CHARACTERIZATION OF THE VIRAL PROTEIN TYROSINE PHOSPHATASE-H2 AND THE METABOLIC CONSEQUENCES OF *MICROPLITIS DEMOLITOR* BRACOVIRAL INFECTION IN *PSEUDOPLUSIA INCLUDENS*

By

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(Under the Direction of Michael R. Strand)

ABSTRACT

The *Microplitis demolitor* bracovirus (MdBV) genome contains a large protein tyrosine phosphatase (PTP) gene family that encodes four enzymes with intact catalytic domains, including *ptp-H2*. Here we report expression of PTP-H2 in *Escherichia coli* cells as non-fusion or thioredoxin fusion proteins. Expressed protein was localized to hemocytes, and the purified nonfusion form of PTP-H2 exhibited classical Michaelis-Menten kinetics. Overall, our results indicate that PTP-H2 is a functional protein, specifically expressed in MdBV-infected hemocytes.

Like many PDV-carrying wasps, *Microplitis demolitor* inhibits development of its host, *Pseudoplusia includens*. Here I report that MdBV infection caused a persistent state of hypertrehalosemia, but reduced nutrient stores at a slower rate than starved larvae. Hemolymph trehalase activity was reduced during infection, and feeding cessation was not related to trypsin activity nor hypertrehalosemia. Overall, my results suggest MdBV causes alterations in metabolism apart from starvation, which prevent the host from achieving critical weight.

INDEX WORDS: polydnavirus, phosphatase, enzyme kinetics, metabolism dysregulation

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B.S., Iowa State University, 2008

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

2011

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May 2011

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Marc Brouillette

ACKNOWLEDGMENTS

Foremost, I would like to express appreciation for my advisor, Mike Strand, who taught me how to think like a scientist and deserves thanks for his tolerance of my "mercurial" nature. I am also indebted to Kevin Clark for all of his active assistance at the bench, and to Mark Brown for his approachability and willingness to offer advice.

The Strand lab quickly became my Georgian family, and I could not imagine a better group of co-workers. I offer special thanks to Jena for being the dedicated glue that holds us all together, and to all the strong women for their friendship, support, advice, and honesty. I would also like to thank to Jai for being my partner in enzyme characterization and Markus for his boundless supply of product information and expertise.

I also would like to thank my parents and Marc for their support, and also Lyric Bartholomay. I would have never have even considered an advanced entomology degree without her contagious enthusiasm.

TABLE OF CONTENTS

CHAPTER ONE

INTRODUCTION

Regulation of a given phosphoproteome has major consequences in all levels of physiology from cellular to organismal. Kinases and phosphatases are enzymes that regulate the phosphorylation status on serine, threonine, and tyrosine residues within proteins, and consequently have high potential as effectors in physiological processes (Moorhead et al., 2009; Tonks, 2006). Many disease phenotypes are the result of cell signaling interruption, and as phosphorylation cascades are usually responsible for the signal amplification and transduction, it is easy to hypothesize pathogen kinases and phosphatases as likely culprits. Several studies have linked physiological abnormalities to pathogenic kinases and phosphatases (Cornelis, 2002; Gruenheid and Finlay, 2003; Mustelin et al., 2005; Nascimento et al., 2006; Sheng and Charbonneau, 1993; Singh et al., 2003). Polydnaviridae is a family of unique insect viruses associated with many parasitoid wasps (Dupuy et al., 2006; Webb and Strand, 2005), and members associated with the wasp family Braconidae have genomes that encode multiple phosphatases (Choi et al., 2009; Desjardins et al., 2008; Falabella et al., 2006; Gundersen-Rindal and Pedroni, 2006; Lapointe et al., 2007; Provost et al., 2004; Webb et al., 2006). The braconid, *Microplitis demolitor,* carries the polydnavirus, MdBV and encodes a large multigene family of protein tyrosine phosphatases (PTPs) (Webb et al., 2006).

Like other polydnaviruses (PDVs), MdBV causes a list of pathologies in its lepidopteran host, *Pseudoplusia includens*, including immunosuppression and developmental arrest (Beckage and Gelman, 2004; Pennacchio and Strand, 2006; Schmidt et al., 2001; Strand, 2009; Webb and

Strand, 2005). Both affected systems are regulated by a complex network of cell signaling pathways, inevitably involving multiple phosphorylation events and cascades. It is not unreasonable, then, to suspect that the largest gene family in the MdBV genome, PTPs, encodes significant players in the manifestation of these conditions.

Developmental arrest is the culmination of appetite and growth suppression as well as metabolism dysregulation. The mechanisms used by MdBV to cause these conditions in its host, though, remain mostly unknown. Viral PTPs make good suspects for developmental arrest; however, there is currently no direct evidence that MdBV PTPs are linked, and much about these genes remains unknown. Recent studies have demonstrated a state of hyperglycemia in *P. includens* larvae during infection and have described the physiological timing of infection required to successfully halt host development (Pruijssers et al., 2009). In order to determine if and how PTPs are involved in these processes, it is first important to establish a basic understanding of the physiological basis for developmental arrest and to build a knowledge base about gene expression and protein function for selected viral PTPs. According to genome sequence analysis, four of the MdBV *ptp* gene family members encode functionally intact catalytic domains, *ptp-H2, -H3, -H5,* and *–N2*. *Ptp-H2* and *ptp-H3* have been demonstrated to be involved in cellular immunosuppression and to localize to focal adhesions in *Drosophila* S2 cells (Pruijssers and Strand, 2007).

Combining data about the physiological consequences of MdBV infection with PTP expression and function profiles would put us in a better position to identify specific roles of viral PTPs in host pathology; therefore, the specific objectives of my thesis are to:

1. Characterize and analyze the kinetics of a recombinant PTP-H2 protein
2. Characterize the basic metabolic effects of MdBV infection

CHAPTER TWO

LITERATURE REVIEW

I will begin by reviewing some general topics related to the following studies including PTPs, polydnaviruses, insect intermediary metabolism and their metabolites, regulatory pathways involving insulin-like peptides (ILPs) and adipokinetic hormones (AKHs), as well as the developmental biology of my host study organism, *Pseudoplusia includens*.

2.1 Protein Tyrosine Phosphatases

Several consequences of dephosphorylating substrates exist, including alterations in polypeptide folding, enzymatic activity, and protein-protein interactions (Larsen et al., 2003). Phosphatases are mostly known for their roles in resetting the players in signaling pathways after stimulation. These enzymes can also initiate signaling (Cohen, 2002) or regulate signal specificity by dephosphorylating specific sites on the cytoplasmic tail of growth factors (Larsen et al., 2003). PTPs have been demonstrated to be involved in several signaling pathways involving cell migration and adhesion (Angers-Loustau, 1999; Beltran and Bixby, 2003; Edwards et al., 1991; Larsen et al., 2003) as well as immune cell signaling (Liu et al., 2007; Pao et al., 2007) apoptosis (Hallé et al., 2007; Junttila et al., 2008), and the JAK/STAT pathway (Xu and Qu, 2008).

Pathogens commonly interfere with signaling pathways in order to disrupt the hosts' ability to mount an attack or to create environments to suit their own developmental and/or metabolic needs. Pathogenic bacteria, such as *Yersinia pestis* and *Salmonella spp.* are known to utilize PTPs, and some of these enzymes have been characterized extensively (DeVinney et al.,

2000; Guan and Dixon, 1993). Protein phosphatases have evolved in separate families, unlike their kinase counterparts, and are dissimilar in both structure and mechanism from each other (Tonks, 2006). PTPs are members of the biggest phosphatase gene family and are defined by the active site signature motif HCX_5R, whereby the cysteine is required to act as a nucleophile on given substrates (Tonks, 2006; Wei-Qing Wang et al., 2003). Originally, four main families were defined based on function: (1) tyrosine-specific, (2) VH1-like dual specificity, (3) cdc25-like, and (4) low molecular weight phosphatases (Fauman and Saper, 1996). This superfamily is now usually divided into three main groups: (1) classical (phosphotyrosine-specific), (2) dual specificity phosphatases, which can also dephosphorylate threonine/serine residues, and (3) low molecular weight phosphatases (Andersen et al., 2001). Classical PTPs contain transmembrane receptor-like proteins and non-transmembrane, cytoplasmic PTPs. MdBV PTPs are classical, cytoplasmic enzymes and, like other cytoplasmic PTPS, can be characterized by sequences that flank the catalytic domain. These regulatory domains may modulate activity either by interfering with the active site or controlling substrate specificity (Tonks, 2006). They also limit substrate availability and specificity *in vivo* by determining the enzyme's subcellular distribution and localization (Mauro and Dixon, 1994).

Ten conserved motifs ranging from low to high conservation are outlined by Anderson et al. (2001). Most importantly are Motif 1 (pTyr-recognition loop which restricts substrate specificity to pTyr), Motif 4 (core structure surrounding PTP loop), Motif 8 (WPD loop), and Motif 9 (catalytic site). X-ray crystal structures (Sun et al., 2003) have demonstrated a strong enough similarity among PTPs to assume that most, if not all, PTPs share tertiary conservation in spite of very low sequence homology. The general PTP framework consists of a seven-stranded, mixed β-sheet flanked by eight α-helices. The main characteristic, though, is the active site loop

which contains the signature HCX_5R motif (Fauman and Saper, 1996). PTPs are recognized by sensitivity to vanadate (Huyer et al., 1997) and insensitivity to okadaic acid, independence from metal ions for activity, and catalytic abolition when serine replaces cysteine at the signature motif (Fauman and Saper, 1996). The enzymatic action of PTPs involves the removal of a phosphate group from a tyrosine residue and is a two step process. Initially, the cysteine residue of the HCX_5R motif mounts a nucleophilic attack on the $P0_3$ group from a phosphotyrosine and generates a phosphocysteine intermediate (Guan and Dixon, 1991). The second step recovers the enzyme by transferring the PO_3 group to a water molecule (Hengge et al., 1995). Just as PTPs often regulate the activity of their substrates, they are subject to regulation themselves. It has been demonstrated that reactive oxygen species target PTPs, which are subject to oxidation due to the conserved reactive cysteine that has an unusually low pKα (Rhee et al., 2003).

2.2 Polydnaviruses

PDVs are unique in that they are the only known DNA viruses to contain segmented genomes, and are always encoded within wasp genomes as proviruses (Brodeur and Boivin, 2004; Webb and Strand, 2005). PDVs only replicate in wasp ovaries (Albrecht et al., 1994; Drezen et al., 2003; Pasquier-Barre et al., 2002), where they accumulate in calyx fluid and are injected with a parasitoid egg during oviposition into a lepidopteran host (Quicke, 1997). Inside the host, PDVs express their viral gene products without further replication (Drezen et al., 2003; Kroemer and Webb, 2004). The relationship between the viruses and their wasps is mutual; the wasp depends on the virus to suppress their hosts' immune system and development (Beckage and Gelman, 2004), whereas the virus depends on the wasp for propagation. PDVs are divided into two genera based on their association with parasitoid wasps. PDVs associated with braconid wasps are called Bracoviruses (BVs) while PDVs associated with Ichneumonid wasps are called

Ichnoviruses (IVs) (Lapointe et al., 2007; Webb et al., 2006) Their alliance with respective mutualists evolved independently (Webb and Strand, 2005; Whitfield and Asgari, 2003) as IVs seem to have derived from an unknown ancestor (Volkoff et al., 2010) and BVs from nudiviruses (Bézier et al., 2009); however, IVs and BVs do show important organizational similarities indicating their analogous functions in parasitism (Pennacchio and Strand, 2006). Since PDV genomes encode almost no structural proteins, their relation to viruses are controversial (Bézier et al., 2009), however, the BVs present today most likely were inherited from a virus that integrated into the genome of a common ancestor around 73.7 ± 10 million years ago (Whitfield, 2002).

M. demolitor contains the PDV, MdBV, and its infection in the host, *P. includens* is the subject of study for this proposal. Many studies have used this *M. demoliter / P. includens* system for furthering our understanding of how PDVs affect parasitoid hosts (Balgopal et al., 1996; Beck and Strand, 2003; Beck and Strand, 2007; Pruijssers et al., 2009; Pruijssers and Strand, 2007; Strand et al., 1999; Strand et al., 1997; Strand, 1994; Strand et al., 2006; Strand and Dover, 1991; Strand et al., 1992; Strand and Noda, 1991; Strand and Pech, 1995; Suderman et al., 2008).

2.3 Intermediary Metabolism in Insects

2.3.1 Trehalose Regulation

Trehalose is present in all insects, and for the majority, it rapidly moves to and from the fat body (Jungreis, 1980) as the dominant sugar circulating in the hemolymph (Becker et al., 1996; Thompson, 2003). Most tissues do not take up disaccharides; glucose uptake was demonstrated to be regulated by passive facilitated diffusion controlled by the concentration gradient between the hemolymph and gut lumen in locusts and cockroaches (Treherne, 1967).

The trehalose transporter of *Bombyx mori*, Tret1, has been demonstrated to also be regulated by passive transport but limits its expression to cells of the fat body, testes, and muscle (Kanamori et al., 2010). The static hemolymph trehalose concentration of insects generally varies between 10 and 50 mM. Lower levels are not unusual, but the lepidopteran and coleopteran insects characteristically have the highest concentrations where trehalose is frequently greater than 100 mM (Thompson, 2003). Unlike this non-reducing disaccharide, glucose is toxic at high levels (Becker et al., 1996). This is most likely why glucose is rarely at concentrations higher than 5 mM and is commonly less than 1 mM. Trehalose concentrations in insect hemolymph are usually between 1 and 2% whereas blood glucose in humans is kept around 0.1% (5.5 mM). Trehalose has the additional advantage that the osmotic effect is only half that produced by an equivalent amount of glucose. The concentration of trehalose is not controlled via homeostasis, like previously thought, but instead exists at highly variable levels depending on environmental conditions, physiological state, and nutrition. This functional regulation has been termed enantiostasis (Thompson, 2003). This type of control could possibly explain to the notorious variability of concentrations even among individuals within a species during the same developmental stage. High concentrations of trehalose and other circulating metabolites make sense for open circulatory systems, as they are necessary due to inefficiency (Friedman, 1985; Mullins, 1985). These high levels may also be needed for their multi-functional roles including cryoprotection, feeding regulation, and dessication resistance. The length of time required for diffusion from the fat body to the site of use is too long for the sugar concentrations present in the closed circulatory systems of vertebrates, and therefore calls for trehalose levels higher in order to answer urgent situations that need sustained a energy source (Weis-Fogh, 1964). Because the distance between the fat body and the site of energy consumption is a rate-limiting

factor, the fat body compensates by being advantageously disseminated throughout the body cavity of the insect.

