MICROPLITIS DEMOLITOR BRACOVIRUS BLOCKS THE ENCAPSULATION RESPONSE OF PSEUDOPLUSIA INCLUDENS

by

JOSEPH GREY BALLENGER

Under the Direction of MICHAEL STRAND

ABSTRACT

The insect immune system consists of two broadly overlapping subsystems. The humoral system consists of germline encoded factors present in the hemolymph, while the cellular immune system consists of several different classes of hemocytes. Insects use hemocytes to fight off foreign invaders by encapsulating them or killing them with reactive oxygen species produced by factors present in the hemolymph. Each response is very tightly controlled in a variety of ways. Few of these regulatory systems are well understood, and crosstalk is a common cofounding factor.

Microplitis demolitor Bracovirus (MdBV) encodes factors which target these regulatory systems to disable the humoral and cellular immune systems. However, the in vivo functions of these factors are ill defined. Thus, the objectives of the project under consideration are to:

1.) Determine if factors encoded by MdBV disable cellular immunity by acting at the branch points of the cellular and humoral immune systems.

2.) Determine if the decline in expression of surface mucins encoded by MdBV correlates with a recovery of the immune system.

INDEX WORDS: *Microplitis demolitor*, *Pseudoplusia includens,* Bracovirus, encapsulation, cellular immunity, humoral immunity, parasitoid wasps, Braconidae, Plasmatocyte Spreading Peptide, PSP,

MICROPLITIS DEMOLITOR BRACOVIRUS BLOCKS THE ENCAPSULATION RESPONSE OF PSEUDOPLUSIA INCLUDENS

by

Joseph Grey Ballenger

Bachelor of Science, Iowa State University, 2010

A Thesis Submitted to the Graduate Faculty of The University of Georgia in Partial Fulfillment of the Requirements for the Degree

MASTER OF SCIENCE

ATHENS, GEORGIA

2012

MICROPLITIS DEMOLITOR BRACOVIRUS BLOCKS THE ENCAPSULATION RESPONSE OF PSEUDOPLUSIA INCLUDENS

by

JOSEPH GREY BALLENGER

Major Professor: MICHAEL STRAND

Committee: KEVIN CLARK
MARK BROWN

Electronic Version Approved:

Maureen Grasso
Dean of the Graduate School
The University of Georgia
December 2012

DEDICATION

I feel I would be remiss if I did not dedicate this thesis to those who had formative influences on me as a scientist. Jane Schmidt of my 5th grade class and the staff of Camp Hantesa assisted me in cultivating my initial interest in entomology, which my parents initially tolerated but later fully embraced. Theodosius Dobzhansky was instrumental in my interest in evolutionary biology, although I credit the instruction of Lyric Bartholomay and Byrony Bonning for making me fall in love with molecular biology. Gwen Pearson and Alex Wild were influential in my interest in popular science writing; Anastasia Bodnar and Karl Haro Von Mogel gave me a platform to share my love of insects with the world. Sarah Thomas and Miles Ingwers were especially loyal friends who assisted me in my time of need.

This thesis is dedicated to my daughter, Aquila Ballenger-Hudson, to whom I have already passed on my love of insects, and her sister Madilae to whom Aquila has already passed on her passion for entomology.

ACKNOWLEDGEMENTS

First and foremost, I would like to express my appreciation to Dr. Michael Strand for giving me the chance to pursue a Master's degree in his laboratory. I feel he took a chance on me when few people would, and it is difficult to express in words the gratitude I feel. The advice he gave me helped me traverse the struggles of graduate school in general, and this project in particular.

I would also like to thank my committee members for their guidance on this project. Dr. Mark Brown's assistance with western blots proved invaluable, as did the assistance of Andrew Nuss and Animesh Dhara from his lab. Dr. Kevin Clark was eager to provide assistance whenever I needed it. I also owe much thanks to Jena Johnson and our laboratory helpers, namely Sarah Thomas, Ian Milton and Spencer Milton for their assistance in maintaining the wasp and caterpillar stocks which formed the backbone of this project.

Throughout this project, I befriended a great many people in my lab who were instrumental in keeping my sanity. Rachel Bottjen, Sarah Johnson, Jayce Brandt, Kerri Coon, Nikki Wren, Miles Ingwers, Kevin Vogel, Gaelen Burke, and many other graduate students formed a wonderful social support system that I feel that I couldn't do without.

TABLE OF CONTENTS

Page

LIST OF FIGURES

Page

Chapter One: Literature Review

1.1) Insect hemocytes adhere to foreign targets during immune reactions and are important targets for disruption by insect parasitoids.

Insect immune responses can be divided into two groups: humoral and cellular responses. Humoral responses are encoded by germline factors and are generally located in insect hemolymph. In insects, hemocytes function as the cellular arm of the insect immune system, acting to phagocytose small invaders such as bacteria and encapsulate larger pathogens such as parasitoids (Strand 2008). During the encapsulation response of the moth *Pseudoplusia includens* foreign bodies, such as parasitoid larvae, are first recognized by one hemocyte type called the granulocytes. This is followed by the binding of a second type of hemocyte, the plasmatocytes, which form a capsule of overlapping hemocytes. *Manduca sexta* provides a contrast to this where capsules contain an unorganized mix of plasmatocytes and granulocytes (Strand 2008). Granulocytes and plasmatocytes are coordinated in their response, as materials from granulocytes cause plasmatocytes to activate and spread. (Lavine and Strand 2002). Oenocytoids are involved in the process of melanization, bursting in response to immune challenge to release phenoloxidase (Strand 2008).

Adhesion of hemocytes to foreign targets involves proteins, which are either secreted or expressed on the cell surface. These proteins can recognize a wide range of pathogens by binding specific molecules present on the surface of pathogens or patterns, which signal deceased cells. Eater is one such receptor expressed on the surface of hemocytes, where it

binds to gram negative and some gram positive bacteria, but not fungi (Chung and Kocks 2011). The phagocytosis of apoptotic cells and gram positive bacteria is accomplished by Croquemort in the hemocytes of *Drosophila melanogaster*, and by its homolog CD36 in human macrophages (Franc et al. 1999; Stuart et al. 2005). Down Syndrome Cell Adhesion Molecule is a protein produced in soluble and cell-bound forms which is differentially spliced in response to pathogen invasion, and can bind to several different classes of foreign targets (Watson et al. 2005; Dong et al. 2006).

Integrins are another class of receptors important to cellular adhesion, as they mediate several different adhesion responses. The activity of these receptors is controlled by a complicated series of kinases and phosphatases housed in focal adhesions inside the cell. Phosphorylation on the cytoplasmic side of beta integrin, followed by the dimerization of alpha and beta subunits and then the formation of focal adhesions are the final steps in integrin activation (Wegener and Campbell 2008). The main players in focal adhesion formation and turnover are Focal Adhesion Kinase (FAK), Src, Paxillin and Talin. FAK autophosphorylates in response to integrin binding then phosphorylates Src with which it forms a complex (Cary et al. 1996). The purpose of FAK and Src in focal adhesions is to act as a scaffold, along with Talin and similar proteins, to recruit other kinases and actin binding proteins (Peng and Guan 2011). Focal adhesions are very dynamic and are constantly recycling, a process apparently regulated in part by the phosphorylation of Paxillin and the recruitment of FAK (Zaidel-Bar et al. 2007). The final step in integrin activation is binding of Talin to the integrin beta subunit (Tadokoro et al. 2003).

While focal adhesion dynamics are very well fleshed out in mammals, some significant questions remain about the differences between mammals and insects. Mammalian FAK

null mutants are lethal, while *Drosophila* FAK null mutants grow and reproduce normally and are indistinguishable from the wild type (Ilic et al. 1995; Grabbe et al. 2004). *Drosophila* FAK retains the phosphorylation site and appears in focal adhesion complexes along with other well-known components such as Talin (Grabbe et al. 2004). The hemocytes of *Ceratitis capita* display lower levels of phagocytosis after uptaking mamallian anti-FAK antibodies (Metheniti et al. 2001). It is possible the function of FAK is slightly different between insects and mammals, with FAK being more important in regulating phagocytosis than regulating muscle attachment in insects.

1.2) The ENF peptides function as cytokines in lepidopteran immunity.

Peptide cytokines mediate communication during insect immune reactions and often serve as an interface between humoral and cellular immune responses in insects. Stress Responsive Peptide of *Spodoptera litura* activates an adhesive state in immune cells and decreases feeding (Yamaguchi et al. 2012). Another is the ligand Spatzle which activates the Toll pathway in insects. (Jiang 2008; Yamaguchi et al. 2012). Hemocyte Chemotactic Peptide is released from blood cells and epidermal cells and assists in targeting hemocytes to areas of wounding or infection in *Pseudaletia separata* (Nakatogawa et al. 2009). A fourth family of cytokines, the ENF peptides, function to promote cell adhesion in Lepidoptera, Coleoptera and Diptera (Clark et al. 1997; Matsumoto et al. 2012; Zhong et al. 2012).

In the *P. includens* precursor to Plasmatocyte Spreading Peptide (PSP), the 23 residue C-terminus is cleaved by an unknown serine protease to produce the active ENF peptide upon exposure to fungal or bacterial cell wall components (Wang et al. 1999; Ishii et al.

2008). After activation, PSP binds to a receptor on the surface of hemocytes. A central part of the signaling pathway of a homolog of *P. includens* PSP, *Pseudaletia separata* GBP, is an adaptor protein called P77 which becomes phosphorylated soon after GBP stimulation (Oda et al. 2010). Many *in vitro* tests of cell spreading in response to PSP stimulation are performed on polystyrene plates (Strand et al. 2000; Clark et al. 2005; Nakatogawa et al. 2009; Yamaguchi et al. 2012). This cell adhesion appears to be heavily dependent on integrins, as incubating the cells in a soluble version of the integrin recognition sequence will almost completely eliminate cell binding (Pech and Strand 1995; Lavine and Strand 2003). Polystyrene is a largely hydrophobic polymer, providing a stark contrast to the highly charged RGD residues of the integrin binding sequence. This implies integrins may act as intermediaries between some pattern recognition receptors and the cells. Thus integrins are the likely endpoints for the PSP pathway, but it's possible that other systems play a role during PSP signaling.

P. includens hemocyte sensitivity to PSP increases throughout development (Clark et al. 2005). The change in sensitivity appears to be related to changes in the concentration of juvenile hormone and ecdysone during development within the instar, as methoprene decreases sensitivity to PSP and 20-hydroxyecdysone increases plasmatocyte sensitivity to PSP (Clark et al. 2005). Transcription of ENF peptides also changes throughout development in *Bombyx mori* (Kamimura et al. 2001). GPB concentrations also change throughout development in *P. separata* (Ohnishi et al. 1995). In P. *includens*, PSP is transcribed in the nervous tissue, fat body and in both plasmatocytes and granulocytes (Clark et al. 1998). These peptides are important in guiding cellular responses, such as phagocytosis, but also play a potential role in inducing the expression of antimicrobial

peptides (Ishii et al. 2010). *B. mori* larvae infected with *Staphylococcus arueus* show increased survival when receiving injections of the *B. mori* ENF homolog Paralytic Peptide (PP), a benefit lost when injected with antibodies raised to BmPP (Ishii et al. 2008). The three classes of hemocytes have different responses to PSP. In *P. includens*, PSP causes plasmatocytes to spread and represses granulocyte spreading (Strand and Clark 1999). Oenocytoids burst in response to ENF stimulation and release an ENF binding protein in *P. separata*. A homolog of this protein is upregulated during infection with a baculovirus in *B. mori* (Matsumoto et al. 2003; Hu et al. 2006).

1.3) Many humoral immune responses are regulated by serine protease cascades.

Serine protease cascades are involved with the activation of the Toll, phenoloxidase and ENF activation pathways (Jiang 2008). These proteases consist of an N-terminal clip domain held together by three cysteine bonds linked to a catalytic C-terminal protease domain (Jiang and Kanost 2000). When activated by another protease in the cascade, the enzymes are cleaved between the clip domain and the serine protease domain to yield the active protease (Jiang and Kanost 2000). These proteases are regulated by serine protease inhibitors (Serpins), which are cleaved by the target protease before forming an irreversible SDS stable complex with the enzyme (Jiang and Kanost 2000; Jiang 2008). Screening of *M. sexta* mRNA libraries using conserved domains of serine proteases have yielded insights into the number of serine proteases, which are a part of lepidopteran plasma, but the full characterization of many pathways is still incomplete (Jiang et al. 2005; Jiang 2008; Strand 2008).

Of the various extracellular serine protease networks in insect hemolymph, the Toll pathway and phenoloxidase (PO) cascade are the best defined (Jiang 2008). *M. sexta* HP14 acts as a pattern recognition receptor and binds to cell wall components from gram positive bacteria and fungi. After binding, HP14 activates itself through proteolytic cleavage, thereby initiating the proteolytic cascade leading to PO activation by cleaving HP21. HP14 requires the pattern recognition receptor BGRP to activate before cleaving HP21 (Ji et al. 2004; Wang and Jiang 2007; Wang and Jiang 2010). In vitro, activated HP14 cleaves recombinant HP21 to yield active HP21 (Wang and Jiang 2007). When incubated in the presence of HP14 activated by curdlan, BGRP2 and HP21, HP21 cleaves Phenoloxidase Activating Proteases (PAPs) 2 and 3 (Gorman et al. 2007; Wang and Jiang 2007). Phenoloxidase itself is activated by PAPs 1, 2, and 3 in *M. sexta* and by PPAE in *B. mori* (Jiang et al. 1998; Satoh et al. 1999; Jiang et al. 2003). PAPs activate prophenoloxidase with the assistance of serine protease homologs, which lack proteolytic activity (Jiang et al. 2003; Yu et al. 2003). The serine protease homologs bind PAP-1 along with PO and immunolectin 2 (Yu et al. 2003). The melanization reaction appears to depend upon the formation of a high molecular weight complex consisting of PO and other components (K. Clark, unpublished data). Purified phenoloxidases from cuticle or insect hemolymph display limited activity towards tyrosine, while insect hemolymph readily utilizes tyrosine as the substrate for the melanization reaction which raises questions about phenoloxidase's role in the immune system (Aso et al. 1985; Hall et al. 1995).

