes in human pathologies such as inflammation, allergies, asthma, and cystic fibrosis (Brinckerhoff and Matrisian, 2002). To further provide evidence that MMP may have deleterious effects on the immune system, we found that inhibition of the MMP activity by the MMP inhibitor, GM6001 significantly increased hemocyte count in hemolymph, regardless of infection (Fig. 18).

3.4.4. MMP inhibition does not affect humoral immune responses to bacterial infection

Because we found that MMP mRNA is up-regulated after *E. coli* infection in the fat body, we hypothesized that MMP plays a role in the fat body-mediated humoral immune responses, AMP and PO activity. The observation that MMP has been shown to cleave and activate the AMP α-defensin in mice (Wilson et al., 1999) provides an additional rationale for studying the role of MMP in AMP activity. We found AMP activity was not affected when

MMP protein expression is inhibited by RNA interference in infected freshly molted 5th instar *M. sexta* (Fig. 14). This result was surprising, because in in *G. melonella*, inhibition of MMP activity by the MMP inhibitor, GM6001 significantly suppressed LPS-induced antibacterial activity of hemolymph (Altincicek and Vilcinskas, 2008) and injection of purified MMP protein stimulates AMP mRNA expression (Altincicek and Vilcinskas, 2006).

Because MMP cleaves many immune-related proteinases (Greenlee et al., 2007; Page-McCaw et al., 2007) and stimulation of PO activity involves multiple proteinases (Kanost et al., 2004), we hypothesized that MMP plays a role in PO activity. We found that the effect of MMP on PO activity in freshly molted 5th instar *M. sexta* depends on the status of infection. PO activity is increased when MMP is inhibited by RNA interference in uninfected freshly molted 5th instar *M. sexta*, but there is no difference in PO activity in infected *M. sexta* (Fig. 15). This indicates that MMP may negatively regulate PO activity under normal, uninfected conditions. A decrease in PO activity during infection is typical, as was observed in honeybees (Laughton and Siva-Jothy, 2010). The results of the AMP and PO activity experiments suggest that although MMP mRNA expression is up-regulated in immune tissues during bacterial infection, MMP does not likely play an active role in mediating humoral immune responses in *M. sexta* to provide survival from infection.

The results indicating MMP is not involved in humoral immune responses in freshly molted 5th instar *M. sexta* were particularly surprising as the one of the main functions of MMP across multiple species involves immunity (Dubois et al., 2002; Greenlee et al., 2006; Gross and Lapiere, 1962; Hong et al., 2011; Page-McCaw et al., 2007; Vanlaere and Libert, 2009; Vu and Werb, 2000). However, because we found that RNA inhibition did not reduce protein expression in the immune tissues hemolymph and hemocytes, perhaps that is why we found conflicting

results regarding the function of MMP in the immune responses we measured. Because MMP mRNA is up-regulated in the hemocytes after *E. coli* infection, we hypothesize that MMP may be involved in cell-mediated immune responses, such as hemocyte migration. MMPs in mice are involved in the cleavage and activation of many chemotactic molecules that guide immune cells to the site of infection (Greenlee et al., 2006; Page-McCaw et al., 2007; Vu and Werb, 2000). Another possible role of MMP in cell-mediated immune responses may include wound healing, such as shown in *T. castaneum* (Mitten et al., 2012). The role of MMP in wound healing starts with MMP secretion from the epithelium at the site of damage (Nagase and Woessner, 1999). Upon release at the wound site, MMP cleaves extracellular matrix proteins and alters cell signaling molecules involved in recruiting immune cells (Nagase and Woessner, 1999). The role of MMP in hemocyte migration and wound healing in insects has not been extensively explored and may explain the up-regulation of MMP mRNA expression in hemocytes in response to bacterial infection.

3.4.5. GM6001 does not affect immune measures

To provide additional data regarding the function of MMP in immunity, we used an alternative MMP inhibitor to RNA inhibition called GM6001 and measured several aspects of immunity, including hemocyte spreading, phagocytosis, and bacterial load (Figs. 19-21). Hemocyte spreading was wide-spread among the hemocytes, but the percentage of hemocytes that spread on the slide did not differ based on whether the hemocytes were incubated with control saline, vehicle, or the MMP inhibitor. Additionally, incubation with these same three treatments did not alter phagocytosis rates of beads either. Taken together, these data imply that in-vitro incubation with MMP inhibitor does not affect hemocyte function. Further, whether caterpillars were injected with vehicle or MMP inhibitor did not affect how well caterpillars were

able to control bacterial load in the hemolymph. Overall, use of the alternative MMP inhibitor, GM6001 did not affect several aspects of immunity, including hemocyte spreading, phagocytosis, and bacterial load. This further provides evidence that the role of MMP is clearly elusive and still needs to be determined.

3.4.6. The role of MMP in other physiological processes

Though a correlation exists between a decline in MMP protein expression and decline in immune responses through 5th instar development in *M. sexta*, we found no evidence of a decline in MMP causing a decline in humoral immune responses. However, these results still raise interesting questions about the role of MMP in other physiological changes during development. In fact, inhibition of MMP resulted in delayed larval growth in *M. sexta* (Vishnuvardhan et al., 2013) and arrested pupal growth in *T. castaneum* (Knorr et al., 2009). As for the specific role of MMP in development, perhaps MMP is involved in the extensive fat body remodeling throughout development, such as is shown with MMP-2 in *Drosophila melanogaster* (Bond et al., 2011).

3.4.7. Other factors mediating the decline in immunity in 5th instar development

The major goal of this study was to determine if MMP mediates development-related changes in immune responses observed in *M. sexta* in the 5th instar. Although our data indicate that MMP may not directly mediate immunity in *M. sexta*, many changes occur within an instar that could affect immunity, including the level of juvenile hormone (JH). Juvenile hormone, a developmental hormone that prevents early molting, peaks early and decreases within an instar until molting (Baker et al., 1987; Nijhout, 1974). JH analog applied topically to early wandering 4th instar silkworm larvae, *B. mori*, resulted in a dramatic increase in gene expression of several AMPs, including cecropin, morincin, lebocin, and neucin, and the gloverin-like proteins 1 and 2

(Tian et al., 2010). In addition, JH analog application increased AMP activity against both *E. coli* and *S. aureus* in a traditional zone of inhibition assay (Tian et al., 2010). Although JH is immunostimulatory in larval *B.mori*, it is unclear how JH might be involved in immunity in *M. sexta*. A better understanding of the role of JH in modulating immunity is necessary and may provide insight into the unidentified mediator of development-dependent decline in immunity.

3.4.8. Conclusions

This study is the first to show that MMP protein expression varies with development in immune tissues and is responsive to bacterial infection in *M. sexta*. Although we did not determine the specific role of MMP in immunity, discovering the function of the evolutionarily conserved MMP in insects may better inform the roles of MMPs in human development and immunity. Also, determining what factors mediate the decline in immune responses throughout development could help in the production of new, more efficient pest control methods that exploit the immune system of insects and reduce the impact on the planet.

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CHAPTER 4. JUVENILE HORMONE ALTERS IMMUNE RESPONSES DURING LARVAL DEVELOPMENT OF THE TOBACCO HORNWORM, *MANDUCA SEXTA*

4.1. Introduction

Insect innate immune responses have been shown to change within a few days in a single instar, a juvenile developmental stage (Booth et al., 2015; Eleftherianos et al., 2008; Tian et al., 2010). Cell-mediated and humoral innate immune responses are more robust in the beginning than the end of the final instar of the tobacco hornworm, *Manduca sexta* (Eleftherianos et al., 2008) and the silk worm, *Bombyx mori* (Tian et al., 2010). Though there is an established link between within-instar development and a decline in immune responses, little research has been done to determine what mediates the development-immunity link. One of the many changes that occur within an instar that could potentially affect immunity is the level of juvenile hormone (JH). Juvenile hormone, a developmental hormone that prevents early molting, peaks early and decreases within an instar until molting (Fain and Riddiford, 1975). JH is most well-known for regulating development and reproduction (Riddiford, 2007), but more recently has been shown to have other unexpected roles, including mediating the immune system. JH analog applied topically to prepupal 4th instar silkworms resulted in a dramatic increase in gene expression of several AMPs, including cecropin, morincin, lebocin, and neucin, and the gloverin-like proteins 1 and 2 (Tian et al., 2010). In addition, JH analog application increased AMP activity against both *Escherichia coli* and *Staphylococcus aureus* in a traditional zone of inhibition assay (Tian et al., 2010). In contrast with the results of these studies, other studies have determined that JH suppresses AMP production, PO activity, and hemocyte concentration and spreading in other insects such as *D. melanogaster*, *Plutella xylostella* (diamond back moth), and *Tenebrio molitor* (mealworm beetle) (Flatt et al., 2008; Kwon and Kim, 2007; Rolff and Siva-Jothy, 2002). Thus,

whether JH is immunosuppressive or immunostimulatory remains unclear. To resolve this conflict, more thorough studies are clearly needed to understand how changes in JH titer contribute to changes in developmental immunity.