The fat body has been described as the functional analog to the vertebrate liver as both store nutrients, detoxify harmful chemicals, and synthesize the metabolites present in circulation (Kilby, 1965). Insect fat body synthesizes many hemolymph proteins, as well as serves as the primary storage site for glycogen (Beenakkers et al., 1985; Candy, 1985) and triglycerides (Arrese and Wells, 1997; Beenakkers et al., 1985; Canavoso et al., 1998; Downer, 1985), which compose greater than 90% of the lipids in reserve (Canavoso et al., 2001). The main type of fat body cell is the trophocyte, and is actively involved in both metabolism and the storage of energy reserves. Metabolic activity, reflected by the total respiration rate of the fat body tissue, is composed of both basal and peripheral metabolism (Keeley, 1978); both of which are hormone-sensitive. The first evidence that intermediary metabolism in insects was under hormonal control was for carbohydrate mobilization in cockroaches (Steele, 1961) and lipid breakdown in locusts (Beenakkers, 1969; Mayer and Candy, 1969). Some neurohormones work by altering the cytochrome composition in mitochondria and changing which substrates are available for oxidative metabolism (Keeley, 1978). Hormones such as ecdysone and juvenile hormone (JH) affect biosynthesis pathways, while adipokinetic (AKH) and hypertrehalosemic hormones (HTH) mediate the synthesis and release of trehalose, which is exclusively synthesized by the fat body (Becker et al., 1996; Candy and Kilby, 1959, 1961).

During feeding stages, dietary sugar serves as a primary source of glucose for the formation of trehalose. Other sources of trehalose are glycogen breakdown and gluconeogenesis (Becker et al., 1996; Becker and Wegener, 1998). The $\alpha 1 - \alpha 1$ glycosidic linkage formation is catalyzed by the trehalose phosphate synthase and involves the condensation of glucose-6-

phosphate with UDP-glucose to form trehalose-6-phosphate. A second reaction, catalyzed by trehalose phosphatase, hydrolyses the phosphate ester, allowing the release of trehalose from the fat body into the hemolymph (Thompson, 2003). Since trehalose is synthesized from glucose phosphates and UTP, it is an energy consuming process (Becker et al., 1996). Glycogen synthesis uses one of the same substrates, therefore as trehalose concentrations rise, it prevents its own synthesis by inhibiting trehalose-6-phosphate synthase. The resulting increase in glucose-6-phosphate activates glycogen synthase and inhibits glycogen phosphorylase, thereby increasing glycogen synthesis (Murphy and Wyatt, 1965). Trehalase is the only known enzymatic mechanism to hydrolyze the disaccharide back into glucose. Multiple isozymes of trehalase exist in insects and are important because trehalose must be reconverted to glucose prior to use in metabolism. Trehalase activity in insect tissues is tightly controlled due to the irreversible nature of the reaction, as the enzyme will hydrolyze trehalose to depletion under some physiological conditions, and to the fact that trehalase and trehalose are present in the hemolymph together (van Handel, 1978). Compartmentalization may be one mechanism of regulation; however, studies with several species have shown trehalase activation is under hormonal control by hypotrehalosemic insulin-like peptides (ILPs) (Thompson, 2003). Other studies with cockroaches demonstrate that pH plays a big role (Downer and Matthews, 1977), and since feeding can strongly affect hemolymph pH (Harrison, 2001), it may play an important part in this regulation.

2.3.2 AKH Signaling

In insects, two neurohemal lobes, the corpora cardiaca (CC) and the corpora allata (CA) are attached to the brain. The CC stores and releases the AKH peptides produced in the brain (Gäde et al., 1997). AKHs are very small peptides, consisting of 8 to 10 amino acid residues,

which act either as hypertrehalosemic hormones or promote lipid mobilization. All are post-translationally modified, forming a pyroglutamic acid at the N-terminus and a carboxyamide at the C-terminus. Consequently, active AKH hormones are blocked peptides. AKH peptides do not vary heavily in sequence and have distinguishing characteristics such as aromatic amino acids at position 4 (usually phenylalanine, but sometimes tyrosine) and position 8 (almost always tryptophan), along with a consistent glycine at position 9, which is used in the octapeptides for amidation. Arginine, lysine, cysteine, and methionine have yet to be found in bioactive AKHs (König et al., 2005). AKH-receptor specificity is higher in species with a single endogenous peptide relative to species with multiple endogenous forms (Gäde et al., 1997). There is much functional diversity in these AKH peptides, among both different species and different life stages within a species (Gäde, 2009). In *M. sexta,* for example, the same peptide increases hemolymph trehalose concentrations in larvae and mobilizes lipids in the adult (Arrese et al., 1996; Ziegler et al., 1990; Ziegler and Schulz, 1986).

AKHs are often cited as functional homologs of vertebrate glucagon even though there is minimal gene structural conservation between them (Kodrík, 2008); therefore, an important function of AKHs is to regulate metabolism involving trehalose, glycogen, and lipids.
In conjunction to countering ILP action, these short peptides also act as stress hormones by activating catabolic reactions while inhibiting anabolic ones. Under stressful conditions, they induce mobilization of energy reserves to supply nutrients to alleviate the effects of the stress and subdue processes that are less important (Kodrík, 2008). Fat body is the critical target tissue for AKH peptides, and the signal transduction pathway is well established in this tissue (Gäde and Auerswald, 2003; Van der Horst, 2001). AKHs induce the release of carbohydrates (trehalose) and lipids (diacylglycerols) into the circulating hemolymph by binding to G_q-protein-

coupled receptors which activate phospholipase C. The inositol triphosphate product causes Ca^{2+} discharge from internal stores synergizing with an influx of extracellular Ca^{2+}. This cation concentration change induces a kinase cascade that ultimately activates glycogen phosphorylase and adenylate cyclase (Gäde and Auerswald, 2003). The regulation of trehalose synthesis by AKH peptides differs among insect species, and the effects can sometimes be difficult to demonstrate (Keeley, 1978). For example, trehalose synthesis was finally demonstrated when flies (*Phormia regina*) were injected with the hormone after being starved for 24 hours (Friedman, 1967). Also, only after the trehalose precursors from feeding are fully consumed does AKH activate glycogen phosphorylase as a source for trehalose synthesis (Gelperin, 1971). AKH peptides may be regulated at some level by a feedback mechanism, as it was demonstrated that trehalose can block the action of adipokinetic hormone by inhibiting the loading of diacyglyceride by lipophorin in the fat body (Lum and Chino, 1990). Much of the research on AKH action is based on its use in adult flight, as it releases energy stores needed for the high-energy demands of the process. AKH peptides also have stage-dependent roles, often involving carbohydrate metabolism during the immature stages, and lipid metabolism during the adult stages as reviewed in (Gäde, 2009). It was also demonstrated that CC removal gave *D. melanogaster* the ability to resist starvation, indicating that rate of energy reserve release can affect survival (Lee and Park, 2004).

2.3.3 ILPs and Insulin Signaling

Insect insulin-like peptides (ILPs) are encoded by multigene families expressed in the brain and other tissues (Wu and Brown, 2006). These secreted peptides serve a variety of functions as hormones, neurotransmitters, and growth factors. *Drosophila melanogaster* ILPs appear to play key roles in metabolism, growth, reproduction, and aging, as evidenced by

molecular genetic studies, and indicate a conservation of the well-studied insulin signaling pathway seen in other organisms (Claeys et al., 2002; Garofalo, 2002; Goberdhan and Wilson, 2003). The discovery of the first insect ILP, bombyxin, led to the subsequent identification of 38 independent ILP genes in the *Bombyx mori* genome (Kondo et al., 1996; Yoshida et al., 1998; Yoshida et al., 1997). Characterization of lepidopteran ILP gene expression has localized transcripts to medial neurosecretory cells in the larval brain (Iwami, 1995; Iwami et al., 1996a; Iwami et al., 1996b; Yoshida et al., 1998; Yoshida et al., 1997). The temporal expression of ILPs is enhanced by circulating sugar levels and decreased by starvation. ILPs are also believed to regulate JH and ecdysone production (Wu and Brown, 2006). Hemolymph ILP titer has been profiled during *B. mori* development using ILP antibodies in radioimmunoassays (Mizoguchi, 1995; Saegusa et al., 1992) and parallels with ecdysteroid titer profiles. This observation may provide evidence for functional cooperation between the two hormones (Mizoguchi, 1994). Nutrients are key signals in the regulation of growth and metabolism, as carbohydrates, fats, and amino acids provide the required energy and resources for building and expanding. When sufficient carbohydrates are available, the insulin signaling pathway is activated. Activation of receptor tyrosine kinases (RTKs) by insulin/ILP and insulin growth factors (IGFs) initiate two separate signaling pathways that regulate metabolism and growth, respectively (Wu and Brown, 2006). Vertebrates express two sister receptors each responsible for one of these pathways, whereas *Drosophila* and other insects have only one, the InR. This lone receptor regulates both processes, and several of the additional pathway players have vertebrate homologs (Klowden, 2007).

In mammals, the extracellular α-subunits of RTKs bind hormone ligands and undergo specific conformational changes that result in extensive tyrosine phosphorylation within the

kinase domains of the membrane spanning β-subunits. In this state of activation, the receptors associate at the intracellular membrane with adaptor proteins and insulin receptor substrate (IRS), which become subsequently phosphorylated. The InR and IRS proteins are mechanistically similar in terms of regulation: both are activated by tyrosine phosphorylation, and are deactivated by PTPs and serine phosphorylation (Taniguchi et al., 2006). The signaling pathways diverge when these proteins then bind and activate a growth factor receptor-bound protein (GRB2) or a phosphatidylinositol-3-kinase (PI3K), affecting growth and metabolism, respectively. GRB2 advances the mitogen-activated protein kinase (MAPK) pathway and promotes cell proliferation, while PI3K (p110) generates a membrane lipid messenger, phosphatidylinositol-3,4,5-triphosphate (PIP3) from PIP2. PIP3 continues the cascade by activating phosphoinositide-dependent protein kinase 1 (PDK1) and Akt/protein kinase B. A dual-action phosphatase, PTEN, acts to mitigate this response by promoting the reverse process while the downstream effects of forward signal transduction leads to the regulation of transcription factors related to metabolism and growth, such as S6-kinase. These activated proteins directly affect other proteins involved in insulin/ILP action, like glucose uptake and lipid synthesis, or gene expression. There are several proteins that block or promote insulin/ILP action at various points within this pathway. This conserved pathway has been explored thoroughly in several different organisms including *D. melanogaster* and has been recently reviewed (Claeys et al., 2002; Goberdhan and Wilson, 2003; Grönke and Partridge, 2010; Luckhart and Riehle, 2007; Taniguchi et al., 2006; Wu and Brown, 2006).

A feedback mechanism that is directly affected by nutrient levels is the fork-head box-containing protein (FOXO) transcription factor (Accili and Arden, 2004). High nutrient levels promote the containment of FOXO in the cytoplasm, away from DNA, which is reflected by a

decrease in the transcription of InR and IRS. Low nutrient levels permit FOXO to localize in the nucleus and initiate factors that decrease cell growth, but concomitantly sensitizing the pathway by activating InR. Regulation of insulin receptors and IRS proteins by PTPs has been demonstrated by PTP1B, which reduces the activity of these proteins by the removal of key phosphate groups required for effective signal transduction (Taniguchi et al., 2006).

Metabolite levels have been used to determine the effects of the insulin/ILP signaling pathway. Under starvation conditions, bombyxin titers decrease in the hemolymph (Masumura et al., 2000). Neck-ligated *B. mori* experienced a dose-dependent drop in trehalose in combination with enhanced trehalase activity in muscle and midgut tissues (Satake et al., 1997). The function of bombyxin is to consume carbohydrates and, unlike vertebrate insulin, not accumulate reserves (Grönke and Partridge, 2010).

2.3.5 TOR Signaling

Amino acids act as the nutritional cues for another signaling cascade that exhibits significant cross talk with the insulin signaling pathway and involves the Target of Rapamycin (TOR) protein and the activation of an overlapping transcription factor, S6-kinase. TOR is a pivotal protein within a complicated signaling network that ultimately coordinates animal size (Sarbassov et al., 2005). In mammalian systems, two TOR-interacting proteins, raptor and rictor, denote separate arms of the pathway. The raptor-TOR pathway is involved in cellular growth through S6K1 and 4E-BP1 and responds to nutrients and growth factors, whereas the rictor-TOR pathway uses Akt/PKB, PKCα, and Rho/Rac to regulate cell survival and metabolism (Sarbassov et al., 2005). How the rictor-TOR complex is regulated is unknown, but overlaps greatly with the ILP signaling pathway. The raptor-TOR complex promotes cellular growth; this arm of the TOR pathway is a crucial component for several major physiological processes, importantly

nutrient metabolism (Peng et al., 2002). In *Drosophila,* the raptor-TOR pathway determines organ and organism size by regulating cell proliferation (Colombani et al., 2003). A reduction in raptor-TOR signaling in the fat body tissue of *Drosophila* results in a notable decrease in body size. The plausible mechanism for this phenomenon is a TOR-dependent fat body production of some soluble unknown enhancers of ILP activity (Sarbassov et al., 2005).

2.4 Lepidopteran Development

2.4.1 Endocrine Regulation of Metamorphosis

Metamorphosis is a complex, endocrine-dependent process that occurs in all holometabolous insects. In general, during the normal course of development, a first instar larva emerges from an egg and grows rapidly, progressing through a series of instars defined by molts until a critical weight is attained in the final instar (Nijhout, 2003; Nijhout and Williams, 1975). ILPs are countered by ecdysone, and the balance between these two hormones determines how insects grow and ultimately determine when an organism will transform into the next instar. The growth of one tissue can be affected by another tissue, indicating that growth factors may mediate competition for resources (Klowden, 2007). Once critical weight has been attained, metamorphosis begins: the larva changes form and rests as a pupa until all transformations are completed. Finally, the sexually mature adult ecloses from the casing and seeks a mate to continue the cycle.