In *M. sexta*, the Toll and PO pathways are both activated by gram positive bacterial and fungal cell wall components. Activation of the Toll pathway results in the synthesis of

antimicrobial peptides, while activation of the PO pathway results in the activation of the enzyme PO. Phenoloxidase in the hemolymph will oxidize tyrosine or dopamine to form o-diphenols which are further processed to form melanin (Zhao et al. 2007; Jiang 2008). Reactive intermediates generated by PO kill bacteria, and the resulting melanin polymer physically isolates pathogens (Zhao et al. 2007; Cerenius et al. 2008). The Toll pathway is only partially characterized in lepidopterans, but HP-6 has been demonstrated to cleave HP-8 and Pro-PAP1. Active recombinant HP-6 and HP-8 are able to induce antimicrobial genes transcribed by the the Toll pathway when injected into *M. sexta* (An et al. 2009). In vitro, a recombinant HP-8 cleaves recombinant prospatzle which results in induced expression of antimicrobial genes in *M. sexta* (An et al. 2010). The C-terminal domain of spatzle then binds to the toll receptor in *M. sexta* to induce the transcription of antimicrobial peptides in *M. sexta* fat body and hemocytes, an action which can be blocked by injecting antibodies specific to the ligand binding domain of the Toll receptor (Zhong et al. 2012).

1.4) Parasitoid wasps subvert host physiology for their own gain.

Parasitoid wasps utilize a variety of methods to subvert host physiology for their own gain. The factors that manipulate host physiology can be derived from the mother during oviposition or from the larva itself in the form of salivary secretions or teratocytes derived from the serosal cells of the wasp egg released into the host after the egg hatches (Strand and Wong 1991; Doury et al. 1997; Deyrup et al. 2006). The most unique methods of immune disruption are found in the superfamily Ichneumonoidea, where many closely related subfamilies form symbiotic relationships with viruses, which serve

to disrupt the immune and metabolic systems of their hosts (Strand 2010). These viruses contain double-stranded DNA genomes separated into multiple segments and are thus known as 'POLYdisperse DNA VIRUSES', or polydnaviruses (PDVs) (Strand 2010). PDVs are associated with the families Ichneumonidae and Braconidae. Within the Braconidae, PDVs are associated with members of the microgastroid complex which is composed of seven different subfamilies. Within the Ichneumonidae they are associated with the subfamilies, Banchinae and Campopleginae. Thus PDVs are divided into two genera, which are most likely polyphyletic. Bracoviruses are symbiotic with the wasp family Braconidae, and Ichnoviruses are symbiotic with Ichneumonid wasps (Strand 2010). The two genera replicate in different ways. Ichnoviruses obtain an outer envelope when they bud through the calyx cells (Huang et al. 2008). Bracoviruses, on the other hand, lyse the calyx cells during replication (Wyler and Lanzrein 2003). In addition to different replication processes and morphology, the two viruses appear to have different evolutionary origins. The viral machinery upregulated during Bracovirus replication is related to nudiviruses (Bezier et al. 2009). Ichnovirus origins still remain poorly understood (Burke and Strand 2012).

PDVs consist of a proviral form integrated into the genome of their hymenopteran hosts, where they replicate in the calyx cells and accumulate in the lumen of the ovary (Wyler and Lanzrein 2003; Huang et al. 2008; Strand 2010). Upon injection of the egg into the wasp's host, the virus integrates into the genome of the organism on which the wasp larva is feeding (Beck et al. 2011). PDVs only replicate in the hymenopteran host, a process which appears to be under strict developmental control (Burke and Strand 2012). PDVs

act as gene vectors in Lepidopteran hosts by producing factors which disrupt physiological systems to benefit the parasitoid wasp's development.

This thesis focuses on the braconid *Microplitis demolitor*, which carries *M. demolitor* Bracovirus and parasitizes larval stage *P. includens.* MdBV virions preferentially infect tissues involved in metabolism and immunity: the fat body, hemocytes, nervous system and gut (Strand et al. 1992; Bitra et al. 2011). The end result of MdBV infection is developmental stasis and immunosupression (Strand 2010). The three main families of proteins most strongly implicated in immunosupression of responses to parasitoids are the Protein Tyrosine Phosphatases (PTPs), the Epidermal Growth Factor-like (EGF) proteins and the two Mucin-like glycoprotein family members Glc 1.8 and 3.2 (Beck and Strand 2005; Beck and Strand 2007; Pruijssers and Strand 2007; Lu et al. 2010). The exact role of these products in subverting immune system physiology will be discussed in later chapters.

Works Cited

An, C. J., et al. (2009). "Functions of Manduca sexta Hemolymph Proteinases HP6 and HP8 in Two Innate Immune Pathways." Journal of Biological Chemistry 284(29): 19716-19726.

An, C. J., et al. (2010). "Proteolytic activation and function of the cytokine Spatzle in the innate immune response of a lepidopteran insect, Manduca sexta." Febs Journal 277(1): 148-162.

Aso, Y., et al. (1985). "Characterization of haemolymph protyrosinase and a cuticular activator from Manduca sexta (L.)." Insect Biochemistry 15(1): 9-17.

Beck, M. and Strand M. R. (2003). "RNA interference silences Microplitis demolitor bracovirus genes and implicates glc1.8 in disruption of adhesion in infected host cells." Virology 314(2): 521-535.

Beck, M. and Strand M. R. (2005). "Glc1.8 from Microplitis demolitor bracovirus induces a loss of adhesion and phagocytosis in insect High Five and S2 cells." Journal of Virology 79(3): 1861-1870.

Beck, M. H. and Strand M. R. (2007). "A novel polydnavirus protein inhibits the insect prophenoloxidase activation pathway." Proceedings of the National Academy of Sciences 104(49): 19267-19272.

Beck, M. H., *et al.* (2011). "The Encapsidated Genome of Microplitis demolitor Bracovirus Integrates into the Host Pseudoplusia includens." Journal of Virology **85**(22): 11685-11696.

Bezier, A., *et al.* (2009). "Polydnaviruses of Braconid Wasps Derive from an Ancestral Nudivirus." Science **323**(5916): 926-930.

Bitra, K., *et al.* (2011). "Transcriptomic profiling of Microplitis demolitor bracovirus reveals host, tissue and stage-specific patterns of activity." Journal of General Virology **92**: 2060-2071.

Burke, G. R. and Strand M. R. (2012). "Polydnaviruses of Parasitic Wasps: Domestication of Viruses To Act as Gene Delivery Vectors." Insects **3**(1): 91-119.

Cary, L. A., et al. (1996). "Stimulation of cell migration by overexpression of focal adhesion kinase and its association with Src and Fyn." Journal of Cell Science 109: 1787-1794.

Cerenius, L., et al. (2008). "The proPO-system: pros and cons for its role in invertebrate immunity." Trends in Immunology 29(6): 263-271.

Chung, Y.-S. A. and Kocks C. (2011). "Recognition of Pathogenic Microbes by the Drosophila Phagocytic Pattern Recognition Receptor Eater." Journal of Biological Chemistry 286(30): 26524-26532.

Clark, K. D., et al. (2005). "Plasmatocyte sensitivity to plasmatocyte spreading peptide (PSP) fluctuates with the larval molting cycle." Journal of Insect Physiology 51(5): 587-596.

Clark, K. D., et al. (1997). "Isolation and identification of a plasmatocyte-spreading peptide from the hemolymph of the lepidopteran insect Pseudoplusia includens." Journal of Biological Chemistry 272(37): 23440-23447.

Clark, K. D., et al. (1998). "Plasmatocyte spreading peptide is encoded by an mRNA differentially expressed in tissues of the moth Pseudoplusia includens." Biochemical and Biophysical Research Communications 250(2): 479-485.

Deyrup, L. D., *et al.* (2006). "Venom from the Ectoparasitic wasp Melittobia digitata (Hymenoptera : Eulophidae) induces paralysis and developmental delay in natural and factitious hosts." Annals of the Entomological Society of America **99**(6): 1199-1205.

Dong, Y. M., et al. (2006). "AgDscam, a hypervariable immunoglobulin domain-containing receptor of the Anopheles gambiae innate immune system." PLoS Biology 4(7): 1137-1146.

Doury, G., *et al.* (1997). "Physiological and Biochemical Analysis of Factors in the Female Venom Gland and Larval Salivary Secretions of the Ectoparasitoid Wasp Eupelmus orientalis." Journal of Insect Physiology **43**(1): 69-81.

Franc, N. C., et al. (1999). "Requirement for croquemort in phagocytosis of apoptotic cells in Drosophila." Science 284(5422): 1991-1994.

Francica, J. R., et al. (2009). "Requirements for cell rounding and surface protein down-regulation by Ebola virus glycoprotein." Virology 383(2): 237-247.

Francica, J. R., et al. (2010). "Steric Shielding of Surface Epitopes and Impaired Immune Recognition Induced by the Ebola Virus Glycoprotein." Plos Pathogens 6(9).

Gettins, P. G. W. (2002). "Serpin structure, mechanism, and function." Chemical Reviews 102(12): 4751-4803.

Gorman, M. J., et al. (2007). "Manduca sexta hemolymph proteinase 21 activates prophenoloxidase-activating proteinase 3 in an insect innate immune response proteinase cascade." Journal of Biological Chemistry 282(16): 11742-11749.

Grabbe, C., et al. (2004). "Focal adhesion kinase is not required for integrin function or viability in Drosophila." Development 131(23): 5795-5805.

Hall, M., et al. (1995). "Proenzyme of Manduca sexta phenol oxidase: purification, activation, substrate specificity of the active enzyme, and molecular cloning." Proceedings of the National Academy of Sciences 92(17): 7764-7768.

Hu, Z.-G., et al. (2006). "Cloning and characterization of Bombyx mori PP-BP, a gene induced by viral infection." Acta Genetica Sinica 33(11): 975-983.

Ilic, D., et al. (1995). "Reduced cell motility and enhanced focal adhesion contact formation in cells from FAK-deficient mice." Nature (London) 377(6549): 539-544.

Ishii, K., et al. (2010). "Insect Cytokine Paralytic Peptide (PP) Induces Cellular and Humoral Immune Responses in the Silkworm Bombyx mori." Journal of Biological Chemistry 285(37): 28635-28642.

Ishii, K., et al. (2008). "Activation of the silkworm cytokine by bacterial and fungal cell wall components via a reactive oxygen species-triggered mechanism." Journal of Biological Chemistry 283(4): 2185-2191.

Ji, C. Y., et al. (2004). "A pattern recognition serine proteinase triggers the prophenoloxidase activation cascade in the tobacco hornworm, Manduca sexta." Journal of Biological Chemistry 279(33): 34101-34106.

Jiang, H. B. (2008). "The biochemical basis of antimicrobial responses in Manduca sexta." Insect Science 15(1): 53-66.

Jiang, H. B. and Kanost M. R. (2000). "The clip-domain family of serine proteinases in arthropods." Insect Biochemistry and Molecular Biology 30(2): 95-105.

Jiang, H. B., et al. (2005). "Molecular identification of a bevy of serine proteinases in Manduca sexta hemolymph." Insect Biochemistry and Molecular Biology 35(8): 931-943.

Jiang, H. B., et al. (1998). "Pro-phenol oxidase activating proteinase from an insect, Manduca sexta: A bacteria-inducible protein similar to Drosophila easter." Proceedings of the National Academy of Sciences of the United States of America 95(21): 12220-12225.

Jiang, H. B., et al. (2003). "Prophenoloxidase-activating proteinase-3 (PAP-3) from Manduca sexta hemolymph: a clip-domain serine proteinase regulated by serpin-1J and serine proteinase homologs." Insect Biochemistry and Molecular Biology 33(10): 1049-1060.

Kamimura, M., et al. (2001). "Molecular cloning of silkworm paralytic peptide and its developmental regulation." Biochemical and Biophysical Research Communications 286(1): 67-73.

Kato, K., et al. (2012). "Membrane-Tethered MUC1 Mucin Is Phosphorylated by Epidermal Growth Factor Receptor in Airway Epithelial Cells and Associates with TLR5 To Inhibit Recruitment of MyD88." Journal of Immunology 188(4): 2014-2022.

Kurucz, E., et al. (2003). "Hemese, a hemocyte-specific transmembrane protein, affects the cellular immune response in Drosophila." Proceedings of the National Academy of Sciences of the United States of America 100(5): 2622-2627.

Lavine, M. D. and Strand M. R. (2002). "Insect hemocytes and their role in immunity." Insect Biochemistry and Molecular Biology 32(10): 1295-1309.

Lavine, M. D. and Strand M. R. (2003). "Haemocytes from Pseudoplusia includens express multiple alpha and beta integrin subunits." Insect Molecular Biology 12(5): 441-452.

Lu, Z., et al. (2010). "Egf1.5 is a second phenoloxidase cascade inhibitor encoded by Microplitis demolitor bracovirus." Insect Biochemistry and Molecular Biology 40(7): 497-505.

Lu, Z., et al. (2008). "The Viral Protein Egf1.0 Is a Dual Activity Inhibitor of Prophenoloxidase-activating Proteinases 1 and 3 from Manduca sexta." Journal of Biological Chemistry 283(31): 21325-21333.

Marmaras, V. J. and Lampropoulou M. (2009). "Regulators and signalling in insect haemocyte immunity." Cellular Signalling 21(2): 186-195.

Matsumoto, Y., et al. (2003). "Insect cytokine growth-blocking peptide triggers a termination system of cellular immunity by inducing its binding protein." Journal of Biological Chemistry 278(40): 38579-38585.