Additionally, all of the studies investigating the role of JH in immunity have only used a single age of insect, either applying JH inhibitor when natural levels of JH are high or applying JH analog when JH levels are low. This method of assessing the role of JH in immunity may lead to flawed results, because it may not take into account variables such as saturation of JH receptors or varying levels of JH receptor expression at various ages (Charles et al., 2011). Further, no studies have investigated the role of JH using functional immunity assays, such as survival and bacterial load in hemolymph. Functional immunity assays have been shown to be a more accurate depiction of immunocompetency compared to using individual indices of immune responses, like PO activity and hemocyte spreading. Individual indices of immune responses have been shown not to correlate with survival of bacterial infection and lower bacterial loads in the hemolymph (Adamo, 2004; Booth et al., 2015). I this chapter, I studied how the age-related changes in JH titer affect immune responses and underlie the decline in immune responses during 5^{th} instar development reported in in chapter 4.

In this study, we hypothesized that age-related changes in JH affect immune responses and underlie the previously observed decline in immune responses during 5^{th} instar development (Booth et al., 2015). To test this hypothesis, we applied either acetone vehicle, JH inhibitor only, JH inhibitor and JH analog, or JH analog only to *M. sexta* at the beginning of the 5^{th} instar when levels of JH are naturally high and to prepupae that have naturally low levels of JH. After JH manipulation, we induced an immune challenge by bacterial infection and assessed both functional measures of immunity (survival from *E. coli* infection and *E. coli* load in hemolymph)

and an individual measure of immunity (AMP activity). In freshly molted 5th instar *M. sexta*, we predicted that application of JH inhibitor would result in a decline of immunity, such as lower survival, higher bacterial load, and weaker AMP activity than *M. sexta* with vehicle applied. Additionally, we predicted that freshly molted 5th instar *M. sexta* that had JH analog applied in addition to JH inhibitor would experience a "rescuing effect", such that the survival, bacterial load, and AMP activity would be similar to those found in *M. sexta* with vehicle applied. The JH inhibitor + JH analog treatment group is essential in the experimental design to isolate the direct effect of eliminating JH and adding exogenous JH on the immune responses. Additionally, we predicted freshly molted 5th instar *M. sexta* with JH analog applied may have even higher survival, lower bacterial load, and stronger AMP activity than *M. sexta* with vehicle applied. In prepupal *M. sexta*, we predicted that application of JH inhibitor would not result in any changes of immunity when compared to *M. sexta* with vehicle applied because there are already low levels of circulating JH. However, this treatment group was essential to include to have a fully factorial experimental design when using a JH inhibitor and JH analog at two ages of *M. sexta* with different levels of circulating JH. In contrast, we predicted that prepupal *M. sexta* that had JH analog applied, regardless of exposure to JH inhibitor, would have higher survival, lower bacterial load, and stronger AMP activity than *M. sexta* with vehicle applied. Our fully factorial experimental design using JH inhibitor and JH analog at two ages of *M. sexta* with different levels of circulating JH tests the prediction that JH is immunostimulatory.

4.2. Materials and methods

4.2.1. Study organism and rearing conditions

Manduca sexta colonies were reared as described in Booth et al., 2015. Briefly, *M. sexta* larvae (Carolina Biological, Burlington, NC) were reared at 25°C under a 16L:8D photoperiod

with *ad libitum* access to an artificial, wheat germ-based diet. *Manduca sexta* were observed daily to determine the age.

4.2.2. Experimental design

To determine the effect of JH on immune responses, we manipulated JH titers at two different life stages of *M. sexta*, freshly molted 5th instar *M. sexta*, when circulating JH is normally high, and prepupal *M. sexta* when circulating JH is normally low (Fain and Riddiford, 1975). *Manduca sexta* at both of these life stages were divided into one of four groups (Table 1) and treatments were applied according to Fig. 22.

Table 1. Experimental groups and treatments. Vehicle in both treatments was acetone. The JH inhibitor was 6-flouromevalonate the JH analog used was methoprene.

Group #	Treatment 1	Treatment 2
1	Vehicle	Vehicle
2	JH inhibitor	Vehicle
3	JH inhibitor	JH analog
4	Vehicle	JH analog

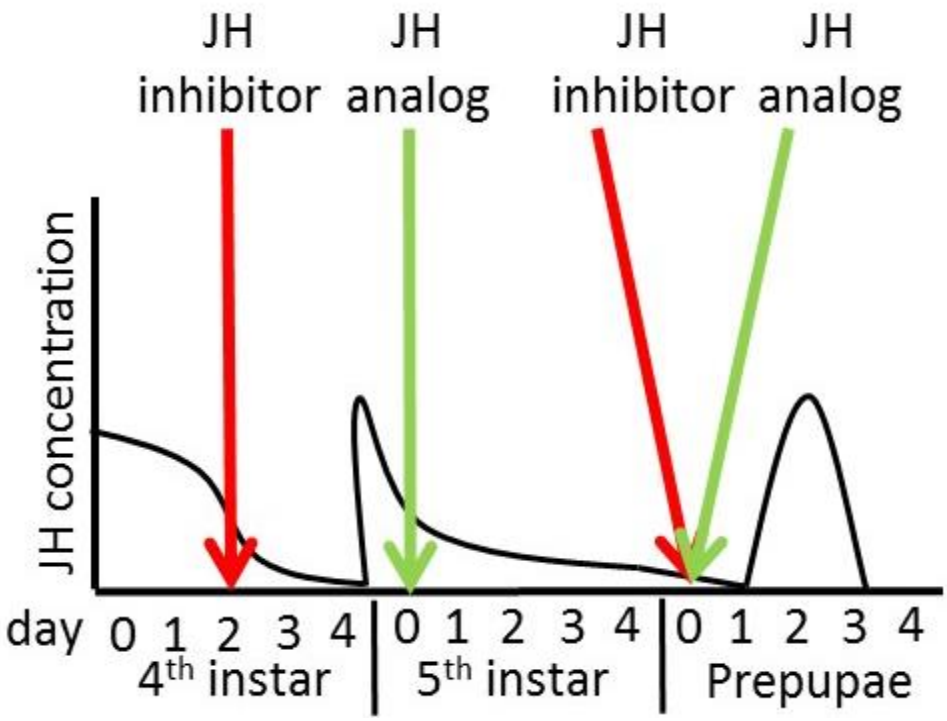

Fig. 22. Experimental groups and timing of treatment application. Two different ages of *M. sexta* were used. For the freshly molted 5th instar *M. sexta*, treatment 1 was applied at a time to inhibit the 5th instar day 0 JH peak (timing modified for 4th instar larvae from (Edwards et al., 1983)) and treatment 2 was applied at a time to simulate the 5th instar day 0 JH peak (as performed in (Nijhout et al., 2006)). For the prepupal *M. sexta*, treatment 1 was applied to caterpillars displaying prepupal characteristics, such as formation of the dorsal vessel, typically 5th instar day 4-6 (as performed in Nijhout et al., 2006).

After JH manipulation of both life stages of *M. sexta*, *E. coli* was injected 1 hour later and three tests of immune responses were performed, as previously described (Booth et al., 2015): survival from infection, bacterial load, and anti-microbial peptide (AMP) activity. Briefly, to assess survival from infection and bacterial load, we infected caterpillars with *E. coli*. To assess survival, caterpillars were checked for mortality at various time points until pupation (7 days post-injection for the prepupal and 12 days post-injection for the freshly molted 5th instar *M. sexta*). To assess bacterial load, hemolymph was collected and spread on LB agar plates 24 h post-injection, and bacterial colonies were counted. To assess AMP activity, caterpillars were injected with lyophilzed *Micrococcus lysodeikticus* (Sigma-Aldrich, St. Louis, MO), hemolymph

was collected 24 h later, AMPs were isolated and loaded into LB agar plates embedded with *E.coli*, and zones of inhibition of bacterial growth were measured.