This major developmental transition results from a hormonal cascade involving three main hormones: juvenile hormone (JH), prothoracicotropic hormone (PTTH), and ecdysone, originally described in *Manduca sexta* (Nijhout and Williams, 1974; Truman and Riddiford, 1974). Ecdysone, which is secreted by activated prothoracic glands (PGs), is responsible for the physiological and physical changes that define the process. PTTH is responsible for PG

activation; however, these glands remain insensitive to PTTH in the presence of JH (Nijhout, 1994). Therefore JH is the master regulator, as a concomitant drop in JH titer is required with PTTH release. JH in circulation is degraded by JH esterase (Hammock, 1985), and the titers of this enzyme in the hemolymph is strongly coupled to nutritional status. Starvation induces a rapid decline of circulating JH into undetectable levels (Browder et al., 2001). The control of ecdysteroidogenesis in PGs has been recently reviewed (Marchal et al., 2010).

In Lepidoptera, larval growth is divided into two simple phases: pre-critical weight and post-critical weight. The duration of the pre-critical weight phase is dependent on a variety of both external and internal factors, including nutrition; whereas the duration of post-critical weight phase is more defined and cannot be stalled by starvation (Nijhout, 2008; Robertson, 1963). It has also been demonstrated that organism size is correlated with PG activity and size, and therefore the signal for the attainment of critical weight may depend on the status of these glands (Mirth et al., 2005). In *Drosophila* it has been demonstrated that low nutrition increases the duration of the post-critical growth period, unlike what is observed in Lepidoptera. This increase in time results in normal sized adults and is apparently due to a reduction in the production of ecdysone via downregulation of the TOR pathway in the PGs (Layalle et al., 2008). The reduction in TOR signaling lessens ecdysone secretion via attenuation of either secretion capacity or PTTH-sensitivity.

Prior studies in *M. sexta* showed that the phosphorylation of ribosomal protein S6, which normally occurs during PTTH signaling in post-critical weight PGs, was inhibited by the drug rapamycin, a TOR kinase inhibitor (Song and Gilbert, 1995; Song, 1994). Rapamycin did not, however, inhibit ecdysone production in PGs induced by dbcAMP in *Drosophila* (Layalle et al., 2008). This is interesting because the rise in cAMP is a previously identified response to PTTH,

which initiates a cascade of kinase activation, including PKA, MAPKs, PKC, and S6-kinase (Rybczynski, 2005; Rybczynski and Gilbert, 2006). The TOR pathway is considered the main activator of S6-kinase (Radimerski et al., 2002; Zhang et al., 2000), and therefore is responsible for ecdysone production regulation (Layalle et al., 2008). The link between TOR and metamorphosis bridges the concepts of nutrition and development transitions, and TOR appears to somehow link PTTH signaling to cAMP accumulation.

2.4.2 *Pseudoplusia includens* Staging Characteristics

My study organism, *P. includens,* is a soybean pest in the southern US and a good host for the parasitic wasp, *M. demolitor*. Because many of my experiments involved observing alterations in growth and development, identifying physiological ages of my specimens was a vital component in experimental design and execution. There are many environmental factors that affect metabolism and growth, consequently, chronological age is not always correlated with physiological age even under artificial conditions. Therefore, proper staging techniques that identify physiological age are of great use and have been previously established (Strand, 1990). *P. includens* larval development usually involves five instars, but can often require six. The first four (or five) instars last about two or three days, whereas the final instar exists for four days. I only used final instar larvae and focused on their developmental markers and time-weight relationships reported in Table 3 and Figure 7, respectively, in (Strand, 1990).

2.5 Background studies

2.5.1 The MdBV Genome Encodes Multiple PTPs

The MdBV genome contains 15 double stranded, circular DNA segments that range from 3.5 (segment A) to 34.5 kb (segment O) (Webb et al., 2006), and the multigene family of PTPs constitutes one of the four major protein families identified. Sequence analysis denoted four of

the thirteen MdBV *ptp* gene family members (*ptp-H2*, *-H3*, *-H5*, and *–N2*) as predicted functional enzymes (Pruijssers and Strand, 2007). Although most MdBV PTP family members are expressed in virus-infected host tissues, many family members exhibit alterations within their catalytic domains suggesting they no longer function as tyrosine phosphatases (Pruijssers and Strand, 2007). Real-time PCR reactions were run from tissue samples taken from *P. includens* at 18 hours post infection (hpi). Isolated tissues included gut, fat body, hemocytes, nervous system, and salivary glands, and their relative mRNA abundance was determined for *ptp-H1, -H2, -H3, -H5, -J1, -J2, -J3, -J4, -N1, -N2,* and *–N3.*

2.5.2 MdBV Infection Inhibits Metamorphosis and Induces Hyperglycemia and Wasting

Pre- and post-critical weight final instars of *P. includens* are differently affected by MdBV infection in terms of growth and development. Our previous studies demonstrated that MdBV infection successfully prevents the onset of metamorphosis, but only when the virus is injected before the attainment of a critical weight (Pruijssers et al., 2009). When MdBV is injected into post-critical weight (275 ± 22 mg at 36 h) larvae, development remains uninterrupted leading to pupation and often adult emergence. When MdBV is injected at a pre-critical weight, larvae stop feeding, fail to gain weight, and do not pupate. In the same study, it was also demonstrated that MdBV-infected prothoracic glands remained refractory to dbcAMP stimulation. Since cAMP is believed to be the secondary messenger of PTTH signaling, this insensitivity indicates that infected pre-critical weight larvae are unable to synthesize and/or release the ecdysone hormone required for metamorphosis. Along with arrested development, MdBV infection also results in an increase in hemolymph sugar concentration and a concomitant decrease in both lipid and glycogen stores (Pruijssers et al., 2009). These metabolic states are

persistent and extend beyond those observed during starvation, indicating a possible role for viral genes further than appetite suppression.

2.5.3 PTP-H2 and PTP-H3 are Involved in Phagocytosis Inhibition and Localize to Focal Adhesions

Expression constructs for four of the most abundantly expressed PTP family members were created and used for functional studies in S2 cells. These experiments confirmed that PTP-H2 and –H3 are functional tyrosine phosphatases, whereas PTP-H1 and PTP-J1 (which lack intact catalytic domains) are not (Pruijssers and Strand, 2007). Moreover, PTP-H2 or –H3 significantly reduced phagocytosis relative to mock controls, while co-expression of these PTPs with Glc1.8 suppressed phagocytosis to even lower levels than Glc1.8 alone. Microscopy studies further revealed that both PTPs localize to focal adhesions suggesting any of the several proteins associated with these sites (Zamir and Geiger, 2001) as potential substrates.

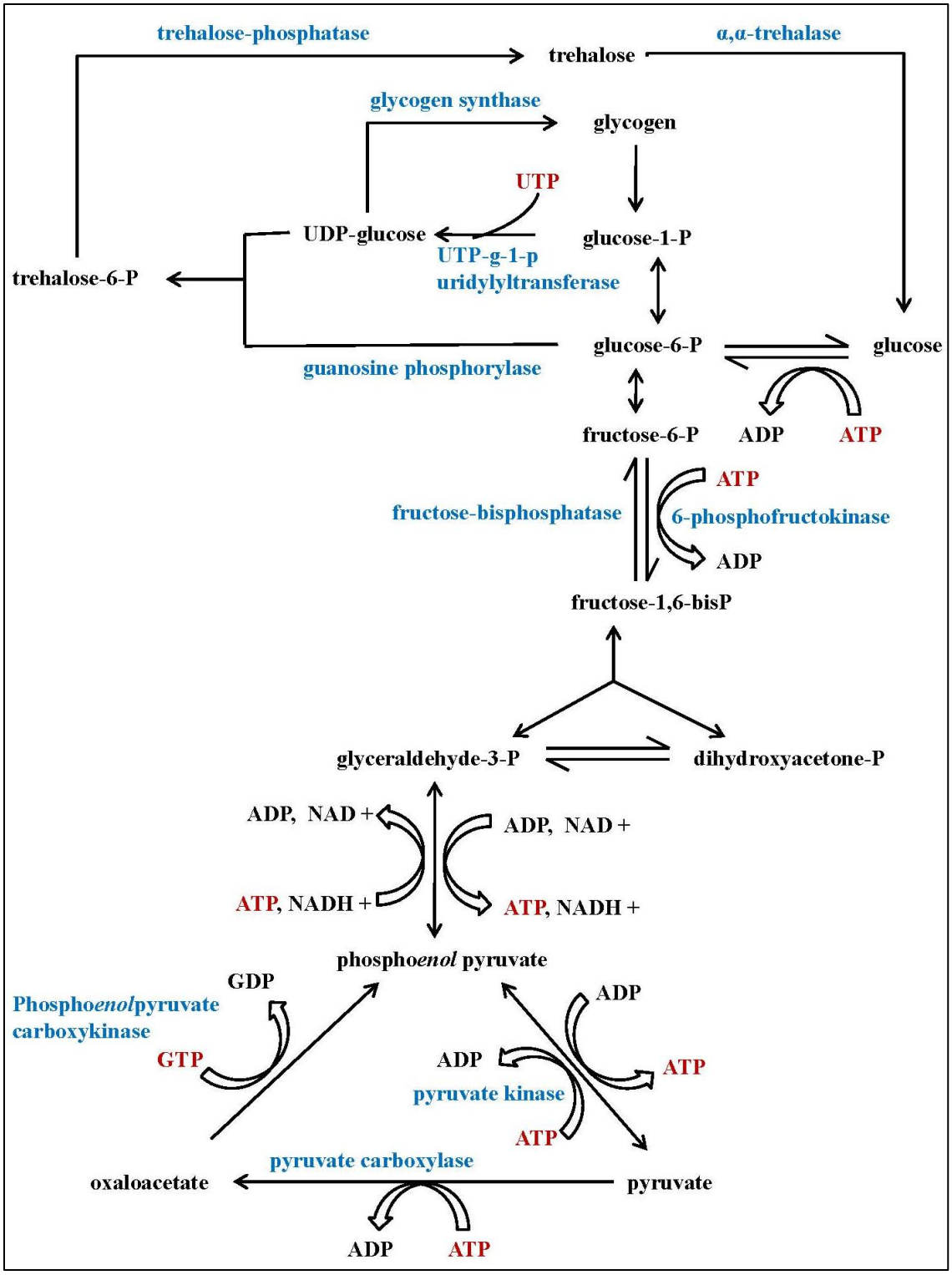

Figure 2.1 Metabolic scheme illustrating the pathways for trehalose synthesis, glycolysis, and gluconeogenesis. Figure modified from Thompson (2003) page 220.

CHAPTER THREE

CHARACTERIZATION AND KINETIC ANALYSIS OF PROTEIN TYROSINE PHOSPHATASE-H2 FROM *MICROPLITIS DEMOLITOR* BRACOVIRUS

Introduction

Tyrosine phosphorylation is widely recognized as an important mechanism regulating signal transduction involving numerous physiological processes including: proliferation, differentiation, apoptosis, gene-transcription, cell motility and morphology, as well as molecular trafficking. The importance of protein tyrosine phosphatases is demonstrated for many examples within the immune system (Mustelin, 1994; Mustelin et al., 2001; Mustelin et al., 2003; Mustelin and Taskén, 2003). Growing evidence supports the idea that PTPs are not redundant molecules that merely reset activated signaling pathways as once thought, but are often highly specific with unique functions as reviewed in (Mustelin et al., 2005).

Studies with both vertebrates and invertebrates have identified a number of functional alterations to the immune system and other physiological processes associated with abnormal PTK or PTP activity (Moorhead et al., 2009; Mustelin et al., 2005; Tonks, 2006). Some pathogens also encode PTPs, which have been hypothesized or shown to function as virulence factors that disable immunological or other host functions (Cornelis, 2002; Gruenheid and Finlay, 2003; Mustelin et al., 2005; Nascimento et al., 2006; Sheng and Charbonneau, 1993; Singh et al., 2003). Given their numerous roles in the immune system, especially in the sensing of extracellular events (Kennelly and Potts, 1996), PTPs are utilized by several pathogenic bacteria and viruses (Galyov et al., 1993; Guan and Dixon, 1990; Kaniga et al., 1996) to interfere

with such signaling. YopH from *Yersinia pestis* (Andersson et al., 1996; Cornelis and Wolf-Watz, 1997; Yao et al., 1999); Sptp from *Salmonella typhimurium* (Gruenheid and Finlay, 2003); MptpA and MptpB from *M. tuberculosis* (Av-Gay et al., 1999; Chaba et al., 2002; Koul et al., 2000; Koul et al., 2001; Peirs et al., 1997; Singh et al., 2003); and BVP from Baculovirus *Autographa californica* (Sheng and Charbonneau, 1993) are all classic examples of virulent PTPs that directly affect host proteins, mostly interfering with cell motility and/or phagocytosis.

Dephosphorylation by YopH of macrophage proteins prevents signaling pathways inducing phagocytosis activation (Andersson et al., 1996; Black and Bliska, 1997; Bliska and Black, 1995; Bliska et al., 1991; Fallman et al., 1995). YopH targets $p130^{Cas}$ and interrupts focal adhesions (Hamid et al., 1999). YopH is also known to interfere with Fyn-binding protein, Fyb (Hamid et al., 1999) along with the scaffolding protein, SKAP-HOM, in ways to inhibit macrophage adhesion (Black et al., 2000) to protect *Yersinia* from engulfment (Grosdent et al., 2002; Visser et al., 1995). In reverse, SptP of *S. typhimurium* is intimately involved with cytoskeletal rearrangements that induce internalization of bacteria into non-phagocytic cells (Fu and Galan, 1999). Virulent *Salmonella* spp. also use SptP to interfere with the activation of MAPKs (Murli et al., 2001), which are important signaling proteins in several pathways, including those activated during immune responses. The biochemical properties of MptpA and MptpB of *M. tuberculosis* are well characterized (Av-Gay et al., 1999; Chaba et al., 2002; Koul et al., 2000; Koul et al., 2001; Peirs et al., 1997), and MptpB has been demonstrated to target myelin basic protein of the cytoskeleton (Koul et al., 2000). Protozoan parasites, such as *Leishmania donovani,* have been demonstrated to utilize PTPs as indirect virulence factors, as first realized by observations involving overexpression of the human PTP1B during infection with the parasite. The PTPs appeared to regulate specific aspects of differentiation toward the

amastigote stage (Nascimento et al., 2003), indicating that enzymes used to promote their own life cycle increased their pathogenicity.