Matsumoto, H., et. al. (2012). “Characteristics of a cytokine family spanning five orders of insects.” Insect Biochemistry and Molecular Biology. 42(6): 446-454

Metheniti, A., et al. (2001). "Involvement of FAK/Src complex in the processes of Escherichia coli phagocytosis by insect hemocytes." Febs Letters 496(1): 55-59.

Nakatogawa, S., et al. (2009). "A Novel Peptide Mediates Aggregation and Migration of Hemocytes from an Insect." Current Biology 19(9): 779-785.

Ohnishi, A., et al. (1995). "Growth-blocking peptide titer during larval development of parasitized and cold-stressed armyworm." Insect Biochemistry and Molecular Biology 25(10): 1121-1127.

Pech, L. L. and Strand M. R. (1995). "ENCAPSULATION OF FOREIGN TARGETS BY HEMOCYTES OF THE MOTH PSEUDOPLUSIA-INCLUDENS (LEPIDOPTERA, NOCTUIDAE) INVOLVES AN RGD-DEPENDENT CELL-ADHESION MECHANISM." Journal of Insect Physiology 41(6): 481-488.

Peng, X. and Guan J.-L. (2011). "Focal Adhesion Kinase: From In Vitro Studies to Functional Analyses In Vivo." Current Protein & Peptide Science 12(1): 52-67.

Persson, C., et al. (1997). "The PTPase YopH inhibits uptake of Yersinia, tyrosine phosphorylation of p130Cas and FAK, and the associated accumulation of these proteins in peripheral focal adhesions." EMBO J 16(9): 2307-2318.

Pruijssers, A. J. and Strand M. R. (2007). "PTP-H2 and PTP-H3 from Microplitis demolitor bracovirus localize to focal adhesions and are antiphagocytic in insect immune cells." Journal of Virology 81(3): 1209-1219.

Satoh, D., et al. (1999). "Prophenoloxidase-activating enzyme of the silkworm, Bombyx mori - Purification, characterization, and cDNA cloning." Journal of Biological Chemistry 274(11): 7441-7453.

Strand MR. 2010. Polydnaviruses. In: *Insect Virology*, Ed: S Asgari, Johnson KN, pp. 171-197. Caister academic press: Wymondham, NR.

Strand, M. R. (2008). "The insect cellular immune response." Insect Science 15(1): 1-14.

Strand, M. R., et al. (2006). "Microplitis demolitor bracovirus inhibits phagocytosis by hemocytes from Pseudoplusia includens." Archives of Insect Biochemistry and Physiology 61(3): 134-145.

Strand, M. R., et al. (1999). "Plasmatocyte spreading peptide does not induce Microplitis demolitor polydnavirus-infected plasmatocytes to spread on foreign surfaces." Archives of Insect Biochemistry and Physiology 40(1): 41-52.

Strand, M. R. and Clark K. D. (1999). "Plasmatocyte spreading peptide induces spreading of plasmatocytes but represses spreading of granulocytes." Archives of Insect Biochemistry and Physiology **42**(3): 213-223.

Strand, M. R., et al. (2000). "Plasmatocyte spreading peptide (PSP1) and growth blocking peptide (GBP) are multifunctional homologs." Journal of Insect Physiology 46(5): 817-824.

Stuart, L. M., et al. (2005). "Response to Staphylococcus aureus requires CD36-mediated phagocytosis triggered by the COOH-terminal cytoplasmic domain." Journal of Cell Biology 170(3): 477-485.

Strand, M. R., *et al.* (1992). "PERSISTENCE AND EXPRESSION OF MICROPLITIS-DEMOLITOR POLYDNAVIRUS IN PSEUDOPLUSIA-INCLUDENS." Journal of General Virology **73**: 1627-1635.

Strand, M. R. and Wong E. A. (1991). "The growth and role of Microplitis demolitor teratocytes in parasitism of Pseudoplusia includens." Journal of Insect Physiology **37**(7): 503-515.

Tadokoro, S., et al. (2003). "Talin binding to integrin beta tails: A final common step in integrin activation." Science 302(5642): 103-106.

Wang, Y. and Jiang H. (2007). "Reconstitution of a branch of the Manduca sexta prophenoloxidase activation cascade in vitro: Snake-like hemolymph proteinase 21 (HP21) cleaved by HP14 activates prophenol oxidase- activating proteinase-2 precursor." Insect Biochemistry and Molecular Biology 37: 1015-1025.

Wang, Y. and Jiang H. B. (2010). "Binding properties of the regulatory domains in Manduca sexta hemolymph proteinase-14, an initiation enzyme of the prophenoloxidase activation system." Developmental and Comparative Immunology 34(3): 316-322.

Wang, Y., et al. (1999). "Biological activity of Manduca sexta paralytic and plasmatocyte spreading peptide and primary structure of its hemolymph precursor." Insect Biochemistry and Molecular Biology 29(12): 1075-1086.

Watson, F. L., et al. (2005). "Extensive diversity of Ig-superfamily proteins in the immune system of insects." Science 309(5742): 1874-1878.

Wegener, K. L. and Campbell I. D. (2008). "Transmembrane and cytoplasmic domains in integrin activation and protein-protein interactions (Review)." Molecular Membrane Biology 25(5): 376-387.

Wesseling, J., et al. (1995). "EPISIALIN (MUC1) OVEREXPRESSION INHIBITS INTEGRIN-MEDIATED CELL-ADHESION TO EXTRACELLULAR-MATRIX COMPONENTS." Journal of Cell Biology 129(1): 255-265.

Wyler, T. and Lanzrein B. (2003). "Ovary development and polydnavirus morphogenesis in the parasitic wasp Chelonus inanitus. II. Ultrastructural analysis of calyx cell development, virion formation and release." Journal of General Virology **84**: 1151-1163.

Yamaguchi, K., et al. (2012). "Enhanced expression of stress-responsive cytokine-like gene retards insect larval growth." Insect Biochemistry and Molecular Biology 42(3): 183-192.

Yu, X. Q., et al. (2003). "Nonproteolytic serine proteinase homologs are involved in prophenoloxidase activation in the tobacco hornworm, Manduca sexta." Insect Biochemistry and Molecular Biology 33(2): 197-208.

Zaidel-Bar, R., et al. (2007). "A paxillin tyrosine phosphorylation switch regulates the assembly and form of cell-matrix adhesions." Journal of Cell Science 120(1): 137-148.

Zhao, P. C., et al. (2007). "Broad-spectrum antimicrobial activity of the reactive compounds generated in vitro by Manduca sexta phenoloxidase." Insect Biochemistry and Molecular Biology 37(9): 952-959.

Zhong, X., et al. (2012). "A Toll-Spätzle pathway in the tobacco hornworm, Manduca sexta." Insect Biochemistry and Molecular Biology 42(7): 514-524

Chapter Two
The Encapsulation Response of *P. includens* Does Not Recover During Parasitism or MdBV Infection

2.1: Introduction

2.1.1 Inhibition of cellular adhesion is important in disabling the encapsulation response

Cell-cell adhesion is important for immune reactions in several ways. To phagocytose small invaders hemocytes must stick to the cells; to encapsulate larger invaders hemocytes must adhere to one another. During parasitization by *Microplitis demolitor*, the encapsulation response is inhibited by infection with MdBV. This lack of response is presumably due to the virulence factors that prevent adhesion, phagocytosis and other immune responses (Strand et al. 1999; Strand et al. 2006). The two major virulence factors, which disrupt cellular immunity are mucin-like proteins and protein tyrosine phosphatases (PTPs) (Beck and Strand 2003; Pruijssers and Strand 2007). PTP H2 is an active phosphatase, which is thought to disrupt hemocyte mediated immunity by stimulating apoptosis of granular cells. PTP H3 has similar effects in that this protein decreases phagocytic ability of cells. Both these proteins very likely interfere with tyrosine phosphorylation dependent signaling pathways, but the manner in which they do so is not yet characterized. Furthermore, the effects of these proteins on the cellular immune system of caterpillars is poorly studied *in vivo*.

Of the responses to parasitism disabled by MdBV, the most important to the development of the parasitoid may be the disruption of cellular immunity. This is accomplished by preventing cells from binding to foreign surfaces. MdBV encodes two mucin-like

glycoproteins with very similar sequences, Glc 1.8 and Glc 3.2 (Webb et al. 2006). Glc 1.8 is expressed at the cell surface of hemocytes and disrupts adhesion of a granulocyte-like cell line from *Trichoplusia ni* as well as plasmatocytes and granulocytes isolated from *P. includens* (Beck and Strand 2005; Strand et al. 2006). Glc 1.8 contains many N and O-glycosylation domains, but only the N-glycosylation domains are required to disrupt adhesion processes (Beck and Strand 2005; MR Strand, unpublished results). The intracellular C-terminus may have a slight role in disrupting adhesion, as cells transfected with Glc 1.8 lacking the intracellular domain display a small but significant increase in phagocytic ability over wildtype Glc 1.8 (MR Strand, unpublished results).

Interference with cellular adhesion is a general strategy used by pathogens to overcome cellular defenses, and by animals to modulate their own immune defenses. Comparisons can be drawn between Glc 1.8 and several other proteins which work to reduce adhesion in humans, insects and other viruses. Potentially informative parallels can be drawn between insects and humans in terms of mucin proteins and disruption of adhesion. Expression of mucins (particularly Muc1) in cancer cells interferes with integrin mediated adhesion, which produces rounded and nonadherent cells (Wesseling et al. 1995). Interestingly Muc1 also interferes with the Toll pathway by sequestering a vital adaptor molecule, MyD88, in its cytoplasmic domain (Kato et al. 2012). Human cells infected with Ebola virus display a protein that causes detachment and cell rounding remarkably similar to Glc 1.8 (Francica et al. 2009). Ebola glycoprotein (EGp) induces a rounded phenotype similar to that seen in MdBV-infected cells (Francica et al. 2009). In addition to this, EGp also shields antibody access to integrins and major histocompatibility complexes (Francica et al. 2010). EGp can also prevent cell-cell

adhesions between cells expressing the protein at the cell surface. Cells can be rescued from this effect and antibody access restored by removing N-linked glycans using PNGase F (Francica et al. 2010). Although it is apparent the carbohydrate moieties of EGp shield molecules important for adhesion and other cell-cell interactions, the results of Francica et al 2010 should be taken with caution as producing the protein with a GPI anchor does not induce the steric effects (Francica et al. 2009). The lack of steric shielding of EGp when expressed as a GPI protein implies the transmembrane domain of the protein may also have some role in this response, perhaps through targeting of EGp to specific areas in the plasma membrane. Parallels to Glc 1.8 and EGp may also be found in the regulation of the insect immune response itself, as the *D. melanogaster* genome encodes a glycophorin-like glycoprotein which appears to act as a negative regulator of immune responses towards parasitoid eggs (Kurucz et al. 2003).

During immunosuppression, *P. includens* hemocytes are prevented from responding to any potential signals (see Chapter 3), but the mechanisms behind these aberrations are not completely understood. Cells infected by MdBV possess a general spherical morphology, but a small subpopulation of cells obtains an elongated spindle-like shape while still remaining apparently unattached to the substrate (Strand and Noda 1991). Transcript abundance of Glc 1.8 fades over time while cell adhesion slowly increases in the later stages of parasitism (Strand and Noda 1991; Bitra et al. 2011). The timeline of hemocyte pathology also manifests differently in granulocytes and plasmatocytes. During natural parasitism, plasmatocytes remain unattached throughout development of the parasitoid until day 7, while the ability of granulocytes to attach to foreign surfaces slowly increases throughout development (Strand and Noda 1991). During MdBV infection, a similar

trend is observed for both hemocyte morphotypes with granulocytes recovering faster than plasmatocytes (Strand and Noda 1991).

These details present a conflicting picture of the current ideas on PDV immunosuppression. The hemocytes of *P. includens* parasitized with *M. demolitor* are prevented from encapsulating the parasite in early stages of infection due to the presumed high expression of Glc 1.8 in the early stages of infection. However, given that the transcription of many of these factors changes over the course of the infection it is unclear whether these factors play a role in immunosuppression for the entire duration of parasitoid development. Although it is clear that Glc proteins are central to the inhibition of cellular adhesion by MdBV, it is unclear what roles proteins like PTPs and other factors play in the inhibition of cellular immunity. The fact Glc 1.8 transcription decreases throughout MdBV infection presents the opportunity to test the hypothesis that Glc 1.8 is the sole factor involved in the disruption of the immune response. In this chapter, I plan to use an observational study to answer the following question:

1.) **Does the expression of Glc 1.8 decrease through development, as implied by the mRNA transcripts?**
2.) **Does this pattern of expression correlate with a recovery of the encapsulation response in later stages of MdBV infection?**

2.2: Materials and methods

2.2.1 Insect Rearing

P. includens larvae were reared in pairs or trios in 1 inch cups with ample diet to the instars specified in the experiments according to previous literature (Strand 1990). Larvae were staged by measuring the head capsule width in accordance with established methods (Strand 1990). All larvae which were destined to be parasitized with *M. demolitor* or injected with MdBV were processed within 24 hours of molting to that instar.

M. demolitor was maintained in the lab in accordance with previous research (Shepard et al. 1983). To parasitize larvae with *M. demolitor*, five newly molted larvae were placed in an arena with 5-10 mated female wasps 7 to 14 days old. Larvae were observed in the arena until they had been stung once by one wasp, at which point they were removed and replaced. Larvae appearing to receive multiple stings discarded.

Calyx fluid was obtained from ovaries of 7-14 day old *M. demolitor* females in culture without access to hosts. Briefly, wasps were dissected in groups of 10-20. Reproductive organs were extracted from the females and ovaries separated from the venom glands and the remainder of the reproductive tract. Calyx fluid was removed from the ovaries by dismembering the ovaries with a sterile syringe in TC-100 and then passing the resulting solution through a 0.2 um filter to both sterilize the solution and remove remaining parts of the reproductive tracts. Larvae were injected with 5 ul solutions of MdBV.