4.2.3. Juvenile hormone inhibitor preparation and application

The JH inhibitor used was 6-flouromevalonate (FMev, Sigma-Aldrich, St. Louis, MO), which was prepared at a concentration of 0.132mg FMev in 5µl of acetone according to manufacturer's protocol (dose modified from (Baker et al., 1986) for 4th instar *M. sexta*). Each caterpillar received 5 µl of the FMev solution, or 10µg of JH inhibitor, pipetted onto the first right proleg. Caterpillars in the vehicle control group received 5 µl of acetone in the same location. The solution was allowed to evaporate from the cuticle before caterpillars were returned to their individual housing.

4.2.4. Juvenile hormone analog preparation and application

The JH analog used was methoprene (Sigma-Aldrich, St. Louis, MO), which was prepared at a concentration of 50 µg methoprene in 5µl of acetone (as performed in Nijhout et al., 2006). Each caterpillar received 5 µl of the methoprene solution, or 50 µg of JH analog, pipetted on the first right proleg (as performed in Nijhout et al., 2006). Caterpillars in the vehicle control group received 5 µl of acetone in the same location. The solution was allowed to evaporate from the cuticle before caterpillars were returned to their individual housing.

4.2.5. Bacterial suspensions

DH5-α *E.coli* used in the survival and bacterial load assays were grown to stationary phase in Luria broth (LB), and the optical densities of the bacterial suspensions were measured using a spectrophotometer (Beckman Coulter, Brea, CA). The bacterial suspensions were centrifuged at 4000 x g for 15 min. Resulting pellets were washed with sterile phosphate buffered saline (PBS, 137mM NaCl, 3mM KCl, 10mM Na_2HPO_4, 2mM KH_2PO_4, pH 7.4) and

centrifuged again at 4000 x g for 15 min. The final *E. coli* bacterial pellet was re-suspended in PBS at a concentration of 1 million CFU per 10 µl. For the AMP activity assay, lyophilized *M. lysodeikticus* (Sigma-Aldrich, St. Louis, MO) were prepared at a concentration of 10 mg/ml in PBS.

4.2.6. Injections

Regardless of injected treatment or age of *M. sexta*, the injection method was the same as we have previously described (Booth et al., 2015). Briefly, larvae were cooled on ice for 5 min and surface sterilized with 75% ethanol. A 25 µl Hamilton syringe (Hamilton, Reno, NV) with a 26 gauge needle was filled with bacterial solution. The needle was inserted subcutaneously into the hemocoel of the caterpillar between the first and second left pro-legs. Once injections were complete, caterpillars were transferred to individual containers with *ad libitum* access to food and monitored daily. Caterpillars for the survival and bacterial load assays were injected with 1 million CFU of *E. coli* in 10 µl of PBS. Caterpillars for the AMP activity assay were injected with 100 µg of *M. lysodeikticus* in 10 µl of PBS.

4.2.7. Survival assay

Survival from *E. coli* infection was assessed at various time points until pupation occurred. Observations of survival were made at 0 h, 4 h, 20 h, 24 h, 28 h, 44 h, 48 h, and then daily for 3-12 days post-infection after JH manipulation of freshly molted 5th instar caterpillars. Observations of survival were made at 0 h, 4 h, 8 h, 20 h, 24 h, 28 h, 32 h, 48 h, and then daily for 3-7 days post-infection after JH manipulation of prepupal caterpillars. Caterpillars unable to right themselves after being placed on their dorsum were considered dead.

4.2.8. Hemolymph collection

Larvae were cooled on ice for 5 min, and surface sterilized with 75% ethanol before hemolymph collection. To collect hemolymph, an incision was made on the first, right pro-leg using a sterilized razor blade, and hemolymph was collected in a 1.5ml Eppendorf tube on ice.

4.2.9. Bacterial load assay

Caterpillars were injected with 1 million CFU of *E. coli* and hemolymph was collected 24 h later. Hemolymph was serially diluted in PBS (10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}) and spread on LB agar plates. The concentration of bacteria was determined via standard colony counts 24 h later.

4.2.10. Anti-microbial peptide activity assay

To determine the activity of the AMP produced as a part of the humoral immune response, a traditional zone-of-inhibition assay was performed as previously described (Booth et al., 2015). Caterpillars were injected with 100 µg of *M. lysodeikticus* and hemolymph was collected 24 h later. AMP were isolated by centrifugation at 7000 x g for 5 min to separate hemocytes from hemolymph plasma. The plasma layer was collected and heated at 95°C for 5 min and spun again at 13,400 x g for 10 min. AMP were isolated in the supernatant. Wells were carved into LB agar plates embedded with DH5α *E. coli.* Five µl of AMP from each caterpillar were loaded into the wells in duplicate. Plates were incubated at 37°C for 1 h, then at 25°C overnight. The next morning, plates were photographed, and the zone of inhibition, the diameter of the area of clearance around the well where the AMPs inhibited bacterial growth, was measured using Image J (Rasband, 1997-2011).

4.2.11. Mass gain after JH manipulation

To provide an additional measure of overall health and response to the JH treatments, *M. sexta* were weighed and treated with either acetone or JH inhibitor as described above as 4th

instar day 1 caterpillars to reduce the 5th instar day 0 peak of JH. Caterpillars were then weighed upon molting into the 5th instar before JH analog application or *E. coli* injection. The difference between masses was calculated to determine the mass gained.

4.2.12. Data analysis

Statistical analyses were performed using IBM SPSS version 21. Analysis of Variance (ANOVA) test was used to determine significant differences among treatments, and Bonferroni post-hoc tests were used to detect differences for all pair-wise comparisons for bacterial load, AMP activity, and mass gain experiments. Differences in survival among treatment groups were assessed by Cox-Regression time-to-event analysis. A p-value < 0.05 indicates statistical significance. Data are expressed as mean ± SEM throughout.

4.3. Results

4.3.1. Immune tests of freshly molted 5th instar *M. sexta*

Infection with *E. coli* was lethal for many freshly molted 5th instar *M. sexta* when JH was inhibited (Fig. 17). In contrast, survival in the other two groups was near 100% (Fig. 17). Application of JH inhibitor with or without JH analog caused approximately 70% mortality compared to *M. sexta* that received the vehicle application (χ^2=8.70, P<0.05; Cox-Regression; n=8 for the vehicle group, n=8 for the JH inhibitor only group, n=6 for the JH inhibitor + JH analog group, n=8 for the JH analog only group). Survival rates did not differ between *M. sexta* with JH inhibitor only or JH inhibitor + JH analog applied (P=1.00, Bonferonni-corrected post-hoc test). Additionally, there were no differences in survival rates between *M. sexta* with vehicle applied or JH analog only applied (P=1.00, Bonferonni-corrected post-hoc test).