Bracoviruses utilize PTPs during infection in lepidopteran hosts, and interfere with prothoracic gland function (Falabella et al., 2006) and well as with cellular adhesion in hemocytes (Ibrahim and Kim, 2008; Pruijssers and Strand, 2007). The encapsidated MdBV genome encodes 13 predicted PTP genes (Webb et al., 2006), each, of which, shares organizational features with the non-transmembrane (cytosolic) subtype 1 (NT1) group of the classical PTP family, while expression studies show that all are expressed in infected *P. includens* except *ptp-D1* (Andersen et al., 2001; Pruijssers and Strand, 2007). Four members (*ptp-H2, -H3, -H5,* and *-N2*) have fully conserved catalytic and flanking domains consistent with being PTPs. In contrast, seven family members exhibit alterations suggesting they are pseudophosphatases (*ptp-H1, -H4, -J1, -J2, -J3, -J4*, and *-N3*), while *ptp-D1* is likely a pseudogene (Pruijssers and Strand, 2007). Prior experimental studies implicate PTP-H2 in suppression of immune cell adhesion and phagocytosis, and also in triggering apoptosis (Pruijssers and Strand, 2007; Suderman et al., 2008). However, it remains unknown whether PTP-H2 or any PDV-encoded PTP is a functional enzyme. In the current study, we produced several recombinant forms of PTP-H2 in bacteria. Enzyme assays using a phosphotyrosine peptide as substrate indicated that PTP-H2 is a tyrosine phosphatase, while immunoblotting experiments showed that PTP-H2 is expressed in several types of MdBV-infected host hemocytes.

Materials and Methods

Insects, MdBV Infection of *P. includens* and Transfection of *Drosophila* S2 Cells

M. demolitor was reared on *P. includens* as previously described (Strand, 1988). MdBV was isolated as previously outlined (Beck et al., 2007), while fifth instar hosts were infected with MdBV by injecting a physiological dose of virus (0.1 wasp equivalents) into the hemocoel using a glass needle (Pruijssers and Strand, 2007). *Drosophila* S2 cells (American Type Culture Collection) were maintained in HyQ medium (HyClone) and passaged as adherent cells in Corning 75-cm^2 tissue culture flasks (Beck and Strand, 2005). The coding sequences for *ptp-h1, ptp-h2*, and *ptp-j1* were cloned into the expression vector pIZT/V5-His and transfected into S2 cells as described (Pruijssers and Strand, 2007). Each of these recombinant proteins contained C-terminal V5 epitope tags.

Cloning and Protein Expression

For bacterial expression of PTP-H2, the full-length ORF was PCR amplified using the primers 5'- GACGACGACAAGATGAGTCGATGCAAATTCAGG-3' (forward) and 5'-GAGGAGAAGCCCGGTCTAGTTATCTTTTAGATGAAG-3' (reverse), pIZT/PTP-H2 as template, and Elongase polymerase enzyme mix (Invitrogen). Primer sequence extensions for LIC cloning are underlined. The resulting product was then directly cloned in frame with the C-terminal His tag of the vectors pET-30 Ek/LIC or pET-32 Ek/LIC (Novagen) using T4 polymerase. We generated the mutant PTP-$H2^{C236S}$, in which the predicted essential catalytic cysteine of PTP-H2 was replaced with a serine, using the Quick Change site-directed mutagenesis kit (Stratagene) together with the primers 5'-GATAGTCGTCCACTCCAGCGCTGGTGTTG-3' (forward) and 5'-CAACACCAGCGCTGGAGTGGACGACTATC-3' (reverse), and pET-30/PTP-H2 as template.

We also generated an N-terminal truncation mutant, PTP-H2ΔC, comprised of the first 117 amino acids of PTP-H2, in pET-32 Ek/LIC using a TOPO pCRII dual plasmid (Invitrogen) that contained the full-length ORF of PTP-H2 as template and the primers 5'-GACGACGACAAGATGAGTCGATGCAAATTCAGG-3' (forward) and RV 5'-GAGGAGAAGCCCGGTCTAAGTATGATGCATCCACAC-3' (reverse). Each of these constructs was confirmed by DNA sequencing, and then expressed by transforming into *E. coli* BL21 (DE3) or Rosetta-Gami 2 (DE3) cells cultured in SOC medium (0.5% yeast extract, 2% tryptone, 10 mM NaCl, 2.5 mM KCl, 10 mM $MgCl_2$, 10 mM $MgSO_4$, 20 mM glucose), supplemented with 10 mg/mL of kanamycin (BL21(DE3)) or 25 mg/mL of ampicillin (Rosetta-Gami (DE3)) to an O.D. of 1.0 at 37 °C. We added 0.1 mM isopropyl-b-D-thiogalactopyranoside (IPTG) to the cultures and grew an additional 18 h at 20 °C. Bacterial cells were harvested by centrifugation at 5000 × g for 10 min and used immediately or stored at -80 °C.

Protein Purification and Antiserum Production

Bacterial pellets from 0.8 L cultures were resuspended in 40 mL of lysis buffer (50 mM Tris-HCl pH 8.0, 300 mM NaCl, 10 mM imidazole). After addition of lysozyme (1 mg/mL) in 50 mM Tris-HCl (pH 8.0), cells were incubated on ice for 1 h followed by sonication with six, 10 s bursts at 200 W using a Branson 450 Sonifier (VWR). The lysate was then centrifuged at 10,000 × g for 30 min, followed by incubation of the supernatant with Ni-NTA Superflow beads (Qiagen) pre-equilibrated with lysis buffer. After washing, attached proteins were eluted with three column volumes of elution buffer (50 mM Tris-HCl pH 8.0, 300 mM NaCl, 300 mM imidazole), followed by desalting and concentration using a Centricon 30 spin column (Millipore, USA). We treated Trx-PTP-H2 with enterokinase (0.1, 1 and 2 units per 100 mg of recombinant protein) in rEk cleavage buffer (20 mM Tris-HCl pH 7.4, 50 mM NaCl, 2 mM

$CaCl_2$) and Thrombin (1.0, 0.4, 0.1 U/100 mg of recombinant protein) in rThrombin cleavage buffer (20 mM Tris-HCl pH 8.4, 150 mM NaCl, 2.5 mM $CaCl_2$). Proteins were quantified using the Micro BCA Protein Assay Kit (Pierce) and visualized after SDS polyacrylamide gel electrophoresis (PAGE) (4-20% precast gels (Lonza)) by staining with Coomassie Blue. Samples were also immunoblotted to PVDF membrane (Immobilon-P; Millipore), and visualized using an anti-His primary antibody (Santa Cruz,1:10,000), horseradish peroxidase-conjugated (HRP) goat anti-rabbit secondary antibody (Jackson Labs, 1:10,000), and chemiluminescent substrate (ECL Advance kit, GE Healthcare) using established methods (Beck and Strand, 2007; Lu et al., 2008).

A polyclonal antibody was produced by immunizing rabbits with 500 mg of purified Trx-PTP-H2ΔC suspended in Freund's incomplete adjuvant. Rabbits were boosted four times at three-week intervals before collection of antiserum. Antiserum was then purified by nitrocellulose-based immunoaffinity chromatography (Robinson et al., 1988). Briefly, 500 mg of rPTP-H2 was resolved on a preparative 4-20% SDS-PAGE gel, transferred to nitrocellulose (Millipore, USA) (Beck and Strand, 2007), and stained with Ponceau S. The blot was then cut into 1 × 0.5 cm strips and dried, followed by washing 1× with glycine buffer (100 mM glycine/HCl, pH 2.5) and1× TBS (20 mM Tris pH 7.4, 500 mM NaCl, 0.05% Tween-20). After blocking with 3% BSA in TBS, 2 mL of the antiserum diluted in 8 mL of TBS was added to the strips for 3 h. The strips were then washed 1× with TBS and 1× with PBS (20 mM sodium phosphate, 150 mM NaCl pH 7.2) followed by dissociation of the antibody-antigen complex using glycine/HCl buffer (pH 2.5). After neutralization with 100 mL of 1 M Tris buffer (pH 8.0), the affinity purified antibody was diluted 1:1 in glycerol and stored at -20 °C.

Substrates and Effectors

Two phosphotyrosine peptides, END(pY)INASL and DADE(pY)LIPQQG, were originally purchased from Promega (Madison, WI). We thereafter synthesized END(pY)INASL on an Applied Biosystems 433 synthesizer using standard Fmoc chemistry. Phospho-Tyr was introduced manually as the Fmoc-Tyr[PO(OBz)OH]-OH derivative (AnaSpec Inc.) by pausing automated synthesis after deprotection of Ile, and adding the resin to a 2.3 mL solution of 1 M 2-(1H-Benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU) and 1 M hydroxybenzotriazole (HOBT) in dimethylformamide (DMF) plus 333 mg (0.58 mmol) of the phospho-Tyr derivative along and 300 mL of DIEA. After 3 h with stirring, the resin was washed extensively with dimethylformamide (DMF), MeOH, and dichloromethane (DCM), dried, and the automated synthesis restarted. The final resin-peptide was cleaved and deprotected for 3 h in 2.5% water, 2.5% triisopropylsilane, and 95% TFA. After removing the resin from the reaction mixture by filtration, the peptide was precipitated in cold t-butylmethyl ether, followed by repeated ether washes and air-drying. The crude peptide was resuspended at a concentration of 1 mg/mL in DMF, acetonitrile and H_2O, and purified by injections onto an HPLC (Rheodyne 9725i manual injector, Hitachi L-6220 pump, Hitachi L-4500A photodiode array detector, and Hitachi D-7000 chromatography software) using a preparatory C18 column (10 mm particle size, 21.2 mm × 25 cm, Jupiter C18, Phenomenex Inc., Torrance, CA). The mobile phase used a linear gradient of HPLC-grade acetonitrile (Fisher) (0-50 min, 10-80%) in H_2O at 5 mL/min. Both the H_2O and acetonitrile contained 0.05% TFA. The desired peak was identified by MALDI-TOF mass spectrometry in negative mode (University of Georgia Proteomic and Mass Spectrometry Core Facility). The purified peptide was then lyophilized, weighed using a microbalance, and dissolved in sodium acetate buffer (pH 4.0) at 5 mM. 6,8-difluoro-4-methylumbiliferyl

phosphate (DiFMUP), a substrate for some PTPs and other phosphatases, and its reference standard, 6,8-difluoro-7-hydroxy-4-methylcoumarin (DiFMU) were purchased from Molecular Probes. A second non-peptidyl substrate, p-nitrophenylphosphate (pNPP), plus several compounds that could potentially affect PTP activity (tetramisole, levamisole, sodium orthovanadate, okadaic acid, heparin, EDTA, NaCl, $CaCl_2$, $CuCl_2$, $MgCl_2$, $MnCl_2$, and $ZnCl_2$) were purchased from Sigma.

Enzyme Kinetics

Activity of PTP-H2, Trx-PTP-H2 and selected mutants toward peptidyl substrates was measured in endpoint assays using a Malachite Green dye solution in half area 96-well plates (Corning) at a final volume of 50 mL (Baykov et al., 1988). Optimal pH conditions for dephosphorylation were first determined at 26 °C in 50 mL reactions containing 450 ng of enzyme, 50 mM substrate, and 50 mM buffer (HCl-Gly pH 3.0-3.5, NaAc pH 4.0-5.5, MES pH 6.0-6.5, MOPS pH 7.0, HEPES pH 7.0, Tris-HCl pH 8.0) for 5-15 min. Reactions were stopped by the addition of an equal volume of Malachite Green solution prepared as described by (Baykov et al., 1988). Plates were then incubated for 30 min at room temperature before measurement at 630 nm using a Biotek plate reader (Synergy 4). The impact of temperature (26-50 °C) and previously mentioned effectors on PTP-H2 activity were determined in sodium acetate buffer (pH 4.0). Activity against DiFMUP was assayed under identical conditions with the exception that we used 5-min continuous assays measuring fluorescence (excit 358 nm, emiss 450 nm) (Welte et al., 2005). Activity toward pNPP was also assayed under identical conditions except reactions were quenched with an equal volume of 3 M NaOH to read spectrophotometrically at 405 nm. Velocities against peptide substrate and DiFMUP were calculated from measurements derived from standard curves of free phosphate and DiFMU

respectively, while Km values were determined by nonlinear regression analysis using Excel. Trx-PTP-H2 and PTP-H2^{C236S} activity toward END(pY)INASL was measured at 26 °C in sodium acetate buffer (pH 4.0).

Immunoblotting and Immunofluorescence Staining of Host Tissues

For immunoblots, transfected S2 cells were collected from medium by centrifugation at 200 × g. Selected tissues (fat body, nervous system (brain plus ventral nerve cord), gut, and gonad) from MdBV-infected and non-infected *P. includens* larvae were collected by dissecting larvae in PBS. After removal of these tissues, the remaining pelt comprised primarily of epidermis plus cuticle was also collected. Hemolymph was collected from larvae by cutting a proleg and separating hemocytes from the plasma by centrifugation at 200 ° g. S2 cells, tissues, hemocytes, and plasma were immediately placed in SDS-PAGE loading buffer and stored at -80 °C (Beck and Strand, 2007). After determining protein concentrations, samples were resolved by SDS-PAGE, transferred to PVDF, and probed with either anti-PTP-H2 (1: 4000) or an anti-V5 primary antibody (Invitrogen, 1: 12,000). Primary antibodies were detected with HRP-conjugated secondary antibodies (1:10,000) and visualized using a chemiluminescent substrate as described above. Hemocytes from MdBV-infected and control larvae were fixed with paraformaldehyde and processed for immunofluorescence microscopy as previously outlined (Beck and Strand, 2005) by double-labeling with anti-PTP-H2 and a rabbit Alexafluor 488-conjugated secondary antibody (Invitrogen), and a commercially available anti-histone-H1 primary antibody (UpState) that labels the nuclei of all cells and a mouse Alexafluor 564-conjugated secondary antibody. Samples were examined using a Leica IRE2 inverted epifluorescent microscope interfaced (Compix, Cranberry, PA) with SimplePCI software and a

Hamamatsu digital camera for image acquisition. Final images were assembled using Adobe Photoshop.