2.2.2 Cell Culture and Glc Expression

Cells were isolated using methods established by previous research (Pech et al. 1994). Caterpillars were bled through a cut in the last abdominal segment. For the Glc expression tests, whole hemolymph was added directly to the TC-100 + 10% FBS and

cells allowed to spread two hours. After two hours, cells were washed 3x in PBS and preserved in 2% paraformaldehyde. Monoclonal antibodies previously prepared to Glc 1.8 were incubated overnight in a 1:2000 dilution. A secondary Alexafluor 546 rabbit anti mouse antibody was used to visualize the specimens. To assess whether cells were expressing Glc 1.8, sets of 100 cells were counted in each sample.

2.2.3 Encapsulation assays

To determine whether the immune system recovers after parasitization by *M. demolitor* and infection with *MdBV,* glass rods were inserted into the larvae just behind the head capsule at 0, 1, 3 and 6 days post-parasitization and infection. 24 hours later, the rods were removed and it was determined if the rods were encapsulated. The glass rods were considered encapsulated if more than half their surface area was covered in hemocytes. Rods were discarded from consideration if trachea or any other evidence of fat body material was present. The larvae were parasitized in petri dishes in groups of 10 with a wasp/caterpillar ratio of 2:1 or 1:1. Larvae destined for timepoint 0 had their glass rods inserted within half an hour of parasitization. If the wasps took longer than 20 minutes to parasitize the group of 10 larvae, these caterpillars were used in later timepoints. To minimize aggressive encounters between parasitized larvae, larvae were reared in pairs as described in Ch. 2.

2.2.4 Statistics

T-tests (Fig. 3.1.1) or Chi-square (Figures 3.1.2 and 3.1.3) tests were performed in JMP by MR. Strand.

2.3 Results and Discussion

2.3.1 The encapsulation response of *P. includens* does not recover after MdBV infection.

Disruption of cellular adhesion in general is a way that many pathogens circumvent the immune system. In the case of the ebola virus, the disruption of cellular adhesions likely serves to disrupt the presentation of immunogenic epitopes to T-cells, thus prolonging the time it takes for the T-cells to respond to infection (Francica et al. 2010). In the case of *Microplitis demolitor* Bracovirus, the role that disruption of cellular adhesion plays is to disable encapsulation response. Encapsulation of the parasitoid would eliminate the host in which MdBV replicates, thus ending the virus lifecycle. As reviewed earlier, a product thought to have a major role in mitigating this reaction is Glc 1.8 but the data presented in this chapter indicates other factors appear to interfere with this response.

In 3rd, 4th and 5th instar *P. includens*, the proportion of cells expressing Glc 1.8 fades as time progresses (Fig. 2.3.1). Between the initial oviposition and 24 hours, the proportion of cells expressing Glc 1.8 increases from barely detectable to 70% of cells or higher in 4th and 5th instar larvae and more than 80% in 3rd instar larvae (Fig. 2.3.1). During this time the parasitized *P. includens* larvae will not form capsules around the glass rods (Fig.2.3.2), which is consistent with Glc 1.8 being a major factor in disrupting adhesion (Beck and Strand 2005). However, as seen in Figure 2.3.1, Glc 1.8 expression fades as time progresses. At the midpoint of *M. demolitor* development, less than half the cells of 4th and 5th instar larvae are expressing this protein on their surface (Fig. 2.3.1). In 3rd instar larvae, this proportion does not appear to change by this midpoint. However, by the

end of the parasitoid's development, the proportion of cells expressing Glc 1.8 has dropped to less than 20% in 4th and 5th instars and to less than 50% in third instars. Despite the lowered expression of Glc 1.8, this does not result in a recovery of encapsulation response (Fig. 2.3.2, 2.3.3). Parasitized larvae are not able to form capsules around glass rods at any timepoint, and the same proportion of larvae encapsulate glass rods in MdBV infected larvae (Fig. 2.3.2 Fig. 2.3.3).

The observation that the encapsulation response does not recover after parasitism raised the question of whether MdBV or another factor released by the teratocytes or the larvae was responsible for this response. The data shown in Figure 2.3.3 indicates that recovery of the encapsulation response does not occur during Bracovirus infection, as roughly the same proportion of larvae were able to form capsules at every timepoint (Fig. 2.3.3). The reasons for this are currently unknown, but the functional data from Strand and Noda 1991 and the transcription data of Bitra et. al 2011 give us some clues as to why this might be. While there is a recovery of granulocyte spreading activity, there is no recovery of plasmatocyte spreading ability (Strand and Noda 1991). Both plasmatocytes and granulocytes are required to encapsulate invaders, which could partially explain this pattern. While the expression of Glc 1.8 drops off during development, the expression of other virulence factors still occurs but at much lower levels than within the first day of infection (Bitra et al. 2011). While Glc 1.8 transcription is transient, the transcription of PTP mRNA levels is more stable. Because the encapsulation response of *M. demolitor* does not recover after Glc 1.8 expression declines, it is likely that the PTPs also interfere with adhesion.

During the course of *Microplitis demolitor* development, capsules are almost completely prohibited from forming around larvae (Fig. 2.3.2). In MdBV infected larvae, capsules are also prevented from forming around targets, but to a much lesser extent (Fig. 2.3.3). In parasitized 4th instar larvae, less than 10% of larvae manage to form a capsule around glass rods at any timepoint (Fig. 2.3.2). In 5th instar larvae injected with the maximum physiological dose of 0.2 wasp equivalents MdBV, the encapsulation rate only declines to 50% (Fig 2.3.3). These results suggest that there are either host-stage effects in MdBV pathology during different stages of host development, or that *M. demolitor* uses a variety of factors which interact to disable the encapsulation response. Host-stage effects have been documented in the *M. demolitor/P.includens* system, with *M. demolitor* experiencing higher mortality in larger hosts (Harvey et al. 2004). This mortality is not related to the immune response, as *M. demolitor* larvae which develop inside later instar *P. includens* grow to a larger overall size which indicates that these hosts are not immunocompentent (Harvey et al. 2004). Thus, host-stage effects are unlikely to account for the discrepancy in the encapsulation response between Figures 2.3.2 and 2.3.3. Despite this, the involvement of other *M. demolitor* derived factors seems likely, as *Spodoptera frugiperda* is susceptible to MdBV infection, yet *S. frugiperda* yields no mature parasitoids (Trudeau and Strand 1998). On the converse, cross-protection experiments with PDVs which exhibit no cellular immunosuppressive activity still yielded adult *M. demolitor* (Kadash et al. 2003).

Immunosuppression is not the only strategy used by parasitoids to escape the immune system. Evasion, rather than suppression, of the immune system is a common tactic used by many parasitoids (Kinuthia et al. 1999; Corley and Strand 2003; Grimaldi et al. 2006).

For parasitic hymenoptera, one factor of particular importance comes in the form of an extraembryonic membrane (EM) which surrounds the parasitoid and prevents the binding of hemocytes (Corley and Strand 2003; Grimaldi et al. 2006). A non-PDV carrying wasp, *Venturia canescens*, has a membrane which contains a mucin-like protein that recruits proteins from the host's hemolymph to create a 'cloak' of host proteins that presumably allow the wasp to blend into the host (Kinuthia et al. 1999). One potential explanation of how *M. demolitor* could persist in a host which still retains an encapsulation response is to hypothesize that *M. demolitor* uses an immunoevasion strategy in addition to immunosuppression. However, an evasion strategy would not fully explain how *M. demolitor* prevents its hosts from encapsulating a separate target, but coat proteins that further disable the hemocyte response would offer a potential explanation. To the author's knowledge, such proteins have not been found in parasitoid wasps, but such proteins have been reported in entomopathogenic nematodes (Wang and Gaugler 1999; Li et al. 2009). Immunoevasive proteins on the EM of the developing *M. demolitor* larvae could potentially assist the parasitoid in evading encapsulation. However, the absence of any factors which have been demonstrated to inactivate hemocytes would not allow the implication of the EM in immunosuppression in the *M. demolitor/P. includens* system.

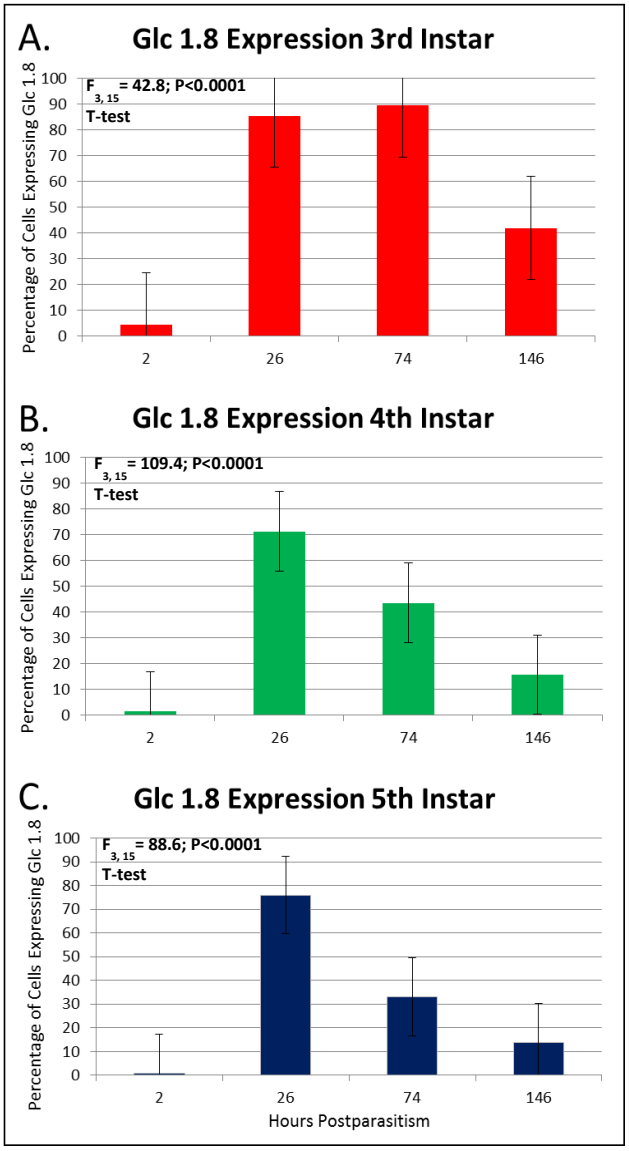
Two potential sources of virulence factors outside of MdBV exist for endoparasitoid wasps. The most likely alternative source of immunosuppressive factors are the teratocytes released from the dissolution of the serosal membrane. In other parasitoid wasps, these have been implicated in immunosuppression (Tanaka and Wago 1990; Andrew et al. 2006). This effect has been reported to be additive but not complete in

Cotesia plutellae, another PDV carrying wasp (Andrew et al. 2006). Teratocytes from *M. demolitor* are quickly encapsulated by the host hemocytes when injected in *the* absence of PDV and venom, but data is deficient as to whether there is an additional immunosuppressive role (Strand and Wong 1991; Andrew et al. 2006).

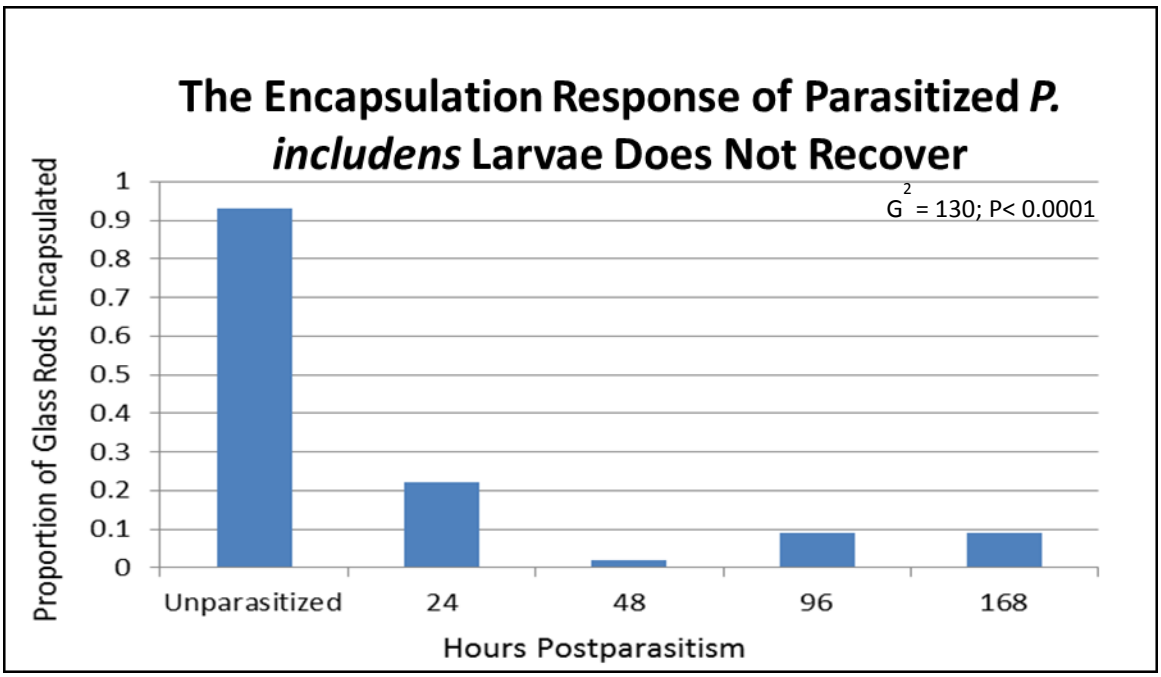
The second source of potential immunosuppressive factors lies within the parasitoid itself. Ectoparasites interact with the immune systems of their hosts through factors in their saliva (Ribeiro and Francischetti 2003). Ectoparasitic wasps use similar factors to ensure that the host will not separate them from their food source through the immune and injury responses (Richards 2012). These factors are generally not well characterized from parasitoid wasps, and separating the effects of endoparasitic wasp secretions from those of the PDVs and teratocytes would be no easy task as the teratocytes of *M. demolitor* seem to be dependent on MdBV (Strand and Wong 1991). However, ectoparasitism is the basal condition for the parasitoid lifestyle in many parasitoid groups including but not limited to the hymenoptera, and multiple shifts to endoparasitism are likely in parasitoids (Eggleton and Belshaw 1992; Pennacchio and Strand 2006). Although it is unclear if shifts to ectoparasitism from the derived endoparasitoid lifestyle have occurred, it seems likely that some strategies designed to manipulate host physiology would be conserved (Pennacchio and Strand 2006). However, it is clear that immunosuppressive activity of certain components of the parasitoid arsenal can vary widely depending on the strategy (i.e. PDV presence) the wasp uses to immunosuppress the host (Strand and Dover 1991; Prevost et al. 2005). While the results of Figures 2.3.2 and 2.3.3 appear to be in conflict with the established *M. demolitor* literature, they are potentially explainable by mechanisms other than interference by MdBV.