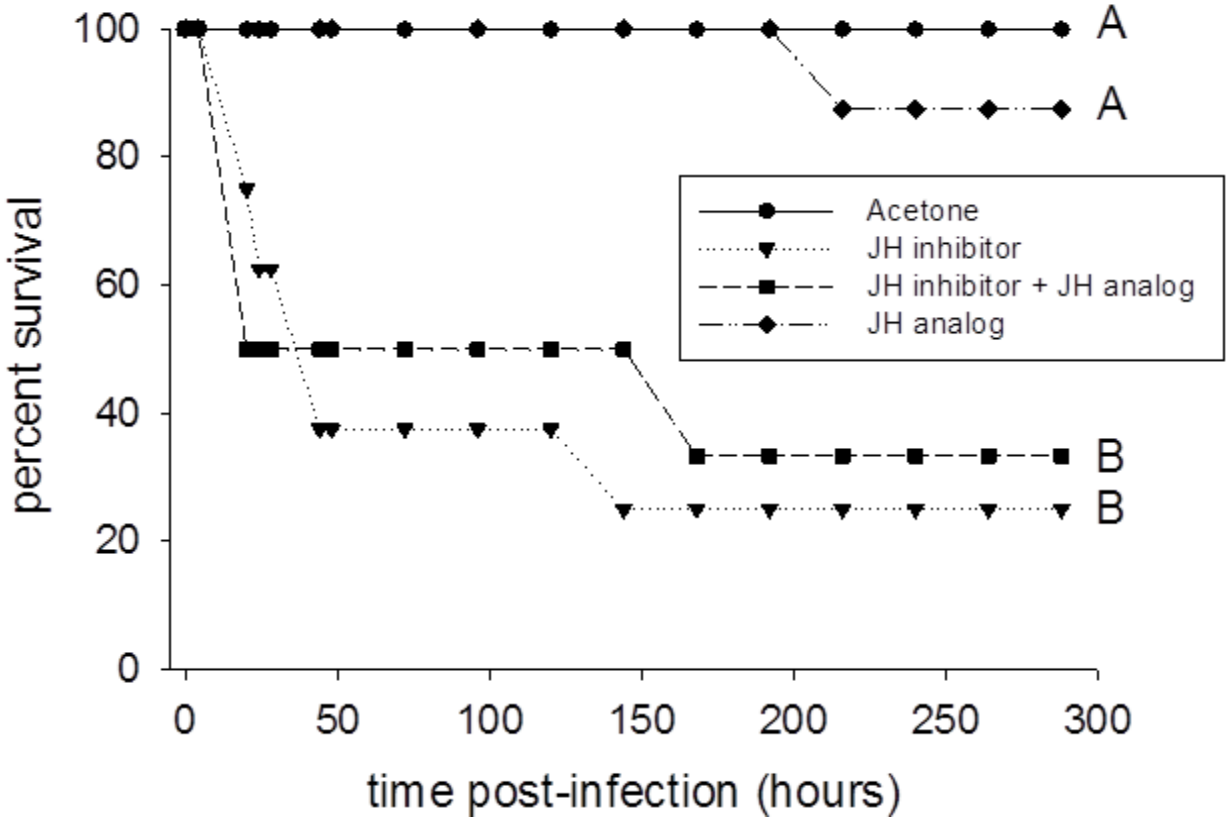

Fig. 23. Survival from *E. coli* infection by freshly molted 5th instar *M. sexta* with acetone vehicle, JH inhibitor only, JH inhibitor + JH analog, or JH analog only applied. Caterpillars were checked for signs of life at various time points post infection. Lines with different letters are significantly different from one another (χ^2=8.70, P<0.05; Cox-Regression).

Freshly molted 5th instar *M. sexta* with JH inhibitor applied had approximately 10-fold higher *E. coli* bacterial loads in hemolymph compared to those without JH inhibitor (Fig. 24, $F_{3,17}$=5.186, P<0.05; ANOVA; n=8 for the vehicle group, n=3 for the JH inhibitor only group, n=3 for the JH inhibitor + JH analog group, and n=9 for the JH analog only group). There were no differences in bacterial load between *M. sexta* with JH inhibitor only or JH inhibitor + JH analog applied (P=1.00, Bonferonni-corrected post-hoc test). Additionally, there were no differences in bacterial load between *M. sexta* with vehicle applied or JH analog only applied (P=1.00, Bonferonni-corrected post-hoc test).

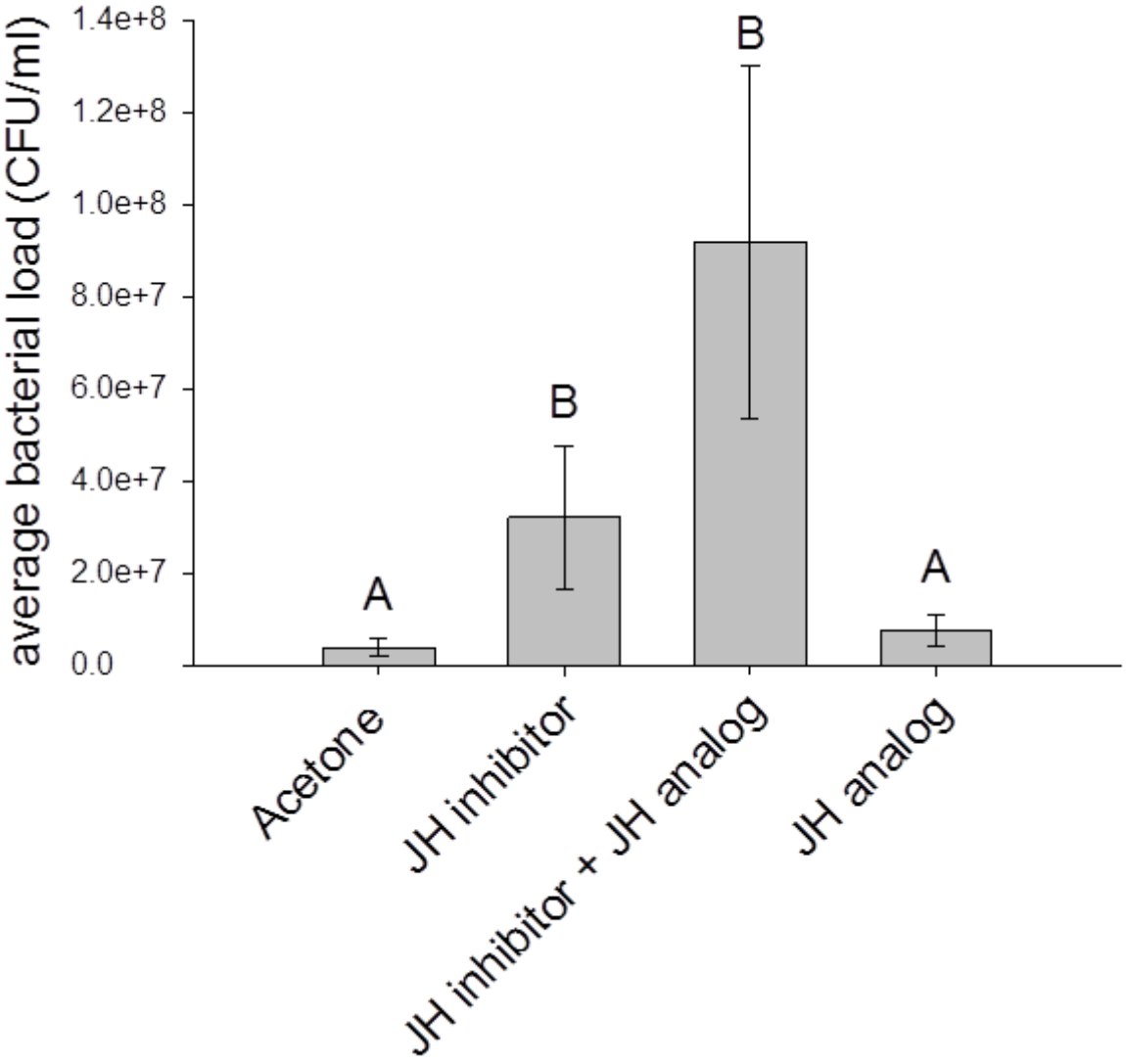

Fig. 24. Bacterial load of freshly molted 5th instar *M. sexta* injected with *E. coli* and with acetone vehicle, JH inhibitor only, JH inhibitor + JH analog, or JH analog only applied. Hemolymph was collected 24 h post-*E. coli* injection, and bacterial load assessed. Bars with the different letters are significantly different from one another ($F_{3,17}$=5.186, P<0.05; ANOVA). Error bars indicate +/- SEM.

AMP activity in freshly molted 5th instar *M. sexta* with only JH inhibitor applied was approximately 20% lower compared to *M. sexta* with vehicle applied (Fig. 25, $F_{3,25}$=3.177, P<0.05 on ANOVA; P=0.05 on Bonferonni-corrected post-hoc test; n=11 for the vehicle group, n=5 for the JH inhibitor only group, n=4 for the JH inhibitor + JH analog group, and n=9 for the JH analog only group). Application of JH analog in addition to JH inhibitor to *M. sexta* rescued

the AMP activity, resulting in no differences in AMP activity among *M. sexta* with vehicle, JH inhibitor + JH analog, and JH analog only applied (P=1.00, Bonferonni-corrected post-hoc test for vehicle vs. JH inhibitor + JH analog, vehicle vs. JH analog only, and JH inhibitor + JH analog vs. JH analog only).