Results

Expression of rPTP-H2, rTrx-PTP-H2, and Selected Mutants in Bacteria

Cloning of ptp-H2 into pET-30 (Ek/LIC) produced a predicted 42.7 kDa protein (PTP-H2) with an N-terminal His tag, while cloning into pET-32 Ek/LIC produced a predicted 55.0 kDa thioredoxin-PTP-H2 fusion protein that also contained a His tag (Trx-PTP-H2) (Fig. 3.1A). Following transformation and induction, analysis of bacterial extracts by SDS-PAGE indicated that proteins of correct size were expressed by pET-30/PTP-H2 in BL21(DE3) cells and pET-32/PTP-H2 in Rosetta-gami 2(DE3) cells (data not presented). Optimization studies indicated that expression of PTP-H2 and Trx-PTP-H2 was highest when bacterial cells were induced with 0.1mM IPTG for 18 h at 20 °C (data not presented). Under these conditions, we detected both recombinant proteins in the soluble exclusion body of *E. coli* cell extracts (Fig. 3.1B, C). After Ni-NTA chromatography and elution with high imidazole buffer, we transferred eluates to Centricon 30 filters for buffer exchange into distilled water and concentration. This prevented irreversible precipitation of PTP-H2 and Trx-PTP-H2. Further purification and removal of endogenous phosphate was achieved by passing each protein through a Sephadex G-25 column. Analysis of these samples by SDS-PAGE and immunoblotting confirmed the presence of near homogeneous PTP-H2 and Trx-PTP-H2 (Fig. 1B, C). From 800 mL of *E. coli* cells, we obtained on average 1.3 mg of purified, soluble PTP-H2 and 2.5 mg of purified, soluble Trx-PTP-H2. PTP-H2 and Trx-PTP-H2 were stable for over 3 months when stored in 20% glycerol (v/v) at -80 °C and 2-3 weeks when stored at 4 °C. We expressed pET-30/PTP-H2^{C236S} and pET-32/PTP-H2ΔC in BL21(DE3) under identical conditions with yields of 1-2 mg of soluble recombinant

protein per 800 mL of *E. coli* cells. PTP-H2^{C236A} and Trx-PTP-H2ΔC were then isolated by Ni-NTA chromatography and stored as described above (Fig. 3.1 B, C).

PTP-H2 Activity Varies with Substrate, pH, and Ionic Strength

Given that no PDV-encoded PTPs have been shown to be functional enzymes, the range of substrates they may be capable of dephosphorylating is likewise unknown. We noted, however, that several NT1 subtype PTPs from mammals are able to dephosphorylate peptidyl substrates and in particular exhibit preferences for phosphotyrosine-containing peptides with N-terminal acidic residues (Barr et al., 2009; Daum et al., 1993; Zhang et al., 1993). We therefore tested two substrates, END(pY)INASL and DADE(pY)LIPQQG, with these characteristics over a broad pH range, because of the tendency for PTPs to vary considerably in their pH optima (Qi et al., 2002; Tonks et al., 1988; Zhang et al., 1993). Our results indicated that PTP-H2 exhibited activity toward END(pY)INASL with a pH optimum of 4.0 (Fig. 3.2 A). In contrast, PTP-H2 exhibited no activity toward DADE(pY)LIPQQG at any pH tested (data not presented). Increasing buffer ionic strength by the addition of NaCl reduced the activity of PTP-H2 toward END(pY)INASL (Fig. 3.2B), while temperatures above 39 °C also decreased activity (Fig. 3.2 C). We next assessed whether PTP-H2 exhibited activity toward two non-peptidyl substrates (DiFMUP and pNPP) that are also recognized by a number of phosphatases including some PTPs (Harder et al., 1994; Montalibet et al., 2005; Qi et al., 2002; Tonks et al., 1988; Welte et al., 2005; Yamada and Sonobe, 2003; Zhao et al., 1994). PTP-H2 exhibited its highest level of activity toward DiFMUP at pH 4.0 and 26 °C but exhibited poor activity toward pNPP under all conditions tested (data not presented). We thus used pH 4.0 and 26 °C to generate kinetic parameters for PTP-H2 using END(pY)INASL and DiFMUP as substrates. Nonlinear regression analyses of the substrate saturation curves yielded a Vmax value of 39.5 pmol/min/mg and a Km

of 74.6 mM for END(pY)INASL, and a Vmax value of 152.3 pmol/min/mg and a Km of 24.1 mM for DiFMUP (Fig. 3.3).

Trx fusion proteins are often expressed in *E. coli* to increase the solubility of recombinant proteins. We similarly used this approach with PTP-H2, because of the difficulty encountered by some investigators in producing soluble full-length forms of some mammalian PTPs (Harder et al., 1994; Qi et al., 2002). As noted above, we successfully expressed a Trx-PTP-H2 fusion protein that yielded on average more soluble protein than obtained for PTP-H2. However, Trx-PTP-H2 exhibited much lower activity toward END(pY)INASL than PTP-H2 (Fig. 3.4). We also were unable to cleave thioredoxin from the Trx-PTP-H2 fusion protein using either enterokinase or thrombin under a variety of conditions (data not presented). Since all classical PTP family members require an essential cysteine residue in the catalytic domain for function (Moorhead et al., 2009; Tonks, 2006), we generated the mutant PTP-H2^{C236S} as a negative control for our enzymatic studies with PTP-H2 and as a candidate substrate trap for use in future studies. As expected, PTP-H2^{C236S} exhibited no activity toward END(pY)INASL (Fig. 3.4). We did not test Trx-PTP-H2ΔC for enzymatic activity, because this mutant lacked the catalytic plus several other domains required for function, and was only generated for the purposes of antibody production.

Some Known Effectors of PTPs Reduce the Activity of PTP-H2

We tested several potential effectors of PTP activity under the standard assay conditions defined above. PTP-H2 activity toward END(pY)INASL was strongly inhibited by vanadate, a potent inhibitor of mammalian PTPs (Swarup et al., 1982), as well as by the divalent cation $CuCl_2$ and heparin which also inhibit the activity of some PTPs (Fernandes et al., 2003; Zhao et al., 1994) (Fig. 3.5). Other divalent cations ($ZnCl_2$, $CaCl_2$, $MgCl_2$, and $MnCl_2$) had little or no

negative effect on activity, while $CaCl_2$ increased activity (Fig. 3.5). PTP-H2 was unaffected by tetramisole and levamisole, which are classical inhibitors of alkaline phosphatases (van Belle, 1972). PTPH2 was also unaffected by okadaic acid, sodium fluoride and sodium citrate, which inhibit some serine/threonine protein phosphatases (Harder et al., 1994), or EDTA which activates PTP1B (Tonks et al., 1988) (Fig. 3.5). However, PTP-H2 did exhibit a small reduction in activity in the presence of tartrate, which inhibits some acid phosphatases (van Etten, 1982) (Fig. 3.5).

PTP-H2 Expression is Restricted to Hemocytes in MdBV-infected Hosts

Previous relative quantitative (rq) RT-PCR studies indicated that PTP family members are differentially expressed in MdBV-infected host larvae with transcript abundance for *ptp-H2, -J1, -J2, J4, -N1,*and *-N2* being highest in hemocytes, while transcript abundance for *ptp-H1, -H3, -H5*, and *-J3* are highest in the fat body or nervous system (Pruijssers and Strand, 2007). We further found that *Drosophila* S2 cells transfected with the expression construct pIZT/PTP-H2 exhibited elevated levels of tyrosine phosphatase activity while cells expressing *ptp-H1* and *ptp-J1*, which are structurally similar to *ptp-H2* but contain alterations that suggest they are pseudophosphatases, did not (Pruijssers and Strand, 2007). Here we repeated our earlier transfection experiments in S2 cells to assess whether the antibody we generated against Trx-PTP-H2ΔC recognized PTP-H2. Following SDS-PAGE and immunoblotting, we found that the antibody strongly and specifically recognized PTP-H2 but not PTP-H1 nor PTP-J1, whereas an anti-V5 antibody recognized each recombinant protein through their shared epitope tag (Fig. 3.6). We then conducted immunoblotting experiments with tissue samples prepared from MdBV-infected and control (noninfected) larvae. Based on primary structure, the predicted molecular mass of wild-type PTP-H2 is 37.9 kDa (Pruijssers and Strand, 2007). Our anti-PTP-H2 antibody

detected a band of similar mass in our infected hemocyte lysate sample but unlike our experiment with S2 cells, we also detected a larger 44 kDa band of similar intensity and a weaker band of ca. 62 kDa (Fig. 3.7A). In contrast, anti-PTP-H2 did not recognize any proteins in hemocyte lysates from non-infected *P. includens*, nor did it recognize proteins in other tissue samples from infected larvae (Fig. 3.7A).

Like other Lepidoptera, circulating hemocytes in *P. includens* consist of four subpopulations (granulocytes, plasmatocytes, oenocytoids, and spherule cells), which are distinguished from one another by morphological, molecular, and functional markers (Gardiner and Strand, 1999; Lavine and Strand, 2002; Pech and Strand, 1996; Strand, 2008). In the absence of infection, granulocytes and plasmatocytes comprise 62% and 31% of the total hemocyte population respectively and rapidly adhere to culture plates in vitro, while spherule cells and oenocytoids comprise only 5% and 2% of the hemocytes in circulation and are non-adhesive (Gardiner and Strand, 1999; Strand and Noda, 1991). MdBV infection, however, prevents granulocytes and plasmatocytes from adhering to foreign surfaces, which results in these cells remaining rounded when placed into culture and are thus distinguished by their differences in size and by use of available granulocyte and plasmatocyte-specific antibody markers (Gardiner and Strand, 1999; Strand and Noda, 1991). To assess the distribution of PTP-H2 in hemocytes and assess whether any differences exist in PTP-H2 expression among hemocyte types, we double-labeled hemocytes from 18 h post-infection host larvae with anti-PTP-H2, and a histone-H1 antibody. Visual inspection of cells by epifluorescent microscopy indicated that 92% of hemocytes from infected larvae were labeled by anti-PTP-H2, whereas no hemocytes from non-infected larvae were labeled (Fig. 3.7B-F). Inspection of cells at higher magnification further

indicated that anti-PTP-H2 labeled the cytosol of granulocytes and plasmatocytes in a punctate pattern, whereas oenocytoids were uniformly labeled throughout the cytoplasm (Fig. 3.7D).

Discussion

Mammals encode more than 100 PTP genes, and their immune cells express more of these genes than any other cell type with the possible exception of neurons (Alonso et al., 2004; Mustelin et al., 2005). Several classical PTPs from mammals and associated pathogens have been functionally characterized and summarized (Barr et al., 2009; Moorhead et al., 2009; Tonks, 2006). Insects and selected pathogens like PDVs also encode multiple PTPs, but much less is known about their roles in immunity or other physiological processes (Baeg et al., 2005; Morrison et al., 2000; Strand, 2010; Zettervall et al., 2004). To our knowledge no insect-encoded PTP has been characterized in terms of enzymatic activity. Prior studies with a predicted PTP, designated PTPA, from *Cotesia congregata* bracovirus (CcBV) and PTP-H2 and PTP-H3 from MdBV provide indirect evidence for functional activity by showing that overexpression of each protein elevated tyrosine phosphatase activity in crude insect cell extracts relative to control cells (Provost et al., 2004; Pruijssers and Strand, 2007). However, direct evidence demonstrating that these proteins are functional PTPs requires data using purified native or recombinant enzymes bioassayed under controlled conditions.

Here we used *E. coli* to express and purify a soluble, full-length form of PTP-H2 from MdBV. Our results indicate that PTP-H2 exhibits Km values using DiFMUP as a substrate that are comparable to a number of PTPs from mammals including PTP1B, CD-45, and SHP-1 (summarized by (Montalibet et al., 2005)). PTP-H2 activity is strongly inhibited by the well-known PTP inhibitor, vanadate, but is largely unaffected by inhibitors of acid, alkaline, and Ser/Thr phosphatases. Similar to known NT1 subtype members, PTP-H2 also displays an

optimum pH in the acidic range with increasing buffer ionic strength reducing activity due presumably to weakened electrostatic interactions between the enzyme and substrate (Qi et al., 2002; Tonks et al., 1988; Zhang et al., 1993). Cytosolic PTPs, including PTP-H2, contain multiple domains with identified or predicted roles in enzyme regulation, subcellular targeting, and substrate recognition (Barr et al., 2009; Pruijssers and Strand, 2007; Seet et al., 2006; Tonks, 2006). While certain classical PTP subtypes, such as members of the R3 and R5 subgroups, are extremely promiscuous and dephosphorylate a diversity of protein substrates, other subtypes exhibit more or highly restricted activity spectra (Barr et al., 2009; Tonks et al., 1988). Specific activity also varies greatly across the PTP superfamily (Tonks, 2006).

The ability of PTP-H2 to dephosphorylate END(pY)INASL is fully consistent with earlier results indicating that hemocytes from MdBV-infected *P. includens* and *Drosophila* S2 cells transfected with pIZT/PTP-H2 exhibit elevated levels of PTP activity using END(pY)INASL as a substrate, whereas no increase in PTP activity was detected using DADE(pY)LIPQQG (Pruijssers and Strand, 2007). Our results are also consistent with the apparent preference of NT1 subgroup members for substrates containing acidic residues N-terminal to the phosphorylation site. The lack of activity toward DADE(pY)LIPQQG, however, indicates that N-terminal acidic residues alone do not fully predict substrate preferences for PTP-H2, and that the pY recognition loop, WPD loop, or other flanking domains influence substrate preferences of this enzyme. The importance of flanking domains for function is further suggested by the large reduction in activity that occurred when PTP-H2 was expressed as a trx fusion protein (Trx-PTP-H2). A similar loss of activity occurred with expression of human PTP-MEG2 as a glutathione-S-transferase (GST) fusion protein (Qi et al., 2002). Other post-translational mechanisms for regulating PTP function include dimerization of receptor PTP family members

and reversible oxidation of both non-receptor and receptor PTPs (Tonks, 2006).

Tissue or cell specific expression provides another level of control for PTP function. Previous studies indicated that ptp-H2 transcript abundance is highest in host hemocytes while our immunoblotting results during the current study fully support this result by showing that we only detect PTP-H2 in infected hemocytes. Our results with S2 cells indicate that our anti-PTP-H2 antibody strongly recognizes PTP-H2 and does not recognize selected other MdBV PTP family members. However, given the observation that anti-PTP-H2 also detects a second protein in infected hemocytes that is larger than the predicted mass of PTP-H2 suggests the possibility that this antibody recognizes another MdBV PTP family member, another MdBV gene product selectively expressed in hemocytes, a host protein significantly up-regulated in infected hemocytes, or some alteration of PTP-H2 itself. Currently, we cannot distinguish between these possibilities although cross-reaction with another MdBV encoded PTP seems unlikely given that the largest member of the family (PTP-H1) has a predicted mass of 39.5 kDa and was not recognized by anti-PTP-H2 when expressed in S2 cells. Given this cross-reactivity, we have to interpret our immunocytochemical data cautiously. Nonetheless, assuming the signal we detect is substantially PTP-H2, our results suggest most hemocytes express this protein which is fully commensurate with prior results showing that 98% of hemocytes are infected by MdBV after parasitism by *M. demolitor* or injection of a physiological dose of virus (Beck et al., 2007; Trudeau et al., 2000). Interestingly, however, the intensity of labeling by our anti-PTP-H2 antibody is greatest in oenocytoids, which are a primary source of phenoloxidase that is required for melanization of hemolymph (Kanost and Gorman, 2008). Thus, in addition to playing a role in disabling adhesion and phagocytosis of granulocytes and plasmatocytes (Pruijssers and Strand, 2007), it is possible that PTP-H2 has other functions in hemocytes like oenocytoids including

possibly contributing to suppression of melanization (Beck and Strand, 2007; Lu et al., 2010; Lu et al., 2008). Now that we have established that PTP-H2 is a functional tyrosine phosphatase selectively expressed in insect immune cells, a key future priority is to identify its natural substrates so that we can understand how this enzyme contributes to immunosuppression.