Another potentially productive area for further research would involve looking at the physical properties of the capsules formed during viral infection. Of the capsules formed despite MdBV infection, many were weak and brittle (JG Ballenger, personal observation). For most capsules formed during MdBV infection, the simple act of attempting to remove the glass rod from the hemocoel with foreceps or manipulating the rod with a probe was enough to remove the capsule from the glass rod. This lack of durability was not evident in capsules formed in healthy individuals. In the MdBV infected individuals it was evident that the hemocytes had recovered enough to form rudimentary capsules, but it was unclear whether the inability of the capsule to mature stemmed from the lack of adhesion of the cells or the inhibition of another factor which resulted in the strengthening of the capsules. It is likely that transglutaminase localizes to the surface of hemocytes and could potentially play a role in the hardening of the capsule (K. Clark, personal observation). Given that EGF 1.0 inhibits transglutaminase activity, it is possible that EGF 1.0 could potentially have an effect on capsule formation or maturity (K. Clark, unpublished results).

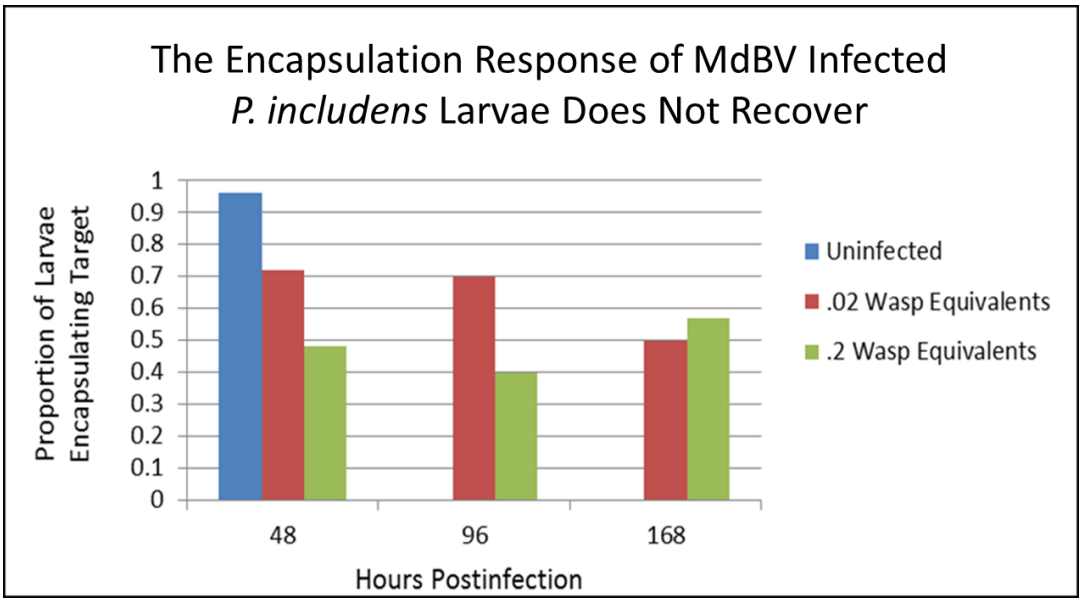
Transcriptome data on *M. demolitor* needs to be generated to assist with many of the future research goals outlined above. Transcriptome analysis of the venom glands, teratocytes, larval salivary glands and MdBV infected larvae would go a long way in identifying additional factors of importance to immunosuppression in *M. demolitor*.



2.3.1 Expression timeline of Glc 1.8. Hemocytes isolated from *P. includens* were stained with Glc 1.8 mouse monoclonal antibody and Alexa 546 conjugated goat anti mouse monoclonal antibody. The percentage of total hemocytes expressing Glc 1.8 changes drastically over the course of parasitoid development with, most cells expressing the protein 24 hours postparasitism and fewer cells expressing Glc 1.8, 6 days postparasitism. 4th and 5th instar hemocytes express Glc 1.8 at a similar rate as 3rd instar, but this expression drops off far more rapidly. For all instars, N=4 for all timepoints. T-tests were performed in JMP, and P-values for all tests between 2 and 26 hours were highly significant (P<0.0001 for third, fourth and fifth instars).



2.3.2 Caterpillars parasitized by *M. demolitor* do not show recovery of encapsulation response. 4th instar *P. includens* were parasitized as described in Materials and Methods or left unparasitized. Glass needles were inserted within 30 minutes of parasitism for Timepoint 0 (N = 45) or at 1 (N = 50), 3 (N = 49), or 6 (N = 47) days postparasitism. The needles were removed 24 hours later by dissecting the larvae in PBS. Needles were considered encapsulated if more than 50% of the needle's surface was covered by hemocytes. Despite decreasing abundance of cells expressing Glc 1.8, the immune response does not recover at any point during the development of the parasitoid. Chi Square tests were performed in JMP, and the encapsulation rate between unparasitized and 24-hours postparasitism individuals was highly significant ($G^2 = 130$, P<.0001).



2.3.3 Caterpillars infected with MdBV do not show recovery of encapsulation response. 5th instar *P. includens* injected with 0.02 or 0.2 MdBV. Glass needles were inserted at 1, 3, or 6 days postparasitism. N for all timepoints was 25, except for .02 wasp equivalents at 168 hours (N=20). The needles were removed 24 hours later by dissecting the larvae in PBS. Needles were considered encapsulated if more than 50% of the needle's surface was covered by hemocytes. Despite decreasing abundance of cells expressing Glc 1.8, the immune response does not recover at any point during infection with MdBV.

Works Cited

Andrew, N.*, et al.* (2006). "Additive effect of teratocyte and calyx fluid from Cotesia plutellae on immunosuppression of Plutella xylostella." Physiological Entomology **31**(4): 341-347.

Beck, M. and Strand M. R. (2005). "Glc1.8 from Microplitis demolitor bracovirus induces a loss of adhesion and phagocytosis in insect High Five and S2 cells." Journal of Virology **79**(3): 1861-1870.

Bitra, K.*, et al.* (2011). "Transcriptomic profiling of Microplitis demolitor bracovirus reveals host, tissue and stage-specific patterns of activity." Journal of General Virology **92**: 2060-2071.

Corley, L. S. and Strand M. R. (2003). "Evasion of encapsulation by the polyembryonic parasitoid Copidosoma floridanum is mediated by a polar body-derived extraembryonic membrane." Journal of Invertebrate Pathology **83**(1): 86-89.

Eggleton, P. and Belshaw R. (1992). "Insect Parasitoids: An Evolutionary Overview." Philosophical Transactions: Biological Sciences **337**(1279): 1-20.

Francica, J. R.*, et al.* (2010). "Steric Shielding of Surface Epitopes and Impaired Immune Recognition Induced by the Ebola Virus Glycoprotein." Plos Pathogens **6**(9).

Grimaldi, A.*, et al.* (2006). "Structure and function of the extraembryonic membrane persisting around the larvae of the parasitold Toxoneuron nigriceps." Journal of Insect Physiology **52**(8): 870-880.

Harvey, J. A.*, et al.* (2004). "Development of the solitary endoparasitoid Microplitis demolitor: host quality does not increase with host age and size." Ecological Entomology **29**(1): 35-43.

Kadash, K.*, et al.* (2003). "Cross-protection experiments with parasitoids in the genus Microplitis (Hymenoptera: Braconidae) suggest a high level of specificity in their associated bracoviruses." Journal of Insect Physiology **49**(5): 473-482.

Kinuthia, W.*, et al.* (1999). "Is the surface of endoparasitic wasp eggs and larvae covered by a limited coagulation reaction?" Journal of Insect Physiology **45**(5): 501-506.

Li, X*., et al.* (2009). "Characterization of immunosuppressive surface coat proteins from Steinernema glaseri that selectively kill blood cells in susceptible hosts." Molecular and Biochemical Parasitology **165**(2): 162-169.

Pech, L. L*., et al.* (1994). "SEPARATION AND BEHAVIOR IN-VITRO OF HEMOCYTES FROM THE MOTH, PSEUDOPLUSIA-INCLUDENS." Cell and Tissue Research **277**(1): 159-167.

Pennacchio, F. and Strand M. R. (2006). Evolution of developmental strategies in parasitic hymenoptera. Annual Review of Entomology. Palo Alto, Annual Reviews. **51:** 233-258.

Prevost, G*., et al.* (2005). "Asobara, braconid parasitoids of Drosophila larvae: unusual strategies to avoid encapsulation without VLPs." Journal of Insect Physiology **51**(2): 171-179.

Ribeiro, J. M. C. and Francischetti I. M. B. (2003). "Role of arthropod saliva in blood feeding: Sialome and post-sialome perspectives." Annual Review of Entomology **48**: 73-88.

Richards, E. H. (2012). "SALIVARY SECRETIONS FROM THE ECTOPARASITIC WASP, Eulophus pennicornis CONTAIN HYDROLASES, AND KILL HOST HEMOCYTES BY APOPTOSIS." Archives of Insect Biochemistry and Physiology **79**(2): 61-74.

Shepard, M*., et al.* (1983). "BIOLOGY OF MICROPLITIS-DEMOLITOR (HYMENOPTERA, BRACONIDAE), AN IMPORTED PARASITOID OF HELIOTHIS (LEPIDOPTERA, NOCTUIDAE) SPP AND THE SOYBEAN LOOPER, PSEUDOPLUSIA-INCLUDENS (LEPIDOPTERA, NOCTUIDAE)." Environmental Entomology **12**(3): 641-645.

Strand, M. R. (1990). "CHARACTERIZATION OF LARVAL DEVELOPMENT IN PSEUDOPLUSIA-INCLUDENS (LEPIDOPTERA, NOCTUIDAE)." Annals of the Entomological Society of America **83**(3): 538-544.

Strand, M. R. and Dover B. A. (1991). "DEVELOPMENTAL DISRUPTION OF PSEUDOPLUSIA-INCLUDENS AND HELIOTHIS-VIRESCENS LARVAE BY THE CALYX FLUID AND VENOM OF MICROPLITIS-DEMOLITOR." Archives of Insect Biochemistry and Physiology **18**(3): 131-145.

Strand, M. R. and Noda T. (1991). "Alterations in the haemocytes of Pseudoplusia includens after parasitism by Microplitis demolitor." Journal of Insect Physiology **37**(11): 839-850.

Strand, M. R. and Noda T. (1991). "ALTERATIONS IN THE HEMOCYTES OF PSEUDOPLUSIA-INCLUDENS AFTER PARASITISM BY MICROPLITIS-DEMOLITOR." Journal of Insect Physiology **37**(11): 839-850.

Strand, M. R. and Wong E. A. (1991). "The growth and role of Microplitis demolitor teratocytes in parasitism of Pseudoplusia includens." Journal of Insect Physiology **37**(7): 503-515.

Tanaka, T. and Wago H. (1990). "ULTRASTRUCTURAL AND FUNCTIONAL MATURATION OF TERATOCYTES OF APANTELES-KARIYAI." Archives of Insect Biochemistry and Physiology **13**(3-4): 187-197.

Trudeau, D. and Strand M. R. (1998). "A limited role in parasitism for Microplitis demolitor polydnavirus." Journal of Insect Physiology **44**(9): 795-805.

Wang, Y. and Gaugler R. (1999). "Steinernema glaseriSurface Coat Protein Suppresses the Immune Response ofPopillia japonica(Coleoptera: Scarabaeidae) Larvae." Biological Control **14**(1): 45-50.

Webb, B. A.*, et al.* (2006). "Polydnavirus genomes reflect their dual roles as mutualists and pathogens." Virology **347**(1): 160-174.

Chapter Three
PSP Processing and Signaling During MdBV Infection

3.1: Introduction

3.1.1 MdBV Encoded Glycoproteins and Phosphatases Disrupt Hemocyte Function.

As reviewed in Chapter One, parasitoid wasps must contend with the cellular and humoral responses of their hosts to complete development. *Microplitis demolitor* uses a symbiotic virus to disrupt these defenses by introducing virulence factors. Two different MdBV gene products, PTPs and Glc proteins, are involved in disrupting this response but their roles are not well understood (Beck and Strand 2005; Pruijssers and Strand 2007; Suderman et al. 2008). The evidence presented in Chapter Two, however, indicates that Glc 1.8 is not the sole factor involved in disrupting the immune defenses.