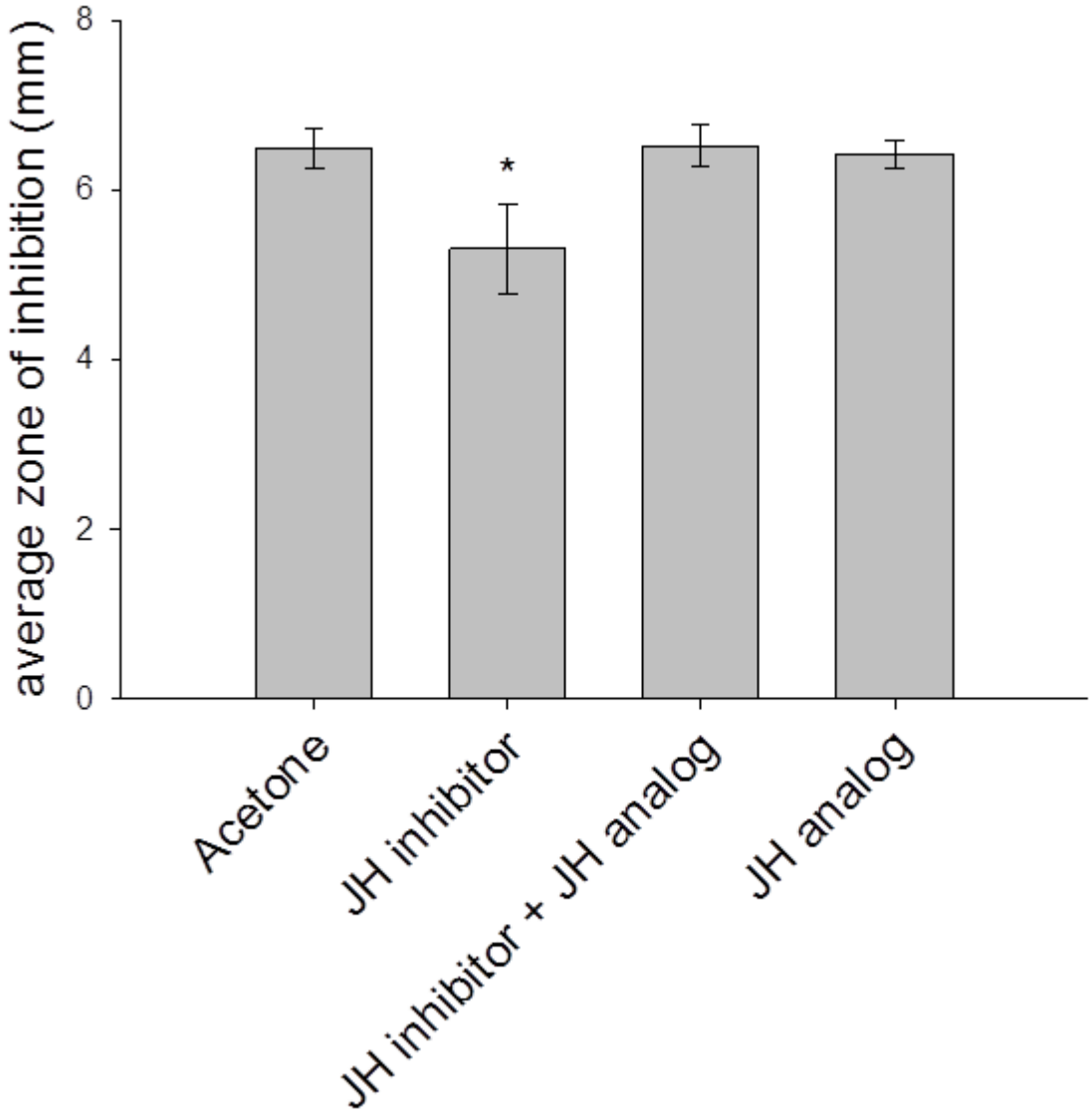

Fig. 25. Anti-microbial peptide (AMP) activity of freshly molted 5th instar *M. sexta* with acetone vehicle, JH inhibitor only, JH inhibitor + JH analog, or JH analog only applied. *M. sexta* were injected with dried *M. lysodeikticus*, and AMP activity was assessed 24 h later. The bar with * indicates a value significantly lower than the acetone vehicle ($F_{3,25}$=3.177, P<0.05; ANOVA). Error bars indicate +/- SEM.

Manduca sexta with JH inhibitor applied as 4th instar day 1 to inhibit the 5th instar day 0 JH peak showed 1/3 of the mass gain from the time of JH inhibition to the time of molting into the 5th instar (before *E. coli* injection) compared to caterpillars that did not have JH inhibitor applied (Fig. 26, $F_{3,26}$=8.031, P<0.01, ANOVA; n=6 for the JH inhibitor + JH analog group, n=8

for all other groups). All caterpillars were the same mass at the time of JH inhibitor application ($F_{3,3}$=0.245, P>0.05, ANOVA; n=6 for the JH inhibitor + JH analog group, n=8 for all other groups).

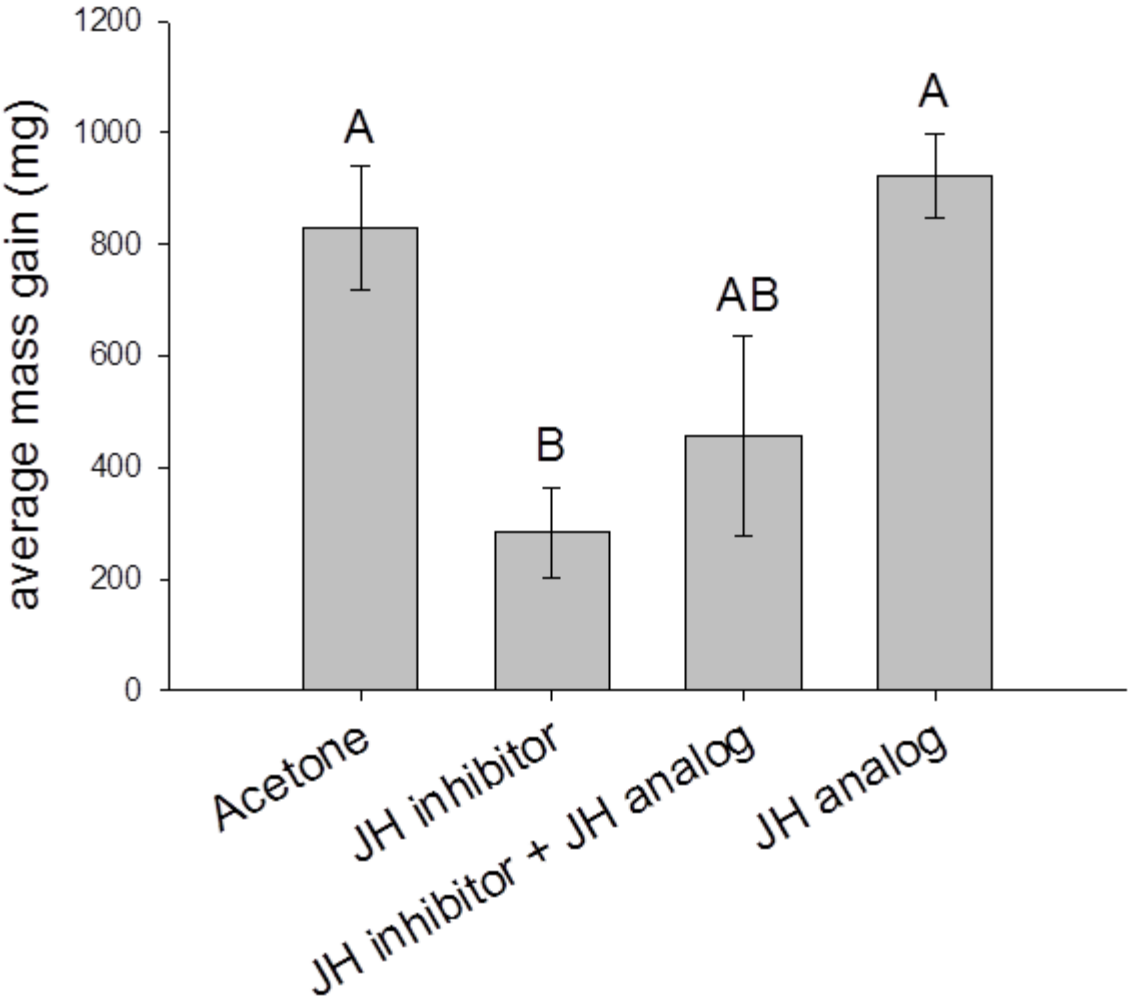

Fig. 26. Mass gain of 4th instar day 1 *M. sexta* with acetone vehicle, JH inhibitor only, JH inhibitor + JH analog, or JH analog only applied. *M. sexta* were weighed as 4th instar day 1 before JH manipulation, weighed again upon molting in to the 5th instar and the mass gain was calculated. Bars with different letters are significantly different ($F_{3,26}$=8.031, P<0.01, ANOVA). Error bars indicate +/- SEM.