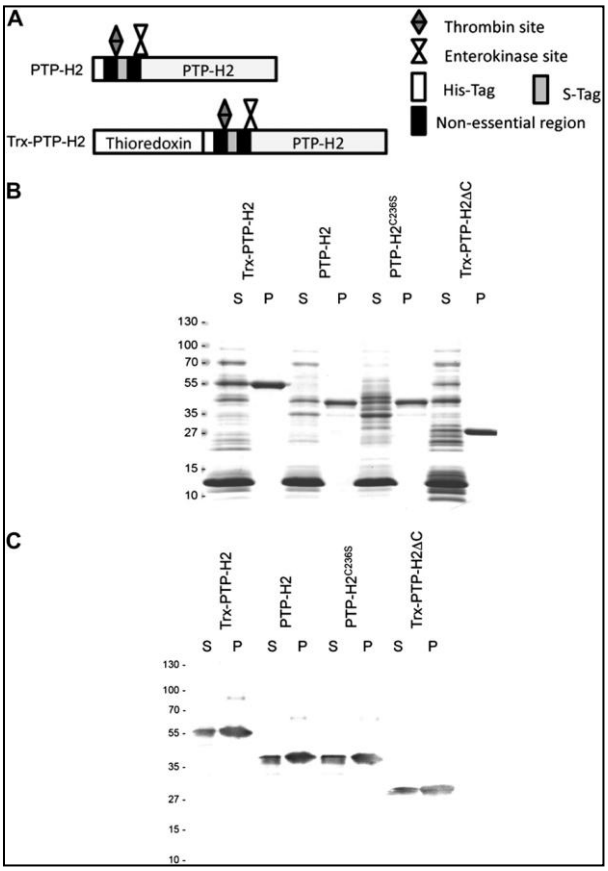

Figure 3.1. Expression and purification of PTP-H2, Trx-PTP-H2, PTP-H2^{C236S}, and Trx-PTP-H2ΔC. (A) Schematic of recombinant PTP-H2 and Trx-PTP-H2 indicating location of epitope tags and cleavage sites for removal of thioredoxin. PTP-H2^{C236S} and Trx-PTP-H2ΔC were generated from these parent constructs (see text). See Pruijssers and Strand (2007) for details on domain structure of PTP-H2 itself. (B) For each expression construct, the soluble exclusion body of *E. coli* was collected after induction with IPTG and the corresponding recombinant protein purified by Ni-NTA chromatography. The soluble lysate (S) and

corresponding eluate after Ni-NTA chromatography (P) was then subjected to SDS-PAGE under reducing conditions followed by staining with Coomassie Brilliant blue. (C) Corresponding immunoblot transferred to PVDF membrane and probed with an anti-His antibody which recognizes the epitope tag present in each recombinant protein. Molecular mass markers (kDa) are indicated to the left of the gel (B) and blot (C).

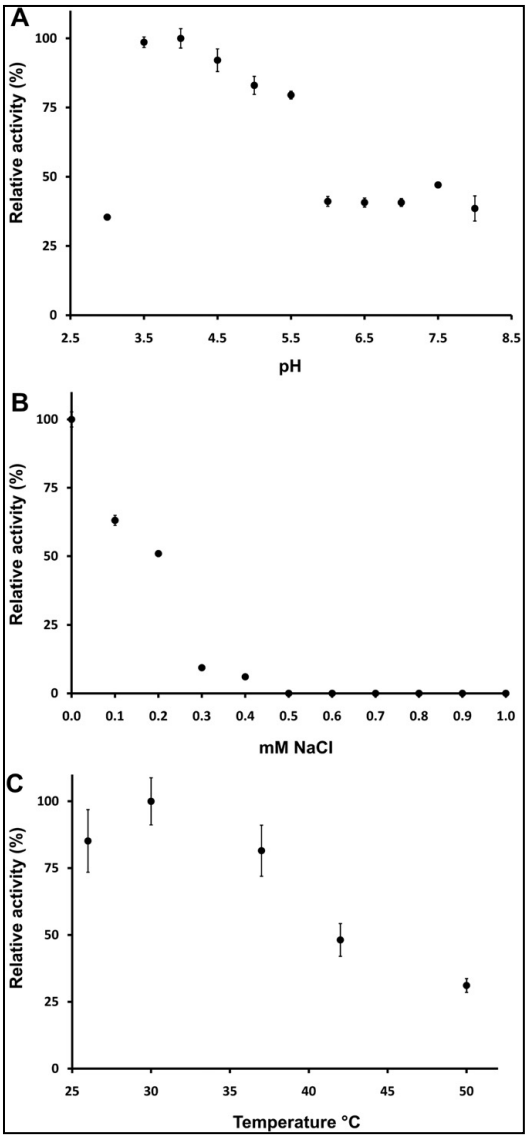

Figure 3.2. Effects of pH (A), ionic strength (B), and temperature (C) on the activity of PTP-H2. Enzyme activity was measured at 10 min in 50 µL reaction volumes that contained 50 mM buffer, 50 µM ENDpYINASL, and 0.45 mg PTP-H2. (A) Reactions were at 26 °C at pH

3.0-8.0 in the following buffers: pH 3.0-3.5 Gly-HCl, pH 4.0-5.5 NaAc, pH 6.0-6.5 MES, pH 7.0 MOPS, pH 7.5 HEPES, pH 8.0 Tris-HCl. (B) Reactions were at 26 °C in NaAc buffer (pH 4.0) containing 0-1 M NaCl. (C) Reactions were in NaAc buffer pH 4.0 at 26-50 °C. Relative activity for each treatment is reported as the mean from assays conducted in triplicate. Error bars ± 1 SD.

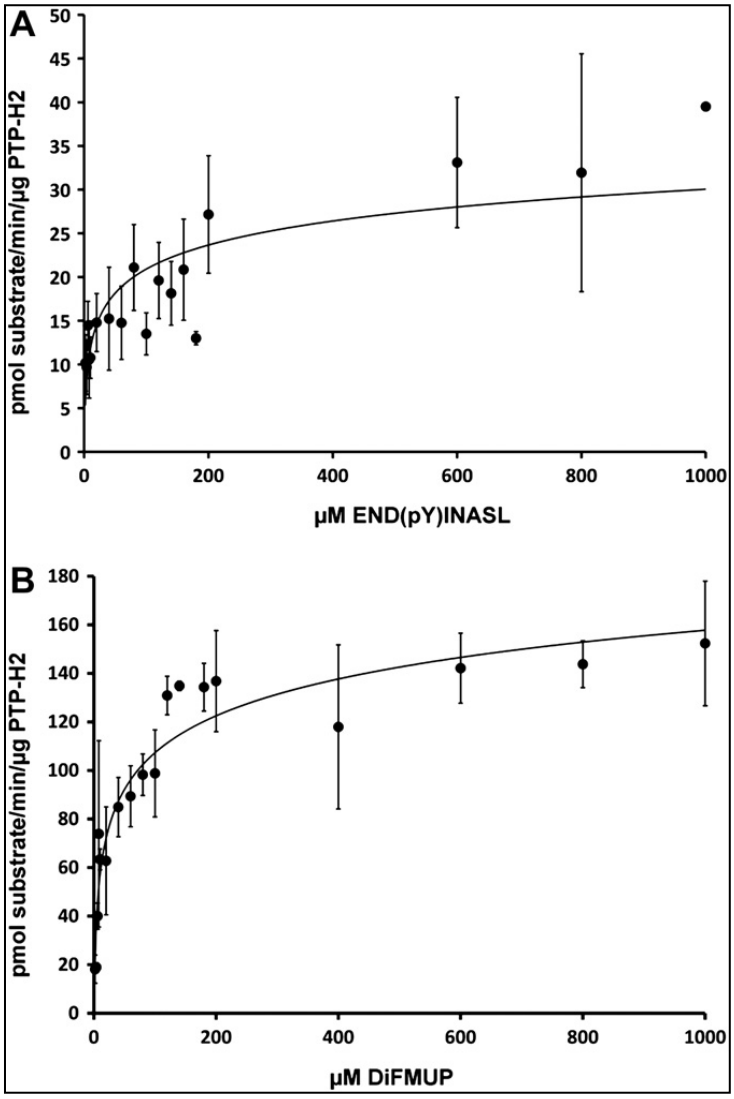

Figure 3.3. Substrate saturation curves for PTP-H2 measured at 10 min. Reactions were performed at 26 °C in 50 µL volumes containing 50 mM buffer, 0.45 mg PTP-H2, and either 0-1 mM END(pY)INASL (A) or 0-1 mM DiFMUP (B). (A) Reactions were measured at 10 min

with 0-1 mM END(pY)INASL. (B) Reactions were measured in a continuous assay for 5 min with 0-1 mM DiFMUP. Activity is reported as the mean from assays conducted in triplicate. Error bars = 1 SD.

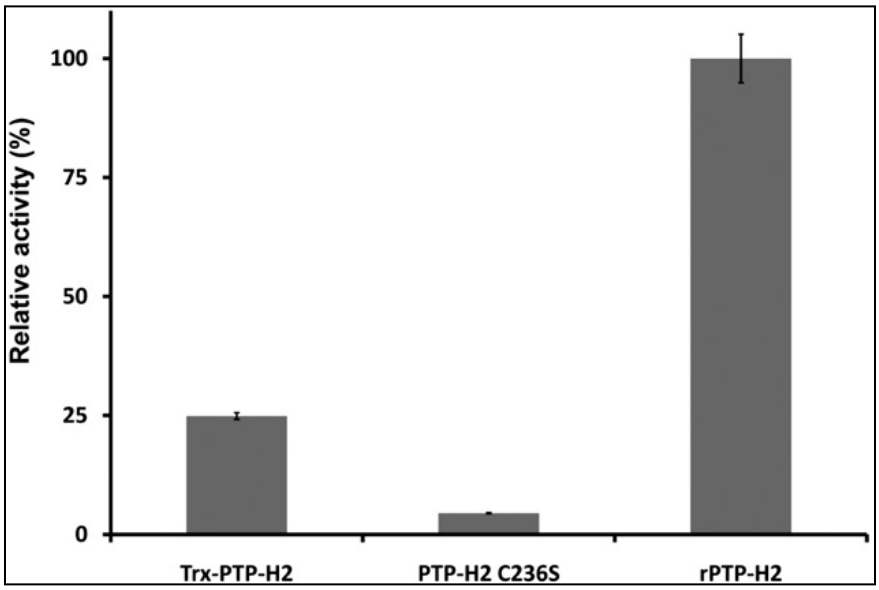

Figure 3.4. Phosphatase activity of Trx-PTP-H2 and PTP-H2^{C236S} relative to of PTP-H2 (control). Activity was measured at 10 min at 26 °C in 50 µL reaction volumes containing 50 mM NaAc buffer (pH 4.0), 50 µM END(pY)INASL, and 0.45 mg PTP-H2. Relative activity for each treatment is reported as the mean from assays conducted in triplicate. Error bars = 1 SD.

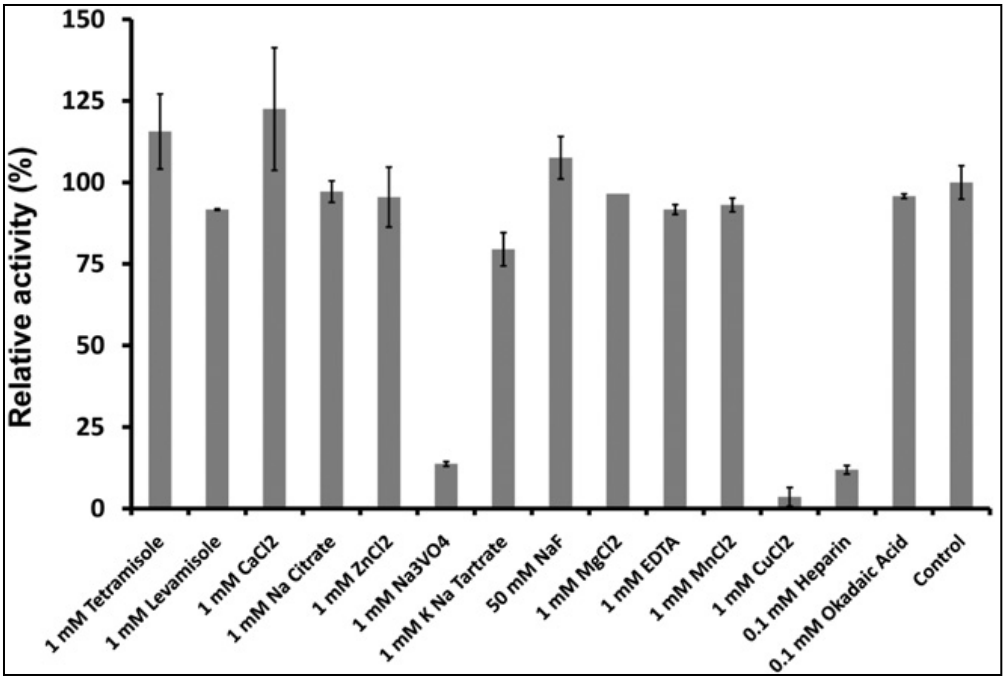

Figure 3.5. Some effector molecules reduce PTP-H2 activity. Activity was measured as described in Fig. 3.4. Each effector molecule was added to the reaction prior to addition of PTP-H2. Relative activity for each treatment is reported as the mean from assays conducted in triplicate. Error bars = 1 SD. The control value shows PTP-H2 activity (100%) in the absence of any effector molecule.