MdBV encodes a pair of protein tyrosine phosphatases (PTPs) which localize to focal adhesions and disrupt phagocytic activity in *Drosophila* S2 cells, a cell line with similarities to immune cells (Pruijssers and Strand 2007). Very little is known about what these proteins are targeting *in vivo* and how they work, but they appear to localize with FAK in transfected Drosophila S2 cells and are likely acting upon proteins which localize to focal adhesions (Pruijssers and Strand 2007). Their effects on cell shape and phagocytic ability are not nearly as severe as those seen with Glc 1.8, but are additive and decrease the amount of phagocytosis by nearly 100% when the two are co-transfected (Pruijssers and Strand 2007). PTP H2 and H3 both reduce the cell's phagocytic ability, and PTP H2 has been implicated in causing apoptosis of

granulocytes during MdBV infection (Suderman et al. 2008). PTP H2 also reduces cell proliferation in SF-21 cells, but the relation of this effect to in vitro effects on hemocytes is ambiguous for two reasons. First because hemocytes are thought to maintain their populations in hemolymph by mitosis, and second because hemocyte counts increase in MdBV infected *P. includens* larvae (Strand and Noda 1991; Strand et al. 1999; Gardiner and Strand 2000). The PTPs of MdBV are closely related to cellular phosphatases which help regulate the cycling of focal adhesions in healthy insect hemocytes (Burke and Strand 2012).

Interfering with the activation of integrin receptors by PTPs is a common strategy employed by pathogenic bacteria. One of the most important virulence factors of *Yersinia pestis* is YopH, a PTP which decreases human macrophage phagocytosis of bacteria by interacting with and dephosphorylating p130Cas and FAK in human macrophages (Persson et al. 1997). Insects contain homologs of these proteins, although their exact roles in the formation of focal adhesions in insect cells not well known (Marmaras and Lampropoulou 2009).

3.1.2 EGF family proteins encoded by *Microplitis demolitor* Bracovirus are serine protease inhibitors which inhibit melanization.

Oenocytoids are not prevented from bursting during MdBV infection, but the products they release are prevented from activating by negative regulators encoded by MdBV. Proteolytic cascades are negatively regulated by several factors including Serpins, which deform the protease's active site and form a covalent bond with the active site serine (Gettins 2002). Just as PTPs are normal parts of insect systems, the

purpose of protease inhibitors in insect hemolymph is to regulate immune responses in order to prevent improper activation at inopportune times and locations. Protease inhibitors can inactivate proteases released by pathogenic organisms, and can function as defensive compounds in addition to their regulatory roles (Eguchi et al. 1994). Insects encode a variety of protease inhibitors. Although Serpins are the best described in terms of humoral immunity, other proteins such as Small Proteinase Inhibitor family proteins (Smapins) have been recorded from lepidopteran larvae in response to fungal infections (Eguchi et al. 1994). Smapin proteins are also used as virulence factors by hookworms, where they function to prevent host blood coagulation during feeding (Zang and Maizels 2001). Pathogens commonly encode proteins that disrupt serine protease pathways, and MdBV also takes advantage of this tactic.

The EGF family members EGF 1.0 and 1.5 encoded by MdBV belong to the Smapin family and inhibit melanization by disrupting PAP activation (Beck and Strand 2007). EGF 1.0 and 1.5 also inhibit the processing of PAP-1, PAP-3 and SPH cofactors essential for phenoloxidase activity (Beck and Strand 2007; Lu et al. 2008; Lu et al. 2010). Recent results indicate EGF 1.0 and 1.5 disrupt melanization by disrupting the formation of the high molecular weight melanization complex (K. Clark, unpublished data). The third member of this family, EGF 0.4, exhibits no antimelanization activity, and its function is currently unknown.

The ENF pathway discussed in Chapter One is dependent on serine proteases, and MdBV encodes serine protease inhibitors. In a similar vein, the activation of adhesion responses are dependent on phosphorylation cascades, while MdBV also encodes

enzymes which are thought to dephosphorylate molecules important to the activation of the adhesion response in the cells. Given the following unknowns, I set out to test the following hypotheses:

1. **Do MdBV encoded factors inhibit PSP processing during immune reactions?**
2. **Do MdBV encoded factors interfere with the phosphorylation of a P77 like entity in *P. includens* cells?**

3.2 Materials & Methods

3.2.1 Western Blotting

Plasma samples from *P. includens* larvae were diluted 1:20 into Laemmli sample buffer under nonreducing conditions unless noted. If noted that samples were processed under reducing conditions, DTT was added to the sample buffer to a final concentration of 50 mM. Samples were boiled for five minutes, then stored at -20^{o}C until run on gels. Gels were either 4-20% or 12.5% (non-gradient) Tris-glycine gels. Gels were run at 150V and transferred at 400 mA. Running time for 4-20% Tris-glycine gels was 90 minutes and transfer time was 90 minutes. Running time for 12.5 % gels was 2.5 hours with a transfer time of 75 minutes. All proteins were transferred to PVDF. Anti-PSP polyclonal antibodies were previously prepared (Clark et al. 1998). Antiphosphotyrosine polyclonal antibodies (anti-PY) conjugated with horseradish peroxidase were purchased from Invitrogen. Goat-anti-rabbit antibodies conjugated to horseradish peroxidase were used as a secondary antibody for the anti-PSP antibodies, while no secondary antibodies were required for the anti-PY antibodies. Gels were blocked either by 5% nonfat milk (anti-

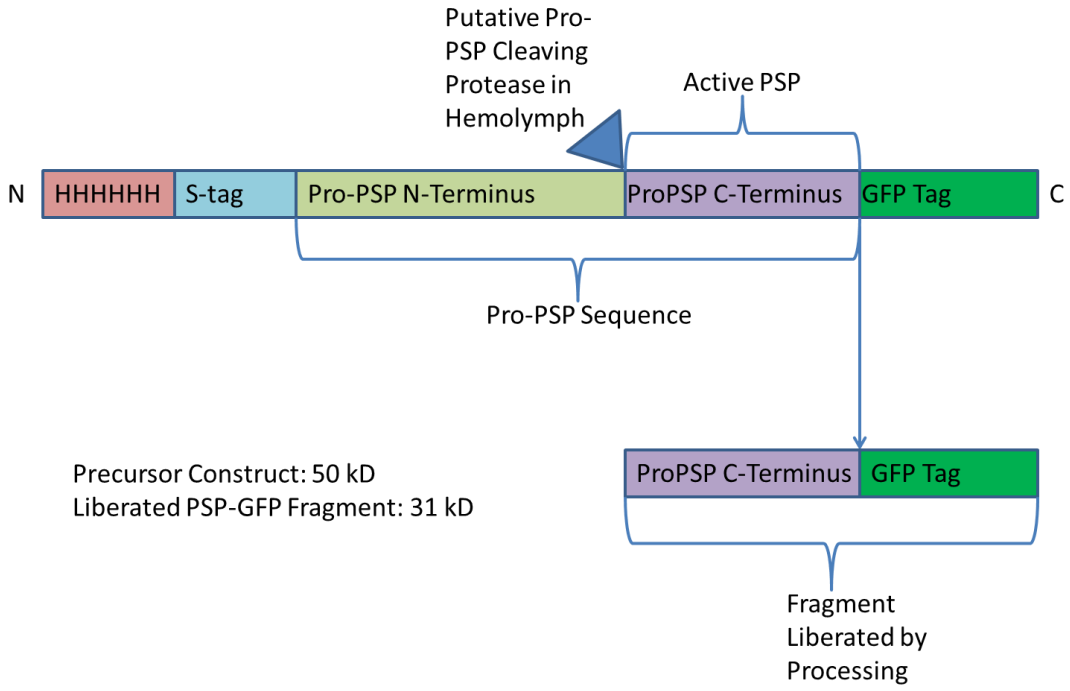
PSP) for 30 minutes or 2% commercially prepared ECL block (anti-PY) for two hours where noted. Blots were developed with ECL select purchased from GE Healthcare.

3.2.2 PSP Processing in hemolymph

Initial PSP processing studies were performed in wandering stage *P. includens*. Fifth instar *P. includens* at 36-48h were injected with 0.2 wasp equivalents MdBV, and bled 24 h later. Controls were naive wandering stage larvae.

To further confirm that PSP was processed, a hexahistidine and GFP tagged recombinant protein was constructed based on the sequence of PSP (Fig 3.2.1). This was encoded on a PET-30 vector and transfected into Rosetta-gami DE3 *E. coli* cells, grown to OD_{600} at 37^{o} C under selection with Chloramphenicol and Kanamyacin at 50 mg/mL. Protein production was induced by adding IPTG to 1 mM, then letting protein be expressed at room temperature. Bacteria were harvested by centrifugation at 5,000 RPM for 5 minutes at 4^{o} C. Pellet was resuspended, incubated in lysozyme at 1 mg/mL for half an hour. Cells then underwent 5 rounds of sonication on ice. The resulting extract was then incubated overnight with Ni-NTA beads.

The Ni-NTA beads were loaded onto a purification column, washed with two volumes of lysis buffer, then gradient purified using 100-250 mM imidazole in 50 mM steps. The 150-200 mM fractions were pooled, then stored in 25% glycerol at a final concentration of 0.3375 ug/ul. To perform the assay using this construct, the construct was incubated at 0.1 ug/ul in hemolymph, and detected with an antiPSP polyclonal antibody as specified above.



3.2.1 Diagram of construct used in determining whether PSP is cleaved in MdBV infected individuals. The construct contains an N-terminal hexahistidine tag used for purification, a C-terminal GFP tag and an N-terminal S-tag for detection. The fragment consisting of the C-terminus of Pro-PSP represents the intact cytokine conjugated to GFP. The intact construct has a calculated weight of 46 kD, but runs at 50 kD on a gel. The fragment liberated by factors in the hemolymph has a calculated weight of 31 kD which corresponds well to its position on the gel.

3.2.3 Cell Culture and Adhesion Assays

Cells were isolated using methods established by previous research (Pech et al. 1994). Caterpillars were bled through a cut in the last abdominal segment into anticoagulant buffer at 5 larval equivalents (LEQ) per milliliter. These cells were centrifuged for 90

seconds at 200 x g, and washed once in anticoagulant buffer. After an incubation period of one hour, cells were again centrifuged for 90 seconds at 200 x g and washed once with TC-100. Isolated hemocytes were counted with a hemocytometer (Fisher Scientific) and plated at a density of 250 cells/mm^2. Adhesion assays were performed in 12 well plates in 1 mL solutions unless noted otherwise. Cells were plated to a standard density of 250 cells/mm^2 and stimulated with either 1% plasma or 10 uM PSP in TC-100. For all timepoints, groups of 200 cells were counted.

3.2.4 Statistics

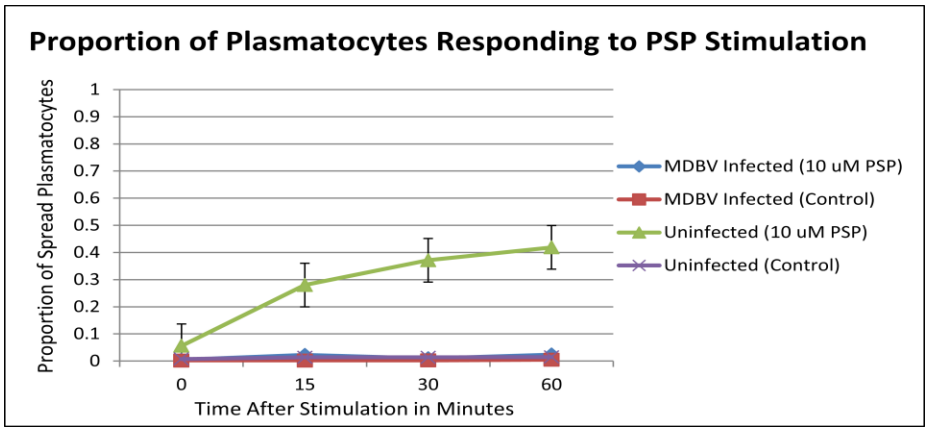
Statistics were performed in JMP. T-tests were performed between each of two groups in Figure 2.1.2.

3.3 Results and Discussion

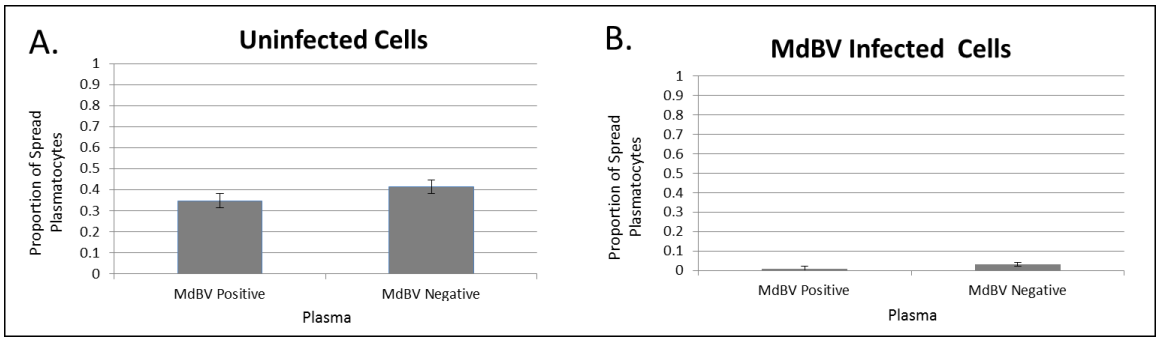
3.3.1 Hemocytes from *P. includens* infected with MdBV do not respond to PSP and other hemolymph factors

Given that PSP is thought to act as an important immune signaling molecule, the first step in the investigation into how PSP signaling is blocked by MdBV was to replicate previous results which determined that MdBV blocked the response to PSP. Roughly 50% of plasmatocytes from 36-48 hour old 5th instar *P. includens* spread within one hour when incubated with 10 uM synthetic PSP (Fig. 3.3.1). Plasmatocytes infected with MdBV or not stimulated with PSP only displayed negligible spreading at a rate only slightly higher but not significantly different from the baseline of uninfected plasmatocytes (3.3.1).