4.3.2. Immune tests of prepupal *M. sexta*

Survival from *E. coli* infection in prepupal *M. sexta* did not depend on JH (Fig. 27). Though there was a 25% reduction in survival from *M. sexta* with JH inhibitor and JH analog applied at about 24 h after *E. coli* infection, then again at 7 days after *E. coli* infection, survival rates were not statistically different from those of *M. sexta* treated with vehicle, JH inhibitor only, or JH inhibitor + JH analog applied (χ^2=0.831, P=0.362; Cox-Regression; n=3 for the

vehicle group, n=4 for the JH inhibitor only group, n=4 for the JH inhibitor + JH analog group, and n=5 for the JH analog group).

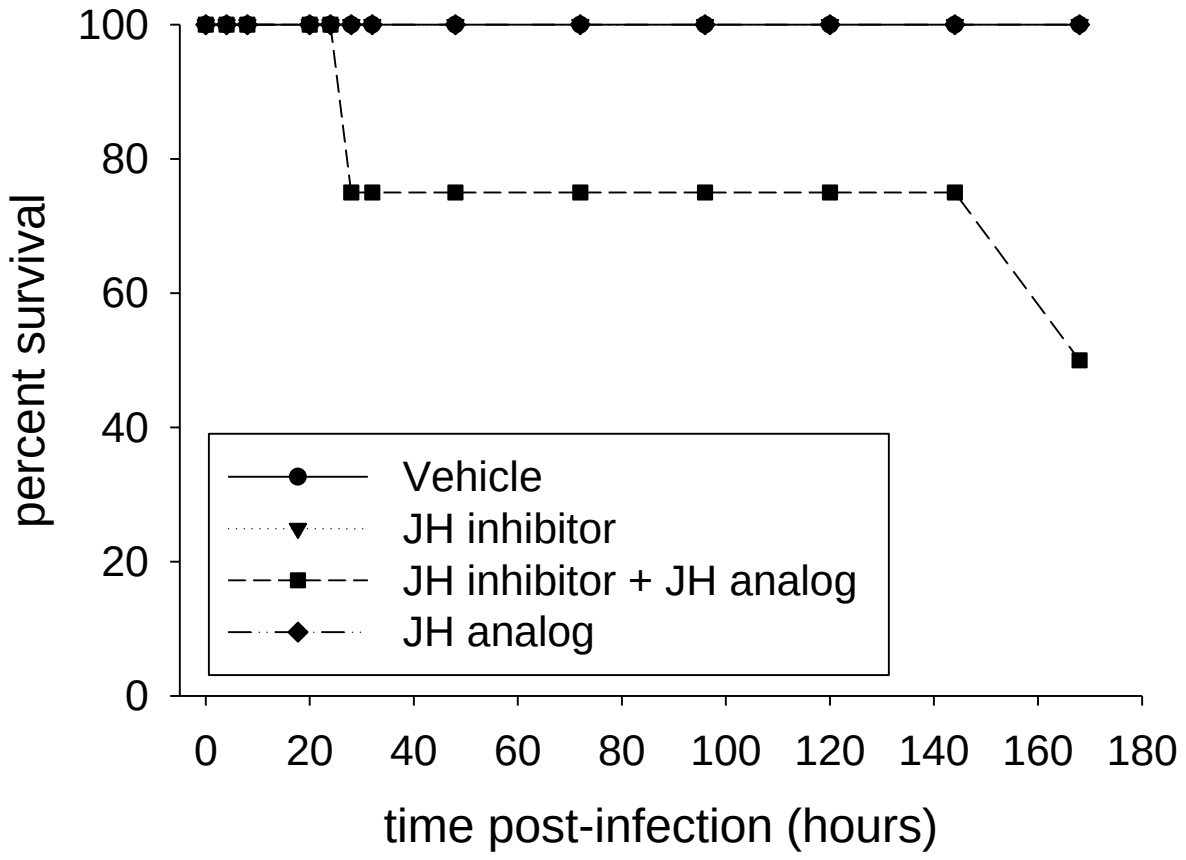

Fig. 27. Survival from *E. coli* infection by prepupal *M. sexta* with acetone vehicle, JH inhibitor only, JH inhibitor + JH analog, or JH analog only applied. Caterpillars were checked for signs of life at various time points post infection. There were no statistically significant differences in survival rates (χ^2=0.831, *P*=0.362; Cox-Regression).

AMP activity in prepupal *M. sexta* with only JH inhibitor applied was approximately 25% lower compared to *M. sexta* with vehicle applied. (Fig. 28, $F_{3,19}$=3241, *P*<0.05 on ANOVA; *P*=0.06 on Bonferonni-corrected post-hoc test; n=5 for the vehicle group, n=6 for all remaining groups). Application of JH analog in addition to JH inhibitor to *M. sexta* rescued the AMP activity, resulting in no differences in AMP activity among *M. sexta* with vehicle, JH inhibitor + JH analog, and JH analog only applied (*P*=1.00, Bonferonni-corrected post-hoc test for vehicle

vs. JH inhibitor + JH analog, vehicle vs. JH analog only; *P*=0.260 for JH inhibitor + JH analog vs. JH analog only).

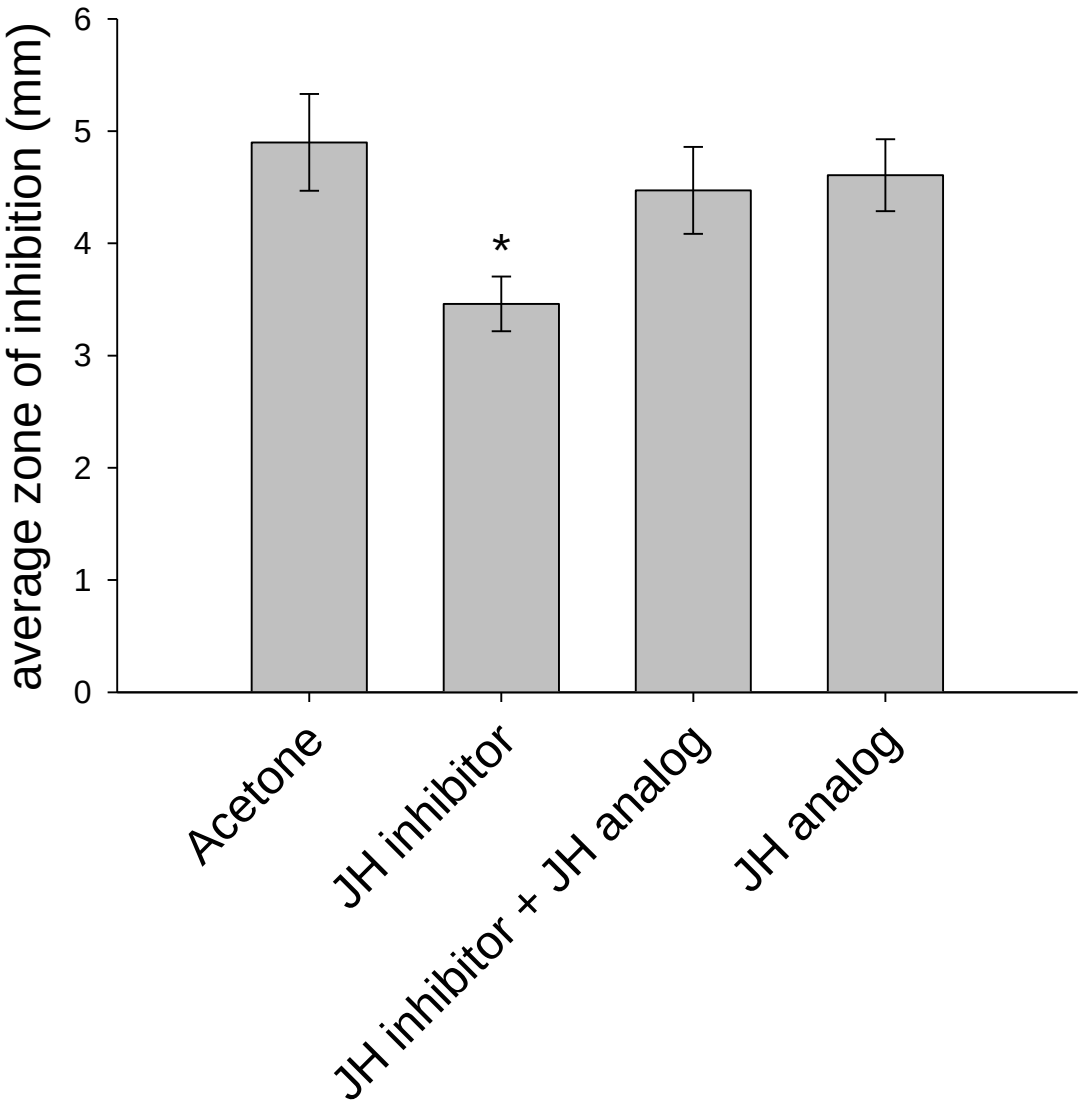

Fig. 28. Anti-microbial peptide (AMP) activity of prepupal *M. sexta* with acetone vehicle, JH inhibitor only, JH inhibitor + JH analog, or JH analog only applied. *M. sexta* were injected with dried *M. lysodeikticus*, and AMP activity was assessed 24 h later. *indicates a value significantly lower than the acetone vehicle ($F_{3,19=}$3.241, P<0.05; ANOVA). Error bars indicate +/- SEM.