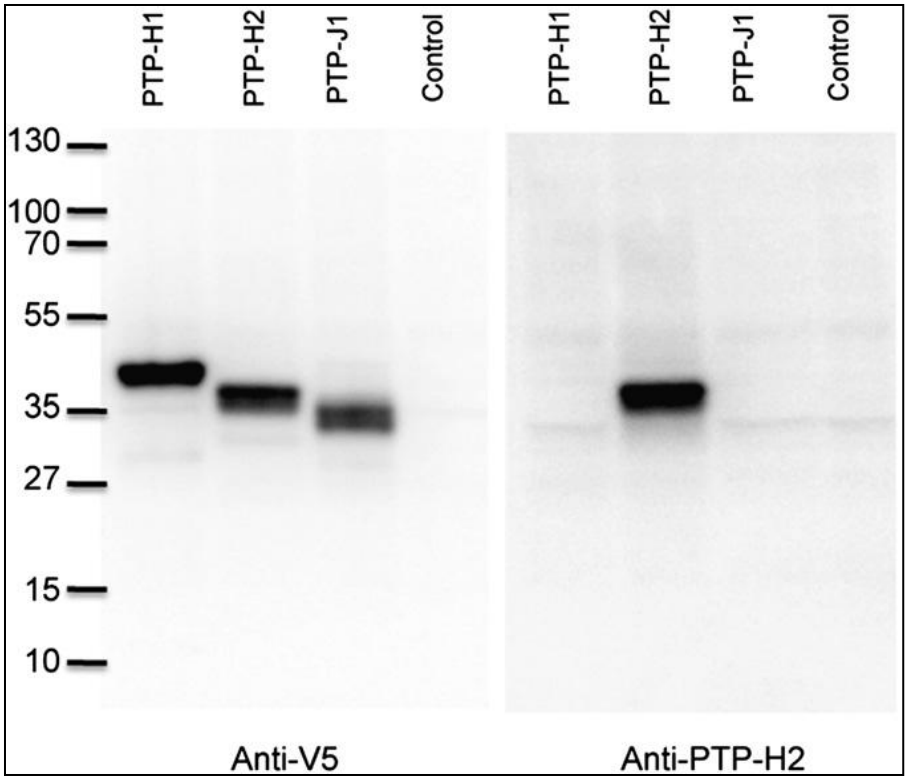

Figure 3.6. Anti-PTP-H2 recognizes PTP-H2 but not other selected PTP family members encoded by MdBV. pIZT/PTP-H1, pIZT/PTP-H2, and pIZT/PTP-J1 were transfected into S2 cells and 36 h post-transfection S2 cell lysates were prepared by adding SDS-PAGE sample buffer and boiling 3 min. The mixtures were then subjected to SDS-PAGE under reducing conditions and immunoblotting using an anti-V5 antibody (left) or anti-PTP-H2 (right). Control lanes contain S2 lysates from non-transfected cells. Molecular mass markers (kDa) are indicated to the left.

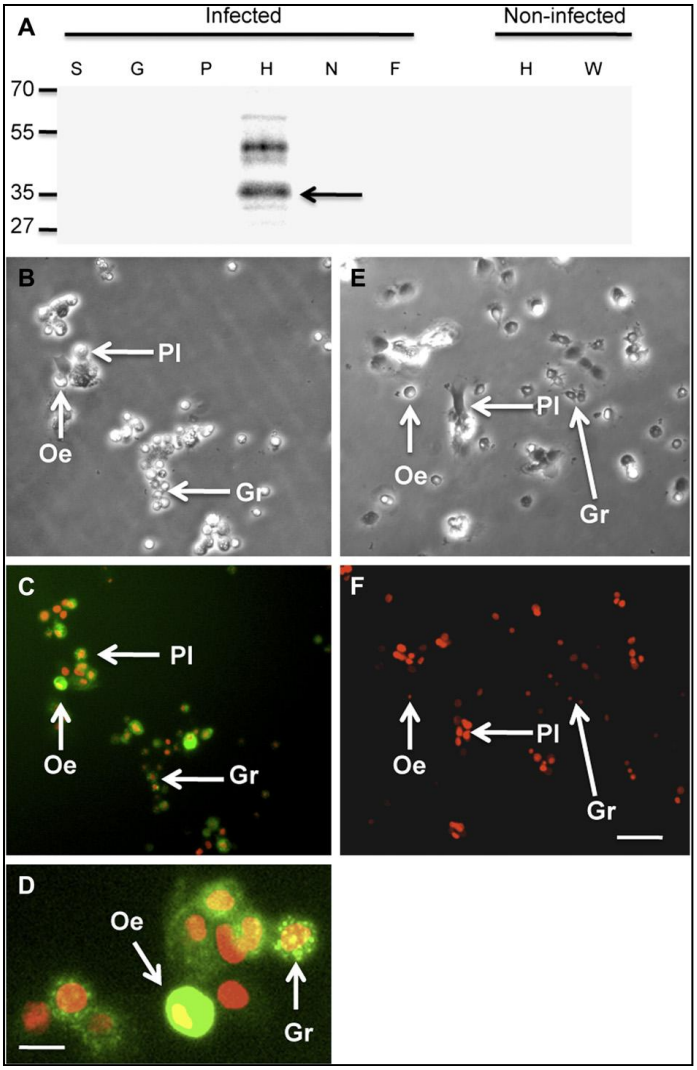

Figure 3.7. PTP-H2 is specifically detected in hemocytes from MdBV-infected *P. includens* larvae. (A) *P. includens* fifth instars were infected with 0.1 wasp equivalents of MdBV. Eighteen hours post-infection, the salivary glands (S), gut (G), plasma (P), hemocytes (H), nervous system (N) and fat body (F) were collected from three larvae in SDS-PAGE sample buffer. Hemocyte (H) and whole body (W) samples were similarly collected from non-infected larvae. Following SDS-PAGE under reducing conditions, samples were immunoblotted using anti-PTP-H2 antibody. Arrow to the right of the infected hemocyte lane indicates a band corresponding to the predicted mass of PTP-H2. Molecular mass markers (kDa) are indicated to the left of the blot. Phase contrast (B) and corresponding epifluorescence (C) micrograph of

hemocytes collected from a host larva 18 h post-infection with MdBV. Cells were then double-labeled with PTP-H2 (green) and histone-H1 antibodies (red). Selected plasmatocytes, oenocytoids, and granulocytes are indicated. Note in (C) that most hemocytes are labeled by anti-PTP-H2. (D) Higher magnification epifluorescence micrograph of infected hemocytes. Note the strong, uniform cytoplasmic labeling of the oenocytoid and punctate labeling pattern of the granulocyte shown in the image. Scale bar in (D) equals 30 mm. Phase contrast (E) and corresponding epifluorescence (F) micrograph of hemocytes collected from a non-infected host larva. Cells were double-labeled as in (B) and (C). Note that the nuclei of all hemocytes are labeled by anti-histone H1 but no hemocytes are labeled by anti-PTP-H2. Scale bar in (F) equals 200 mm with the images shown in (B), (C) and (E) being of the same magnification.

CHAPTER FOUR

METABOLIC CONSEQUENCES OF *MICROPLITIS DEMOLITOR* BRACOVIRAL INFECTION IN *PSEUDOPLUSIA INCLUDENS*

Introduction

Hymenopteran parasitoids that carry polydnaviruses are among the most important mortality agents of insects (Pennacchio and Strand, 2006). (Lawrence, 1986) described all parasitoid wasps as members of one of two groups based on their interaction with their respective hosts: regulators and conformers. The regulators, such as *Microplitis demolitor*, induce developmental arrest or alternation in the host usually by interfering with hormone signaling, while the conformers do not affect the host's development. A direct action of a PDV on host endocrine targets has remained largely unconfirmed, except for *Campoletis snorensis* PDV, which destroys host prothoracic glands in *Heliocoverpa virescens* (Dover et al., 1988). This demonstration is looked upon with caution, though, because the host prothoracic glands differ in their sensitivity to cytopathic effects in a stage-dependent manner (Dover et al., 1995). Parasitized hosts usually show reductions in circulating ecdysteroid titers that are associated with slowed growth and failure to pupate (Beckage and Gelman, 2004; Pennacchio and Strand, 2006). The temporal correlation between PDV expression and the onset of host developmental arrest make it tempting to speculate, although PDV transcripts have yet to be demonstrated to have a direct effect on such alterations in endocrinology. The majority of the current literature describing the physiological effects observed during PDV infections have overlooked the simplest hypothesis that failure to pupate is an indirect response to alterations in host growth or

nutritional physiology (Pennacchio and Strand, 2006; Thompson, 1993) and put all efforts into searching for PDV-produced molecules that are directly responsible for the reductions in ecdysteroid titers by altering control of prothoracicotropic hormone (PTTH) (Coudran et al., 1990; Falabella et al., 2003; Grossniklaus-Burgin et al., 1998; Kelly et al., 1998; Pennacchio et al., 1998a; Pennacchio et al., 2001; Tanaka et al., 1987; Tanaka and Vinson, 1987; Zitnan et al., 1995).

Lepidoptera, as other insects, regulate development by sensing physiological age as determined by nutritional status along with other environmental factors and hormones (Nijhout, 1994, 2003). Insects do not grow as adults, and are therefore responsible for acquiring all necessary nutrients for growth during the larval or nympal stages. Studies with Diptera indicate the final size an insect achieves is regulated by the prothoracic glands (Mirth et al., 2005), and is a function of a species-specific threshold (critical weight) that must be attained during the final instar to molt to a pupa without developmental delay (Nijhout and Williams, 1975). When an immature insect attains such a critical weight, the prothoracic glands become sensitive to PTTH, and actively release ecdysteroids upon receiving the signal. These neuropeptides induce the cessation of feeding and initiate premetamorphic behaviors, while a second release of ecdysteroids causes the larva to pupate (Gilbert et al., 2002; Riddiford, 1976). Less understood is how insects assess when a size threshold like critical weight has been reached. Positive regulators include nutrient acquisition and metabolic activity mediated through the insulin and TOR signaling pathways, whereas juvenile hormone (JH), ecdysteroids and the glucagon-like adipokinetic hormones (AKHs) can repress growth under conditions of stress such as starvation (Baker and Thummel, 2007; Mirth and Riddiford, 2007; Oldham and Hafen, 2003; Takaki and Sakurai, 2003; Truman et al., 2006).

Lepidoptera larvae must feed almost incessantly, except during molting periods, in order to accumulate large enough amounts of reserves for pupal and adult life (Ziegler, 1985), and since all animals must persistently expend energy for even the most basic metabolic processes, they resort to the utilization of energy reserves during even very brief periods of fasting. Considering that *P. includens* larvae cease feeding completely during MdBV infection, understanding the effects starvation has on metabolism are important to determine in order to identify the physiological effects, other than appetite suppression, that are directly targeted in host metabolism during infection. During starvation, hemolymph glucose levels decrease rapidly. Injections of glucose into starving larvae can prevent the activation of fat body glycogen phosphorylase (Siegert and Ziegler, 1983b; Ziegler, 1985) therefore crashing glucose concentrations may trigger the secretion of AKH from the CC, which in turn activates glycogen phosphorylase (Siegert and Ziegler, 1983a; Ziegler, 1985). The percentage of phosphorylase in the active form doubles within only one hour of starvation and increases to a maximum of *ca* 45% by three hours followed by a slow decline to levels observed during feeding periods. Starvation also causes a quick decline in synthetic activity, as demonstrated by the strong decrease in respiration (Ziegler, 1985), while the energy charge remains high at least for 24 hours. Gluconeogenesis from injected amino acids is even less active (Roth and Ziegler, unpublished NMR data) in starving larvae.

One metabolic consequence of PDV infection repeatedly demonstrated in earlier studies is induced hypoglycemia, often more specifically, hypertrehalosemia (Dahlman, 1975; Dahlman and Vinson, 1975; Dahlman and Vinson, 1980; Thompson, 1982). Altered carbohydrate levels, however, are not uncommon in invertebrate hosts during infections by metazoan parasites. Although the specificities of such changes are variable, depletion of carbohydrate reserves is

regularly observed during parasitization by schistosomes (Gordon et al., 1971; Ishak et al., 1975; Rutherford and Webster, 1978; Schwartz and Carter, 1982), and trematodes (Manohar and Venkateswaro Rao, 1976, 1977a, b). Previous studies on nutritional physiology established that parasitized hosts also have a significant reduction in food conversion efficiencies (Thompson, 1983), suggesting that increased gluconeogenesis may be responsible for the elevated carbohydrate reserves of parasitized *Trichoplusia ni.*

As summarized in chapter two, previous work in *P. includens* determined that MdBV infection causes a rapid degradation of energy reserves while inducing a persistent state of hyperglycemia (Pruijssers et al., 2009). The foremost purpose of my work was to further investigate the metabolic alterations created during this infection. My results indicated that the utilization of energy reserves in the form of glycogen and triglycerides is actually slowed during infection compared to starvation alone while the hyperglycemia is a reflection of the inability of an infected larva to hydrolyze the disaccharide for use.

Materials and Methods

Insects and Host Staging

Pseudoplusia includens Walker 1858 larvae were reared on an agar-based artificial diet in plastic rearing cups at 27±1 °C with a light: dark photoperiod of 16h: 8h as described previously (Strand, 1990). For some experiments, *Pseudoplusia includens* larvae were physiologically staged using previously established morphological criteria (Strand, 1990). Other experiments used larvae with individually recorded ecdysis times that were subjected to treatment based on number of hours past eclosion during final instar, while other experiments were based on total larval mass alone. *Microplitis demolitor* Wilkinson 1934 is a solitary endoparasitoid that

produces the polydnavirus, MdBV, which accumulates in the calyx fluid of the ovaries. Wasps were reared as described previously by (Strand, 1988).

MdBV Collection and Host Infection

MdBV was collected from wasps as previously described (Beck and Strand, 2003; Strand et al., 1992). The amount of MdBV collected from the reproductive tract of a single adult female was defined as one wasp equivalent. One wasp equivalent on average equals 1 x 10^{10} virions and wasps inject 0.1-0.01 wasp equivalents per host during oviposition (Beck et al., 2007). For this study, 0.1 wasp equivalents of MdBV suspended in TC100 cell media (Sigma, St. Louis, MO, USA) were injected into prolegs of CO_2 anesthetized *P. includens* final instars at specific times post-ecdysis using a pulled glass needle mounted on a micromanipulator (Beck et al., 2007).