To determine if hemocytes would react to any uncharacterized factors present in plasma, cells from 36-48 hour old *P. includens* were incubated in 1% plasma from larvae 24 hours postinfection with MdBV (Fig. 3.3.2). Plasmatocytes from healthy individuals spread to a comparable degree when incubated with 1% plasma from healthy and MdBV infected individuals (3.3.2 A). MdBV infected plasmatocytes were not rescued with plasma from uninfected individuals and did not spread in response to plasma stimulation regardless of source (Fig. 3.3.2 B). The fact that uninfected plasmatocytes spread in response to MdBV infected plasma indicates signaling factors present in plasma were not appreciably changed by MdBV infection. The lack of a rescue effect in MdBV infected plasmatocytes indicates that changes in plasma composition are not responsible for the lack of an adhesion response in plasmatocytes from *M. demolitor* parasitized individuals.



3.3.1 MdBV-infected hemocytes do not respond to PSP stimulation. MdBV infected hemocytes were stimulated by 10 uM PSP and counts 200 of plasmatocytes were performed directly after PSP or TC-100 was added at time point 0, then at 15, 30 and 60 minutes (N = 4 repetitions). Plasmatocytes from healthy individuals spread in response to PSP stimulation in a time-dependent manner, while plasmatocytes from MdBV infected individuals did not respond to PSP stimulation.



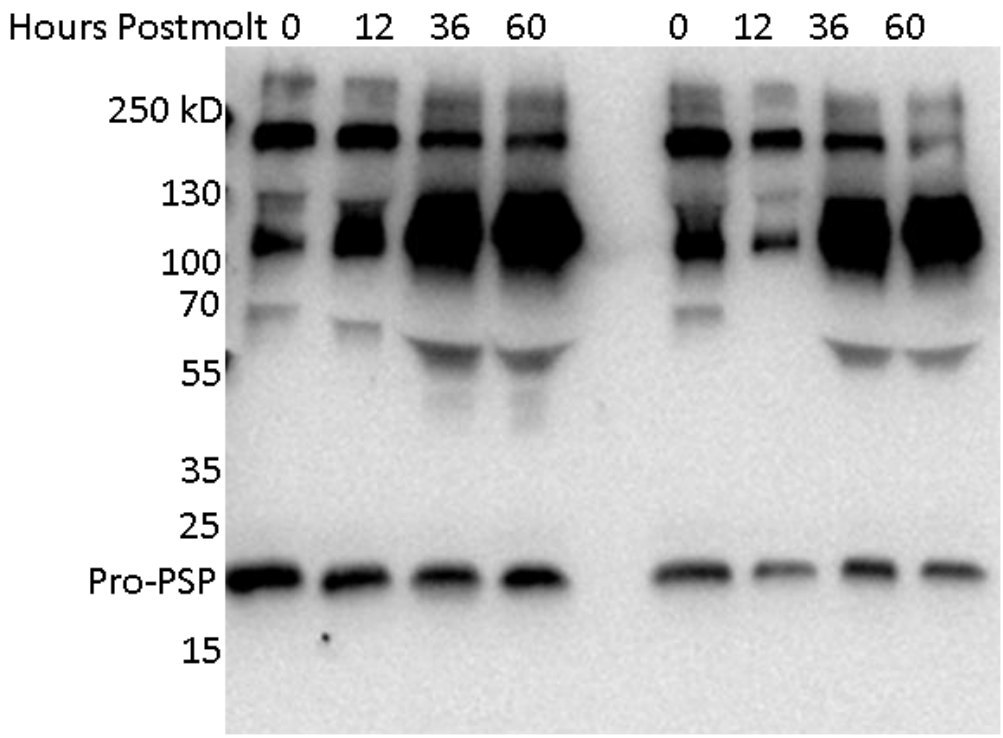
3.3.2 Cross-incubation of plasma does not rescue hemocyte response. Cells from healthy and infected individuals were incubated with plasma from healthy and infected individuals for one hour, then the proportion of spread plasmatocytes was counted(200 plasmatocytes counted, N= 4). Plasmatocytes from MdBV infected individuals did not spread when incubated in 1% plasma from healthy individuals (B). In contrast, plasmatocytes from healthy individuals spread when incubated in plasma from MdBV infected individuals (A). These results indicate that inhibition of activation of plasma factors in hemolymph is not a significant part of MdBV pathology, and that uncharacterized factors present in hemolymph are still unable to influence hemocyte spreading during MdBV infection. Results between groups in Fig. A and B were not significant (T-test, $\alpha = 0.01$).

3.3.2 PSP is expressed and processed in 5th instar healthy and MdBV-infected larvae

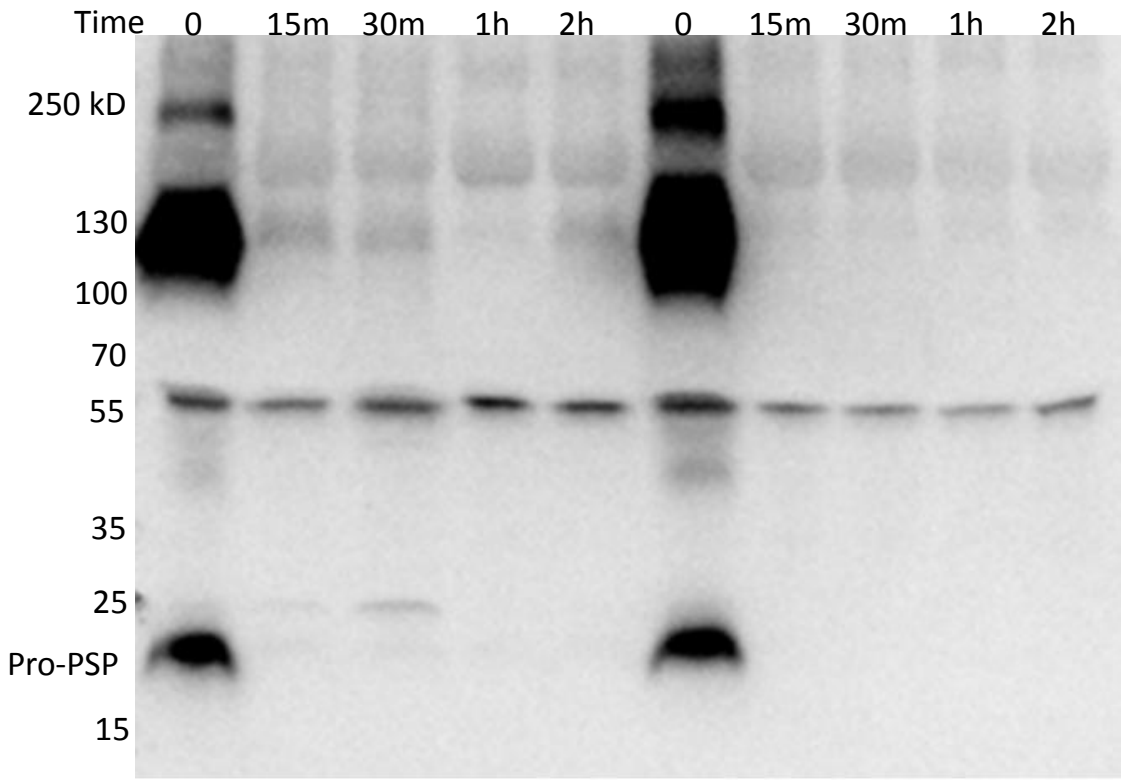
As a first step in characterizing the production and processing of pro-PSP in MdBV infected caterpillars, the production of PSP must be characterized. Pro-PSP shows up as a 20 KD band, with several additional bands appearing (Fig. 3.3.3-3.3.5). As seen in Fig. 3.3.3, pro-PSP is produced throughout the fifth instar with the amount present in the

hemolymph staying somewhat constant, perhaps with a slight increase being observed. Proteolytic activation is accomplished by injury of the larva, and after injury PSP is quickly cleaved (Fig. 3.3.4, Fig. 3.3.5). The 2.5 kD peptide which results from PSP cleavage cannot be detected on a western blot. Thus this experiment is based on the loss of Pro-PSP signal as the PSP epitope is lost when Pro-PSP is cleaved to active PSP (Fig. 3.3.4, Fig. 3.3.5).

In both healthy and infected individuals, PSP is rapidly cleaved upon injury (Fig. 3.3.5). The amount of Pro-PSP present in the hemolymph of healthy individuals can vary considerably (Fig. 3.3.3), but the amount of Pro-PSP detectable in hemolymph is not appreciably different between MdBV infected and healthy individuals (Fig. 3.3.5). Pro-PSP is cleaved within 15 -30 minutes from the time it is extracted from caterpillars (Fig. 3.3.4, Fig. 3.3.5). Interestingly, the 250 kD and 130kD band observed in Figures 3.3.3-3.3.5 disappears within the same time frame of Pro-PSP. These 250 kD and 130 kD bands are dose-dependently outcompeted in blots with PSP added to the blocking medium and disappear when the sample is processed under reducing conditions (Data not presented). Several experiments were undertaken to determine the nature of these proteins but results were ultimately inconclusive. Current investigations into PSP cleavage by K. Clark failed to confirm the presence of these bands, indicating they may be nonspecific artifacts. The 50 kD band observed in Figs. 3.3.3-3.3.6 is reactive to the secondary antibody used to develop the blots and is not out competed by PSP (Data not presented).



3.3.3 Pro-PSP is present in the hemolymph of *P. includens*. Hemolymph was harvested from *P. includens* 5th instar larvae directly after the moult (0), then at 12, 36, or 60 hours postmolt. Hemolymph from *P. includens* was diluted 1:20 in SDS and boiled immediately after collection. Pro-PSP was detected through Western blots; two repetitions are shown. Pro-PSP is visible as a band in the 20 kD range. Although there may be some variation between individual larvae, as larval development goes on the amount of PSP stays relatively constant and does not appear to change drastically (N = 4). There are a number of bands which are detected by the PSP antibody. The bands which show up the most reliably in plasma are the 250, 100 and 50-70 kD band. The disappearance of the 250 kD and 100 kD bands is correlated with the disappearance of Pro-PSP upon injury, while the 50-70 kD band is most likely a nonspecific band (See Figs. 3.3.3-3.3.5**,** discussion).



3.3.4 Pro-PSP is processed after injury in the hemolymph of *P. includens*. Processing of Pro-PSP to PSP was monitored in hemolymph by Western blot. Upon injury in healthy individuals, Pro-PSP is rapidly processed in the hemolymph (N=4). Tests from two larvae are shown. In wandering stage larvae, Pro-PSP becomes undetectable 15 minutes after injury. Assay is based on loss of PSP at the 20 kD band. Several bands cross-react with the antibody used in this assay. The disappearance of the bands at 250 kD, 130kD and 100 kD is correlated with the disappearance of Pro-PSP, while a band at roughly 50 kD appears to be unprocessed in the hemolymph and is most likely non-specific. See discussion for details.

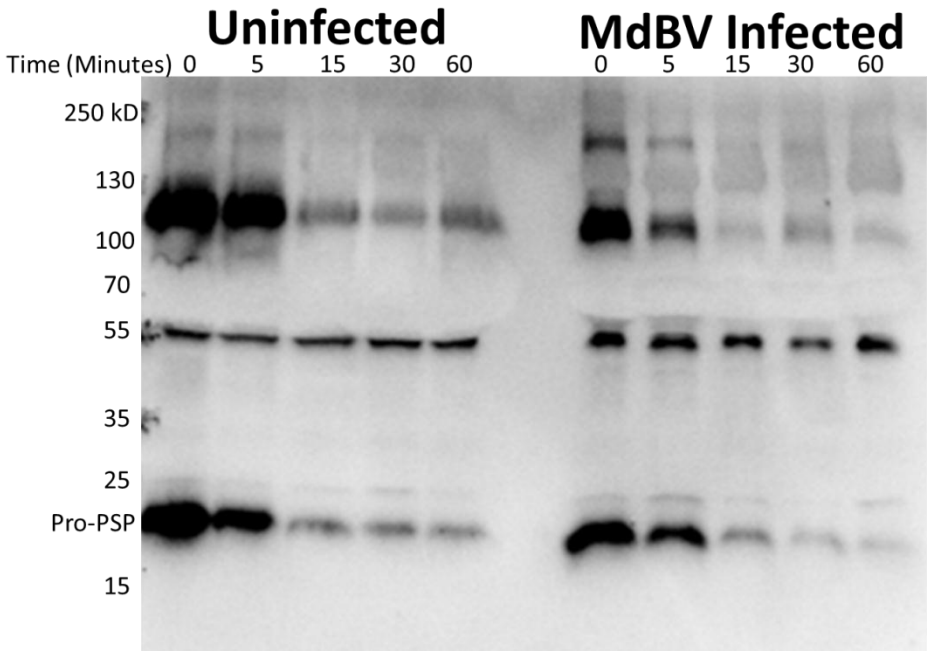
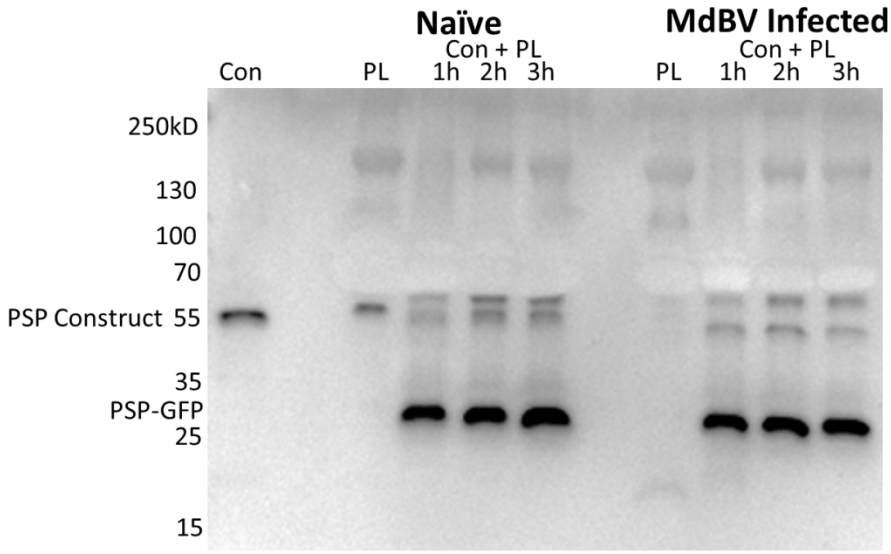