4.3.3. Comparing freshly molted 5th instar and prepupal *M. sexta*

Performing factorial ANOVA statistics with age of *M. sexta* as a factor in addition to treatment showed that age of the *M. sexta* affected AMP activity. Regardless of the treatment applied, prepupal *M. sexta* showed lower AMP activity than the freshly molted 5th instar *M. sexta* (ANOVA; χ^2=0.012; P<0.05). However, the age of *M. sexta* did not affect how treatment affected survival (ANOVA; χ^2=3.752; P>0.05).

4.4. Discussion

In this study, we tested the hypothesis that JH is involved in immune responses of *M. sexta*. Our study is novel, because it is the first to incorporate the development-dependent nature of JH release. To test the role of JH in immunity, we used a fully factorial experimental design using both JH inhibitor and JH analog in freshly molted 5th instar *M. sexta* when circulating JH levels are high, as well as prepupal *M sexta* when circulating JH levels are low. Our results are exciting because they help illuminate the role of JH in a conflicting body literature about the immunostimulatory or immunosuppressive nature of JH.

4.4.1. Immune tests of freshly molted 5th instar *M. sexta*

Our results are the first to show that JH is necessary to survive an infection, control bacterial growth in hemolymph, and mount an AMP activity immune response (Figs. 23, 24 and 25) and match our predictions that JH is immunostimulatory. When JH inhibitor is applied to freshly molted 5th instar *M. sexta*, survival from *E.coli* infection decreases until pupation is reached (Fig. 23). Decreases in survival coincide with increases in *E. coli* bacterial load in hemolymph resulting from inhibition of JH (Fig. 24). Accordingly, AMP activity is also negatively affected when JH inhibitor is applied to freshly molted 5th instar *M. sexta* (Fig. 25). The immunostimulatory effects of JH on AMP activity concur with previous findings that

showed JH analog application increased AMP activity in prepupal silkworms, *B. mori* (Tian et al., 2010). Additionally, it is noteworthy that the decreased AMP activity due to JH inhibition was rescued upon application of the JH analog (Fig. 25). The rescuing effect of JH analog on AMP activity confirms that the deleterious effects of JH inhibition on AMP activity are truly due to JH and not from any other unexpected effects of the JH inhibitor, which inhibits the mevalonate pathway necessary for the synthesis of juvenile hormone (Staal, 1986).

Interestingly, application of JH analog did not show a rescuing effect when testing the role of JH in survival and bacterial load in freshly molted 5th instar *M. sexta*. Because we predicted that JH would have an immunostimulatory effect, we expected that caterpillars treated with JH analog only would have higher survival, lower bacterial loads, and higher AMP activity than caterpillars with vehicle applied. However, there were no significant differences in survival, bacterial load, or AMP activity in caterpillars with vehicle or JH analog only applied. Because these indices of immune responses were assessed in freshly molted 5th instar *M. sexta* when circulating levels of JH are high, we speculate that the JH receptors may have been saturated and that no additional exogenous amount of JH would elicit further physiological effects. In addition, because survival and bacterial loads are functional measures of immunity, it is likely that there are many immune responses working together to help the organism fight infection (Adamo, 2004). Therefore, it is possible that it is more difficult to find the role of a single hormone (i.e., JH) using a broad measure of immunity (i.e., survival, bacterial load) than it would be in a specific measure of immunity (i.e., AMP activity). Either way, the effect of JH inhibition on immune function is consistent, because when AMP activity is low due to JH inhibition, survival is low and bacterial load is high.

We also were able to confirm that the JH inhibitor had an effect on *M. sexta* by measuring their mass gain between the time of JH manipulation as 4th instar day 1 caterpillars to the time of molting into the 5th instar (before *E. coli* injection). Caterpillars that did not receive JH inhibitor gained 3 times the mass of caterpillars that did receive JH inhibition (Fig. 26). This verified that the JH inhibitor was having a physiological effect on the caterpillars and was likely reducing JH titers.

4.4.2. Immune tests of prepupal *M. sexta*

In addition to testing the role of JH in immune responses in freshly molted 5th instar *M. sexta* when circulating JH levels are high, we also assessed immune responses in prepupal *M. sexta* when circulating JH levels are naturally low. In contrast to the experiments run in freshly molted 5th instar *M. sexta*, we predicted that there would be no effect of the JH inhibitor on immune responses (because there is little JH at the prepupal developmental stage), and that there would be a positive effect of JH analog application on immune responses. However, there was no statistically significant effect of either the JH inhibitor or JH analog on the survival of prepupal *M. sexta* to *E.coli* infection (Fig. 27). We speculate that because the vehicle control *M. sexta* already had 100% survival, adding additional exogenous JH would not result in higher than 100% survival from infection, explaining why JH analog did not have a positive effect on survival, according to our predictions. Indeed, prepupal *M. sexta* are shown to have high survival from infection, so this result is not entirely surprising (Booth et al., 2015).

Interestingly, there was a negative effect of JH inhibitor on AMP activity in prepupal *M. sexta*, when there is little circulating JH to be inhibited (Fig. 28). The decrease in AMP activity due to JH inhibition was rescued upon application of JH analog, which again confirms that the deleterious effects of JH inhibition on AMP activity were truly due to JH and not from any other

unexpected effects of the JH inhibitor. Despite low levels of circulating JH during the prepupal developmental stage, this implies that there is still some JH that could be inhibited, which was manifested in lower AMP activity. As with seen with the experiments testing the role of JH in freshly molted 5th instar *M. sexta*, the role of JH in immune function in preupal *M. sexta* is consistent because when AMP activity was low due to JH inhibition, bacterial loads were high. Although the consistent role of JH in immune function does not expand to survival, perhaps this provides evidence that there are many aspects of immunity that contribute to survival from infection, not just AMP activity. When taken together, our fully factorial study shows that JH is required to mount an immune response against bacterial infection.

4.4.3. JH as a mediator development-immunity link

One of the driving factors in this study was to determine if JH mediates the established link between 5th instar development and decline in immune responses in *M. sexta* (Booth et al., 2015). Although our data indicate that there is a link between JH manipulation and survival, bacterial, load, and AMP activity at various points in 5th instar development, they still do not integrate perfectly to explain this phenomenon. JH was found to be necessary to survive infection and control bacterial load in the hemolymph in freshly molted 5th instar *M. sexta*, when they typically have low survival and high bacterial load (Booth et al., 2015). Therefore, our data indicate it is not high levels of JH that can explain the poor survival and bacterial load in freshly molted 5th instar in *M. sexta*. Though reviewed more thoroughly by Booth et al. (2015), there is a disparity between the functional indexes of immunity (i.e., survival and bacterial load) and specific immune responses (i.e., AMP activity). Specifically, freshly molted 5th instar *M. sexta* had high AMP activity despite exhibiting low survival from infection and high bacterial loads. Because our fully factorial experimental design shows that it is specifically JH that is important

in mounting an AMP activity response, the high levels of JH found in freshly molted 5th instar *M. sexta* may explain the high AMP activity observed at this stage (Booth et al., 2015; Eleftherianos et al., 2008; Tian et al., 2010). In the other developmental stage in which immune responses were assessed, prepupal *M. sexta* show high survival, but also surprisingly low AMP activity. Because our results with prepupal *M. sexta* show that JH is important in stimulating AMP activity, it is possible that the low levels of JH found in prepupal *M. sexta* may explain the low AMP activity observed in this developmental stage. Our results, taken together, show that fluctuating levels of JH with development may help explain the fluctuating AMP activity levels with 5th instar development, but not necessarily fluctuating rates of survival from infection or bacterial load. Although not assessed in this study, fluctuating levels of JH with development may also help explain other fluctuating indexes of immunity with 5th instar development, such as phagocytosis, nodulation, and phenoloxidase activity (Eleftherianos et al., 2008).