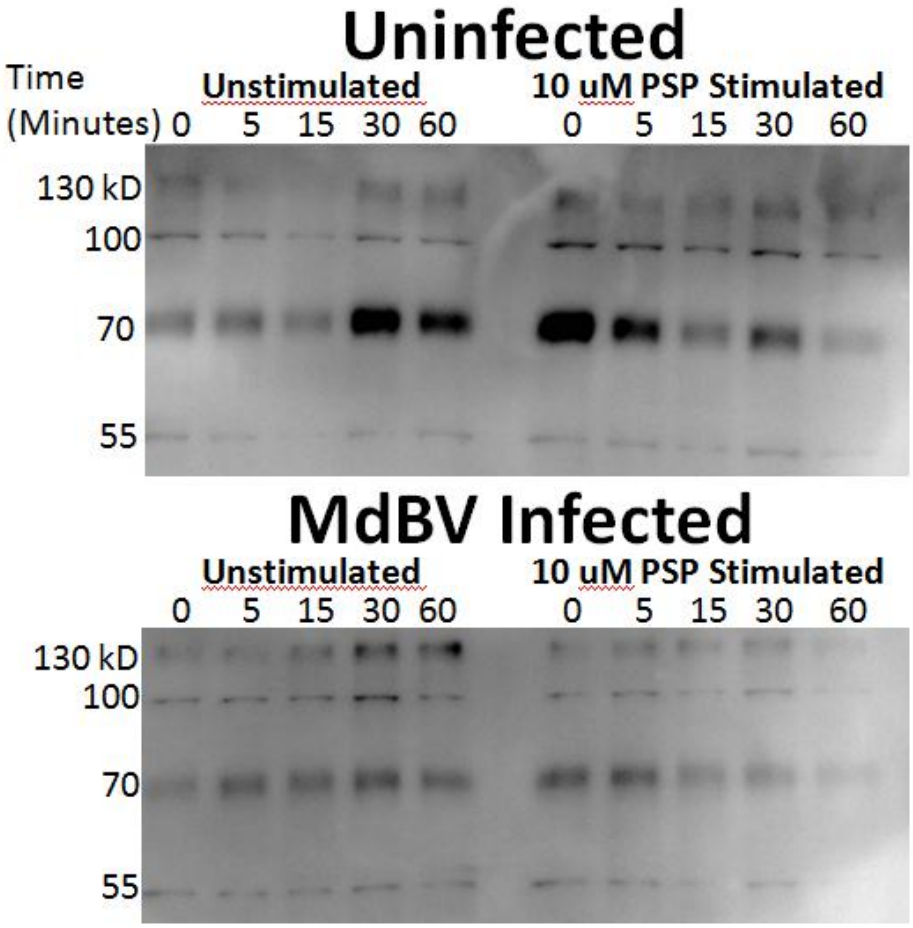
Fig. 3.3.5 Pro-PSP is cleaved in MdBV infected individuals. In MdBV infected and healthy individuals, Pro-PSP is still processed after injury in a manner comparable to one another. Pro-PSP at the 20 kD band is almost completely gone by 60 minutes in both infected and uninfected individuals (N = 3). Bands at the 250 kD and 100 kD range disappear in conjunction with the loss of Pro-PSP, while the bands at the 50 kD range remain relatively constant. See discussion for details.



3.3.6 Artificial Pro-PSP substrates are processed in *P. includens* plasma. Upon injury in *P. includens* 5th instar larvae, the cascade which results in the activation of PSP from Pro-PSP is rapidly activated. The artificial PSP construct is labeled Con; plasma labeled PL. When combined, the two are labeled Con+PL. A band of similar size to the Pro-PSP construct (Con) shows up in plasma without the construct (PL). The band in the PL column appears to be a slightly higher molecular weight than the construct when incubated with plasma as seen in the Con+PL column. When added to plasma (Con+PL), the construct detailed in Fig. 3.2.1 is cleaved to a 30 kD protein as a result of PSP cleaving activity in plasma. When incubated at 0.1 ug/ul, the construct is almost completely cleaved within 1 hour of introduction and samples taken at subsequent timepoints (1, 2, 3h) do not show a significantly larger amount of PSP-GFP product despite the fact some of the construct apparently remains uncleaved (N = 2). Results from infected individuals were similar, with the construct being almost entirely cleaved within 1h of introduction.

3.3.4 Intracellular signaling of PSP is not Affected by MdBV Infection

The next step in characterizing whether the signaling of PSP is intact in insect hemocytes is to determine if the known signaling pathways are intact. GBP signaling in *P. separata* involves the phosphorylation of a tyrosine residue on an adaptor protein P77 soon after stimulation with PSP in vitro (Oda et. al 2010). To determine if there is a P77-like entity in *P. includens*, Western blots were performed on *P. includens* hemocytes using phosphotyrosine antibodies.



3.3.7 Phosphorylation cascades are seemingly intact in *P. includens* hemocytes.

Proteins containing phosphotyrosine residues were detected using Western blots. The band corresponding most closely to P77 in *P. includens* hemocytes appears in MdBV infected larvae, and appears to be phosphorylated in response to PSP. In *P. includens* hemocytes, a band at 70 kD appears to be differentially phosphorylated in *P. includens* hemocytes stimulated by 10 uM PSP, as indicated by the darkened bands at timepoints 0 and 5 (N = 2). Interpretations of results involving the presence of a protein with functional similarities to P77 in *P. includens* should be taken with caution; see discussion for details.

PSP is a highly important molecule in the course of events leading to a cellular immune response. In response to wounding or to the introduction of peptidoglycan or glucans, PSP is cleaved from its putatively inactive precursor causing an immune response in a subpopulation of plasmatocytes (Pech and Strand 1995; Clark et al. 1997; Wang et al. 1999; Ishii et al. 2008). This results in the activation of an intracellular signaling cascade involving an adaptor protein, ultimately resulting in integrin activation and a spreading response (Oda et al. 2010). *In vitro,* this was measured as a spreading response on cell culture plates (Fig. 3.3.1, Fig. 3.3.2). *In vivo,* this was measured as the encapsulation response towards glass rods (Fig. 2.3.2, Fig. 2.3.3).

PDV infection results in a broad suppression of the cellular response in *Pseudoplusia includens* (Strand and Noda 1991). To begin my investigation, I focused on the best characterized cytokine pathway involved in this response, the PSP pathway. Because sensitivity to PSP changes throughout development, I began my experiments by determining whether the amount of PSP changes in the hemolymph during development (Clark et al. 2005). After this, I moved on to determine whether PSP was cleaved in MdBV infected individuals (Figs. 3.3.4-3.3.6) . As MdBV encodes several phosphatases, I then attempted to determine whether MdBV acted by disabling components of the intracellular PSP signaling pathway (Fig. 3.3.7). A band differentially phosphorylated in response to PSP stimulation was detected in Fig. 3.3.7, but because the protein was not purified and its sequence was not determined there is no basis for calling it a P77 homolog at this time.

During the course of MdBV infection, hemocytes are prevented from responding not only to PSP but to hemolymph in general (Fig. Fig. 3.3.1, Fig. 3.3.2.). The data presented here indicate that the proteolytic activation of cytokines is not interfered with, and we were unable to uncover evidence that phosphorylation of a P77-like entity was interfered with during MdBV infection (Figs. 3.3.4-3.3.7). However, the encapsulation response was completely blocked during parasitism by *M. demolitor* (Fig. 2.3.2). Thus, *Microplitis demolitor* blocks the encapsulation response *in vitro* but not by interfering with any component of PSP signaling *in vivo*. During infection with MdBV and parasitization with *Microplitis demolitor* Pro-PSP is still activated by proteolytic cleavage in response to injury much as it is in healthy individuals (Fig 3.3.5, Fig. 3.3.6). In a similar manner, the tyrosine dependent signaling pathway appears to be intact during PSP signaling (Fig. 3.3.7). Although PSP signaling is an important response to parasitoid wasps, the PSP pathway does not appear to be specifically targeted by any MdBV target as is the PO pathway (Beck and Strand 2007). Although Glc 1.8 plays a big role in disrupting adhesion, other factors likely play a role in this reaction. See Chapter Two for details. The finding that Pro-PSP is still cleaved during infection sheds light on how PSP is activated. The PSP cascade culminates in PSP activation by an unknown protease, which is part of an uncharacterized proteolytic pathway (Strand 2008). The PSP and Pro-PO pathways are activated upon injury and may have a common mechanism of activation (Strand 2008). During MdBV infection, Pro-PO is prevented from being activated through the inhibition of its activating proteases (Beck and Strand 2007). Because EGF 1.0 and 1.5 block the phenoloxidase activating proteases, the fact cleavage is intact through MdBV infection indicates the Pro-PSP pathway may not be dependent on the

activity of phenoloxidase activating proteases. If the PO and PSP pathway share a common point, the common components are probably upstream of this point in the pathway.

Another interesting find during the course of this study is that the expression of PSP appears to stay relatively constant during development in healthy *P. includens* larvae (Fig. 3.3.3), while the sensitivity to PSP increases during the final instar due to hormonal regulation (Clark et al. 2005). In contrast, the melanization response in *M. sexta* decreases as the insect develops (K. Clark, unpublished observations). This is intriguing as these results demonstrate that parts of the insect immune system are specifically regulated at certain points during development.

The goals of this study at the beginning of the chapter were to investigate whether MdBV encoded factors inhibited Pro-PSP processing or if MdBV encoded factors interfered with the phosphorylation of a P77-like entity in *P. includens* cells. The ultimate question this answered was whether *M. demolitor* disrupted the encapsulation response through interfering with cellular signaling. Through the course of this study, I have demonstrated that Pro-PSP is cleaved in MdBV-infected larvae (Fig. 3.3.5, Fig. 3.3.6). No evidence was found that a P77 like entity is blocked by MdBV encoded factors (Fig. 3.3.7). Instead, the results of this study suggest that PSP is blocked through either interference with integrin activation by phosphatases which act on focal adhesions, or through Glc 1.8 which acts by preventing adhesion to the substrate. The data in Chapter Two suggest that other mechanisms other than interference with adhesion by Glc 1.8 are responsible for disrupting the encapsulation response *in vivo.*

Works Cited

Beck, M. H. and Strand M. R. (2007). "A novel polydnavirus protein inhibits the insect prophenoloxidase activation pathway." Proceedings of the National Academy of Sciences **104**(49): 19267-19272.

Clark, K. D*., et al.* (2005). "Plasmatocyte sensitivity to plasmatocyte spreading peptide (PSP) fluctuates with the larval molting cycle." Journal of Insect Physiology **51**(5): 587-596.

Clark, K. D*., et al.* (1997). "Isolation and identification of a plasmatocyte-spreading peptide from the hemolymph of the lepidopteran insect Pseudoplusia includens." Journal of Biological Chemistry **272**(37): 23440-23447.

Clark, K. D*., et al.* (1998). "Plasmatocyte spreading peptide is encoded by an mRNA differentially expressed in tissues of the moth Pseudoplusia includens." Biochemical and Biophysical Research Communications **250**(2): 479-485.

Eguchi, M*., et al.* (1994). "AMINO-ACID-SEQUENCE OF AN INHIBITOR FROM THE SILKWORM (BOMBYX-MORI) HEMOLYMPH AGAINST FUNGAL PROTEASE." Journal of Biochemistry **115**(5): 881-884.

Ishii, K*., et al.* (2008). "Activation of the silkworm cytokine by bacterial and fungal cell wall components via a reactive oxygen species-triggered mechanism." Journal of Biological Chemistry **283**(4): 2185-2191.

Oda, Y*., et al.* (2010). "Adaptor protein is essential for insect cytokine signaling in hemocytes." Proceedings of the National Academy of Sciences of the United States of America **107**(36): 15862-15867.

Pech, L. L. and Strand M. R. (1995). "ENCAPSULATION OF FOREIGN TARGETS BY HEMOCYTES OF THE MOTH PSEUDOPLUSIA-INCLUDENS (LEPIDOPTERA, NOCTUIDAE) INVOLVES AN RGD-DEPENDENT CELL-ADHESION MECHANISM." Journal of Insect Physiology **41**(6): 481-488.

Pech, L. L*., et al.* (1994). "SEPARATION AND BEHAVIOR IN-VITRO OF HEMOCYTES FROM THE MOTH, PSEUDOPLUSIA-INCLUDENS." Cell and Tissue Research **277**(1): 159-167.

Strand, M. R. (2008). "The insect cellular immune response." Insect Science **15**(1): 1-14.

Strand, M. R. and Noda T. (1991). "Alterations in the haemocytes of Pseudoplusia includens after parasitism by Microplitis demolitor." Journal of Insect Physiology **37**(11): 839-850.

Wang, Y*., et al.* (1999). "Biological activity of Manduca sexta paralytic and plasmatocyte spreading peptide and primary structure of its hemolymph precursor." Insect Biochemistry and Molecular Biology **29**(12): 1075-1086.

Zang, X. X. and Maizels R. M. (2001). "Serine proteinase inhibitors from nematodes and the arms race between host and pathogen." Trends in Biochemical Sciences **26**(3): 191-197.

Zhang, S*., et al.* (2011). "The protein P23 identifies capsule-forming plasmatocytes in the moth Pseudoplusia includens." Developmental and Comparative Immunology **35**(4): 501-510.