4.4.4. Conclusions

Our results corroborate other studies showing that changes in JH levels affect immune responses, crucial information for scientists. Natural fluctuations in JH should not be overlooked as a confounding factor in experimental design, especially since *M. sexta* are one of the most common model species in insect physiology studies. Solely comparing studies using the same age of *M. sexta* with the same circulating JH levels may not be enough to provide an accurate portrayal of *M. sexta*'s immunity or physiology as a whole. In addition, understanding the developmental physiology of *M. sexta* and how JH regulates immunity throughout development will allow for better understanding of how hormones contribute to immune function. It would be interesting to determine the mechanism behind the link between JH and immunity. For example, one would be able to determine how JH affects the immune system by using transcriptomics to

see what types of genes JH alters. For society, determining the factors causing development-dependent changes in immunity could help in the production of new, more efficient pest control methods and reduce impact on the planet.

4.5. References

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CHAPTER 5. CONCLUSIONS

Research from Chapter 2 determined that insect innate immune responses are developmentally plastic to such a degree that immune responsivity can even change within a single larval instar. Specifically, the humoral immune responses AMP and PO activity peak at the beginning of the 5th instar of *M. sexta* and decrease throughout the remainder of that instar. I investigated two possible factors that could mediate the development-immunity link, matrix metalloproteinase (MMP) and juvenile hormone (JH). In experiments outlined in Chapter 3, I studied how MMP protein levels change during 5th instar development and if that impacts AMP and PO activity. Because I found that MMP protein levels in the immune tissues of hemolymph and fat body were highest at the beginning of the 5th instar, I predicted that inhibiting MMP mRNA expression in freshly molted 5th instar *M. sexta* would decrease AMP and PO activity. I found that inhibiting MMP mRNA expression actually increased PO activity and had no effect on AMP activity. The high levels of AMP and PO activity at the beginning of the 5th instar in *M. sexta* could not be explained by high MMP protein expression observed at that same stage of development. In fact, because MMP negatively affected PO activity, I suspected that there may be other factors strongly linked to PO activity that could help explain its heightened function at the beginning of the 5th instar, despite MMP negatively regulating it. In Chapter 4, I investigated the role of another factor, JH, which could mediate the development-immunity link. Although I did not investigate how JH affects PO activity, I predicted that because JH is highest at the beginning of the 5th instar when AMP activity is highest, inhibiting JH in freshly molted 5th instar *M. sexta* would decrease AMP activity. Matching our predictions, inhibiting JH at the beginning of the 5th instar decreased AMP activity, providing evidence that JH could be mediate the development-AMP activity link. To further support this claim, adding exogenous JH back to

caterpillars that had been treated with JH inhibitor showed an increase in AMP activity comparable to caterpillars that had received control treatments. Collectively, my dissertation research found a link between 5th instar development in *M. sexta* and a decline in the humoral immune responses, that MMP does not mediate the heightened AMP and PO activity observed at the beginning of the 5th instar, but JH does mediate the heighted AMP activity observed at the beginning of the 5th instar.

The other aspect of the insect immune system that I studied in Chapter 2 included the functional immune assays, survival and bacterial load. I showed that despite low AMP and PO activity at the end of the 5th instar, *M. sexta* were actually able to survive from infection better and have lower bacterial loads in the hemolymph at the end of the 5th instar than at the beginning of the 5th instar. In Chapter 3, I studied how MMP is involved this development-survival link. Because I found that MMP protein levels in the immune tissues of hemolymph and fat body were lowest at the end of the 5th instar when high survival from infection is observed, I predicted that inhibiting MMP mRNA expression in freshly molted 5th instar *M. sexta* would increase survival from infection. Our predictions were met when we observed that caterpillars without MMP survived infection better than control caterpillars. Although I did not study how MMP affected bacterial load, I investigated how JH affected bacterial load as well as survival from infection in Chapter 4. Because JH is lowest at the end of the 5th instar when high survival and low bacterial load is observed, I predicted that inhibiting JH in freshly molted 5th instar *M. sexta* would result in increases in survival and decreases in bacterial load. In contradiction to these predictions, we found inhibiting JH in fact decreased survival from infection and increases in bacterial load. Collectively, my dissertation research found a link between 5th instar development in *M. sexta* and an increase in functional immune measures, MMP does mediate the heightened survival

observed at the end of the 5th instar, but JH does not mediate the heighted survival or low bacterial load observed at the end of the 5th instar.

A peculiar observation made from this dissertation research is the lack of consistency between functional immunity measures (e.g., survival, bacterial load) and specific indices of humoral immunity (e.g., AMP and PO activity). For example, in Chapter 2, despite high AMP and PO activity at the beginning of the 5th instar in *M. sexta*, survival from infection is low and bacterial load in the hemolymph is high. Although the two measures of functional immunity and the 2 indices of humoral immunity are consistent between each other (e.g. high survival coincides with low bacterial load), they are not consistent among each other (e.g. high AMP activity does not coincide with high survival). These results are puzzling because of the assumption that active immune responses will clear an infection. It is possible that the ability to survive an infection depends on a vast number of immune responses working together, which does not translate into all immune responses with peak activity upon an immune challenge. Surprisingly, disparity between functional immunity measures and specific indices of humoral immunity are not always disparate as observed in Chapter 2. In Chapter 3, inhibiting MMP mRNA expression resulted in an increase in PO activity and an increase in survival. In Chapter 4, inhibiting JH resulted in a decrease in AMP activity and a decrease in survival and an increase in bacterial load. Unlike Chapter 2, results from Chapters 3 and 4 show consistency between functional immunity measures and specific indices of humoral immunity. My results emphasize the importance of using several measures of immunity when assessing how immunity varies with any factor being studied. Because the immune response to infection is so complex, measuring all aspects of the immune response is difficult, but assessing a few different types of immunity is

better than a single measure. By performing several types of immune assays, interpretation of results can be greatly improved.

For scientists, these studies emphasize the need to control for age in experimental design. *M. sexta* are one of the most common model species in insect physiology studies because of the large tissue amounts that can be isolated. Solely comparing studies using the same age of *M. sexta* may not be enough to provide an accurate portrayal of *M. sexta*'s immunity as a whole. It is also not fully understood why there is a change in immune responses during development from an ecological perspective. It is possible that freshly molted insects need high humoral immunity because their cuticle is not yet sclerotized fully and may be vulnerable to wounding. On the other hand, it is possible that the change in habitat from a caterpillar feeding on a leaf to a pupa burrowing underground may warrant a need for greater resistance to infection, such as seen in the prepupal caterpillars. It would also be interesting to see if this same pattern of change in immune responses with development is consistent across other larval instars or into pupation and adulthood.

Furthermore, I determined that the function of MMP in *M. sexta* is perhaps more complex and enigmatic than first thought. Though MMP gene expression is upregulated during infection in immune tissues, I found that MMP negatively affected PO activity. This was surprising because MMP in insects has been shown to be important in surviving infection and wound healing. Overall, my study did not determine the clearly elusive role of MMP. Because MMP gene and protein expression is found in the immune tissues, perhaps MMP is involved in other aspects of immunity my study did not measure, such as cell-migration, wound healing, and fat body remodeling. This may still be important to inform the roles of MMPs in humans because

MMP is an evolutionarily conserved proteinase and many insect immune responses are evolutionarily conserved as well.

In addition, understanding how JH regulates immunity throughout development will allow for better understanding of how hormones contribute to immune function. It highlights the concept that physiological systems do not function in isolation, but rather, work together for organismal function. For society, determining the factors causing development-dependent changes in immunity could help in the production of new, more efficient pest control methods and reduce impact on the planet. For example, JH is currently a target of pesticides, such as Pestinal. With this research, scientists can understand the full impact of a pesticide on the function of an organism. In another example, identifying when an insect is at its most vulnerable state due to the effect of hormones on immune function could allow for pesticides that exploit the immune system. The pesticide *Bacillus thuringiensis* that attacks the immune system of insects could be applied at specific times to make give the effect more impact.

My dissertation research determined a link between development and immunity and found that JH is responsible for the high AMP activity of freshly molted 5^{th} instar *M. sexta*. It would be interesting to investigate if the decline in immunity within a single instar is observed in other life stages of insects, such as across other larval instars, pupae, and adults. It would provide additional insight to determine the molecular mechanisms behind how JH affects immunity, such as examining changes in gene transcription of immune genes. Another important future direction would be to explore the evolutionary benefits of changes in immunity with development, such as changes in energy reallocation. Overall, expanding the span of development in which immunity is studied, determining the mechanisms behind what underlies the development-immunity link, and determining why this link exists would enlighten the field of insect immunity immensely.