Analysis of Metabolite Reserves

P. includens larvae were anesthetized on ice before hemolymph was collected by cutting a proleg into anticoagulant buffer (98 mmol l^{-1} NaOH, 146 mmol l^{-1} NaCl, 17 mmol l^{-1} EDTA, 41 mmol l^{-1} citric acid; pH 4.5; 1:1 v/v). Hemocytes were removed by centrifugation for 1 min at 1000 × g. The supernatant (plasma) was diluted 5 fold in water, boiled 5 min, centrifuged 2 min at 15,000 × g, and stored at 4 °C. A small sample of plasma was removed prior to boiling and diluted 50 fold in water to use in a Bradford assay using Coommassie reagent (Pierce) for total protein content determination. Hemolymph blood sugar titers were measured using a glucose oxidase assay (Sigma) according to manufacturer's instructions. Briefly, boiled plasma samples were divided into two parts: one for glucose measurement and the other for trehalose measurement. The latter samples were diluted 10 fold in phosphate-buffered saline (PBS) to a total volume of 25 µL and added to an equal volume of porcine kidney trehalase (Sigma; 1:20 in PBS) into Eppendorf tubes and incubated at 37 °C for 3 h, while the former samples were kept at

4 °C. Aliquots of both hydrolized and unhydrolized samples were then added to 96-well plates and brought to a total volume of 100 μL with glucose oxidase/ horse radish peroxidase/Amplex Red (Invitrogen, Carlsbad, CA, USA) and incubated in the dark at room temperature for 30 min before reading absorbance at 560 nm with Biotek Synergy 4 plate reader (Winooski, VT, USA). Unhydrolized samples determined glucose concentrations, while subtracting these concentrations from the hydrolyzed sample concentrations determined trehalose concentrations.

Total glycogen and triglycerides in larval tissues were measured as previously outlined (Telang and Wells, 2004). Briefly, larvae used for the hemolymph samples were dissected in PBS to remove the gut and reproductive organs. The remaining tissues were placed in 300 μL methanol/ saturated Na_2SO_4 (2:1 v/v) and frozen at -20 °C. A microseparation procedure was used to separate glycogen and triglycerides from the whole-body homogenates before samples were measured colorimetrically using modified anthrone-sulfuric acid and vanillin-phosphoric acid assays, respectively.

Normalization curves for hemolymph protein, glycogen, and triglycerides were produced by collecting hemolymph and microseparating whole-body homogenates of feeding stage, final instar larvae that were selected based on total wet mass. Normalization for hemolymph sugars was determined by collecting hemolymph from final instar larvae selected based on estimated age using staging characteristics outlined (Strand, 1990).

Time Courses

P. includens larvae were collected from mass rearing cups during apolysis of the penultimate instar, and were observed for eclosion into the final instar each hour. Individuals were collected, weighed, and treated 18 h post eclosion, and were maintained individually in plastic rearing cups with nutrient-free agar in lieu of artificial diet. Treatments included

starvation, neck ligation, MdBV infection, and neck ligation + MdBV infection. Neck-ligated individuals were CO_2 anesthetized prior to ligation with sewing thread. Five larvae from each treatment group were collected and processed for metabolite measurements (as described above) every 12 hours for four days. Five larvae were processed immediately at 18 h post eclosion without treatment to serve as the zero time point for all treatments.

Dose Response Curves

P. includens larvae were collected from mass rearing cups during apolysis of the penultimate instar, and were observed continuously for eclosion. Immediately after ecdysis, larvae were weighed and injected with a respective dose of MdBV per treatment group (0, 0.001, 0.01, 0.1, 0.5 w.e.). Larvae were maintained individually in rearing cups and fed *ad libitum* with artificial diet for the remainder of the experiment, except for larvae used as starvation controls, in which larvae were maintained individually in rearing cups with nutrient-free agar. Wet masses of larvae were measured at specific times using an analytical balance (Ohaus, Waukegan, IL, USA). The total wet mass of diet a larva consumed was indirectly estimated by collecting and weighing the fecal pellets (frass) every 12 h. Larvae were processed for hemolymph sugar, glycogen, and triglycerides as described above at the final time point. Normalizing calculations were based on the weight measurements collected at the final time point.

Trehalase Activity Assays

Six fifth instar *P. includens* larvae (between 24 and 36h post eclosion) were injected with 0.1 w.e. MdBV and maintained individually on agar cups. The collected hemolymph from three larvae was pooled for each 48 and 72 h post infection treatment groups, of which 25 μL was added to an equal volume PBS and centrifuged at 1000 × g for 2 min to remove hemocytes. 40 μL of the supernatant (plasma) was then transferred to a 0.5 mL 30 kDa Amicon Spin Filter

(Millipore) and mixed with 460 µL PBS by repeated pipetting. Samples were centrifuged at 14,000 × g for 10 min at room temperature after which 475 µL PBS was added to the remaining volume, and centrifuged as before. A final reduced volume of 25 µL was achieved and combined with 62.5 µL 40 mM trehalose and 162.5 µL PBS followed by incubation at 37 °C for 1 h. The reaction tubes were then boiled 5 min to stop further hydrolysis, centrifuged at 12,000 × g for 10 min at 4 °C, and analyzed for glucose production by the glucose oxidase assay (Sigma) as described above.

Hemolymph Trehalose Clearance *In Vivo*

Feeding stage fifth instar *P. includens* larvae were CO_2 anesthetized and injected with 5 µL of a modified Pringles saline (154.0 mM NaCl, 2.7 mM KCl, 1.7 mM $CaCl_2$, and 1 M trehalose) performed as described for injections with MdBV. Hemolymph was collected from individuals at specific times post injection and hydrolyzed with trehalase as described above to determine trehalose concentrations.

Trypsin Activity Assays

P. includens larvae were selected as described for the trehalase activity assays; three larvae were collected per treatment group per time point. The treatment groups included starved, neck ligated, MdBV infected, neck ligated + MdBV-infected, along with a fed control group that was selected at the time of dissection. Each treatment group was dissected at either 24 or 72 h post treatment in PBS, in which the guts were carefully removed and placed in 500 µL 20 mM Tris-HCl/$CaCl_2$ buffer, pH 8.0. Samples were then quickly sonicated to complete homogenization, centrifuged at 13,000 × g for 12 min at 4 °C, and diluted 10 fold in buffer to a total volume of 100 µL. Trypsin standards were also in 100 µL aliquots. 200 µL of 4 mM trypsin substrate, BapNA (Sigma), was added to each of the samples and standards in Eppendorf

tubes and allowed to incubate in the dark at room temperature for 15 min. Aliquots of 75 µL were added in triplicate to 96-well plates to be read at 405 nm with Biotek Synergy 4 plate reader (Winooski, VT, USA). Trypsin activity was calculated relative to fed-control samples for independent reads.

Data Analyses

Correlation coefficients were calculated for all metabolite normalization curves using linear regression analysis in Microsoft Excel (2007). Comparisons of hemolymph sugar concentrations, frass weight, and trypsin activity were made by one-way analysis of variance (ANOVA) with a *post-hoc* Holm-Sidak multiple comparison procedure using SigmaPlot 11 (Systat Software, Inc., Chicago, IL, USA). Time course data, including protein, glycogen, and triglyceride measurements, were compared using a two-way analysis of variance (ANOVA) with JMP 7.0 (SAS Institute, Cary, NC, USA).

Results

PDV infection induces a state of starvation in the host, so in the interest of identifying potential viral targets for the true induction of developmental arrest, I designed to characterize the metabolic condition that exists during MdBV infection independent of starvation. Because of the pivotal roles metabolism plays in development, any direct effects MdBV has on these pathways or metabolites may have a significant role in creating this stalled state. Starvation, whether pathogenically induced or not, requires the affected organism to utilize its energy reserves for survival. If MdBV infection causes a change in the rate or pattern in which these energy reserves are consumed, these differences should be easily demonstrated by comparing metabolite content between infected and uninfected larvae that have both been removed from diet. To answer this, I constructed a time course experiment that assessed the levels of different

metabolites at time points throughout the course of infection and/or starvation to determine the patterns and rates of metabolite consumption or production. Because previous reports of PDVs and their associated effects on host physiology have widely hypothesized a pathogenic influence in endocrine-mediated signaling (reviewed by Beckage and Gelman, 2004), I also included ligation treatments to determine if any or all of these metabolic effects involve head factors, such as hormones and neuropeptides. But first, there were some basic metabolite questions to be addressed.

Each Metabolite Type Has a Unique Relationship with Total Larval Weight

To compare metabolite utilization among *P. includens* larvae that had a notable variance in initial weight, I first needed to understand the relationships individual metabolite types had with total weight. Glycogen and triglycerides are the two major storage forms of energy maintained in the fat body of insects and are utilized for major processes such as molting, locomotion, and metamorphosis in developing larvae (Arrese and Soulages, 2010). *B. mori* larvae were demonstrated to use excess dietary glucose primarily for lipid synthesis during the early stages of the final instar, and for glycogen synthesis in the later phases (Inagaki and Yamashita, 1986), indicating that unique metabolite/total weight relationships may exist for these different storage forms. Additionally, hemolymph content, in terms of amino acids, proteins, glucose, and trehalose, has been demonstrated as a valuable source of nutritional status information and may serve important functions in feeding and metabolism regulation (Simpson and Raubenheimer, 1993). These circulating metabolites may also exhibit weight or age dependence, and must also be considered for normalization. For the following set of experiments, then, I developed a suite of normalization curves for each of the metabolites with larvae collected between 12 and 24 h post eclosion that were between the weights of 100 and 280

mg (Fig. 4.1). I considered the differences in metabolites strictly weight-dependent effects because all larvae selected for the experiment were in the same developmental stage of feeding, and significant physiological shifts most often, if not always, occur at developmental transitions in healthy insects.

Linear regression analysis demonstrated that hemolymph protein concentration ($y = 0.0263x^{\wedge 1.2099}$, $R^2 = 0.7361$; Fig. 4.1 A), triglyceride mass ($y = 6.5283x^{\wedge 0.9329}$, $R^2 = 0.645$; Fig. 4.1 C), and glycogen mass ($y = 0.0007x^{\wedge 2.5046}$, $R^2 = 0.8733$; Fig. 4.1 E), are all weight dependent. What is unique about each of these metabolite types, though, is the relationship each has with total weight. Relative to the rate of larval growth, hemolymph protein increases at a slightly higher rate ($y = 0.00008x + 0.0644$, $R^2 = 0.0759$; Fig. 4.1B), triglyceride mass increases at a slightly slower rate ($y = -0.0023x + 5.1171$, $R^2 = 0.0147$; Fig. 4.1 D), while glycogen mass increases at a significantly higher rate ($y = 0.0162x + 0.9497$, $R^2 = 0.7533$; Fig. 4.1 F).

Together these data suggest that the amount of each metabolite an individual will possess at a given weight is predictable, but not linear. Therefore, I used each respective normalization curve for the remainder of metabolite measurements in this study by deriving a "starting point" for each larva (Fig. 4.1 A, C, E). I did not consider larval weights in hemolymph sugar measurements, as hemolymph sugar concentrations, especially trehalose, are highly variable among individuals, but remain relatively constant throughout the feeding stage of development, independent of total weight (Fig. 4.2). The concentrations of circulating carbohydrates, unlike proteins, are most heavily dependent on fluctuating parameters such as stress and environmental factors, as well as on developmental stage.

MdBV Infection Reduces the Rate of Energy Storage Consumption during Starvation

Pruijssers et al. (2009) reported that MdBV infection induces rapid degradation of energy stores in *P. includens* larvae by mobilizing glycogen and triglycerides beyond the rates observed for starvation alone. According to my observations, however, infected larvae significantly outlive starving larvae, in spite of the fact that infected larvae are also starving. In order to accomplish such a feat, it would seem that overall rate of energy storage consumption would need to be decreased, not increased, as longevity is often associated with a reduced metabolic rate (Voorhies, 2002). I decided to repeat this work, and for this time course experiment, all treatments were denied diet to more properly control for the effects of starvation.

Unlike previously reported, the rate of glycogen ($F_{35,134} = 11.4868$; $p < 0.0001$; Fig. 4.3 D) and triglyceride ($F_{31,122} = 19.7574$; $p < 0.0001$; Fig. 4.3 E) consumption appeared slower during infection and/or ligation than during starvation alone, especially in terms of triglycerides. The rate of glycogen consumption overall does not appear much different among treatments beyond 24 h, but because glycogen is the first reserve to be utilized during major physiological processes, it seems plausible that glycogen would deplete much earlier than triglycerides and could possibly be better assessed at earlier time points. Therefore, my results of overall energy storage utilization are in accordance with my longevity observations in that metabolism rates decline during MdBV infection. As previously mentioned, hemolymph metabolites serve as another, more accessible form of energy, but also play important roles as regulatory molecules for metabolism and feeding behavior. Measurements of these circulating metabolites were also measured in the time course experiment and reflect diverse roles during infection.

Glucose Drops, Trehalose Rises, and Proteins Remain Constant during MdBV Infection

While storage molecules such as glycogen and triglycerides are expected to decrease during the course of starvation, circulating metabolites such as protein, glucose, and trehalose that serve alternative roles involved in regulation may be less predictable. Interestingly, each of the three metabolites investigated had unique responses to the treatments. Hemolymph protein concentrations did not demonstrate any clear effect during any of the treatments, as the majority of the reported concentrations hovered around their predicted normal concentrations ($F_{23, 95}$ = 5.0768; $p < 0.0001$; Fig. 4.3 A). The statistical significance derived from the data only reflect the differences between the two ligation treatments, therefore I consider hemolymph protein levels to remain unaffected by starvation or infection at least in terms of total amount of protein. There remains an obvious possibility for a significant shift in the diversity or population of specific proteins; however, that is outside the scope of this study.

Hemolymph sugar concentrations, as expected from previous reports on BVs, differed greatly from normal conditions when ligated, starved, or infected (Fig. 4.3 B, C). For all treatments, glucose concentrations dropped immediately, and remained very low for the remainder of the time course (Fig. 4.3 B); whereas trehalose concentrations rose steadily for the first 24 h of treatment and remained very high for the remainder of the time course for all treatments except starvation, where trehalose rises slightly during the first 12 h, and steadily declines to near zero by 48 h (Fig. 4.3 C). Statistics could not determine the significance of these graphs since hemolymph sample replicates were pooled for analysis, however, the dynamic difference between trehalose concentrations in infected treatment groups versus those in starved treatment groups is clear. The drop in glucose is likely an effect of starvation for all controls, as it is the first source of energy. The steady state of hypertrehalosemia was strongest in both

ligation treatments, and slightly less dramatic in infected larvae. This indicates that infection, in terms of trehalose metabolism, is much more similar, but not identical, to larvae lacking head factors than it is to either larvae under normal conditions or larvae experiencing simple starvation. These data support previously reported hypotheses involving dysregulation of hypoglycemic head factors, and also suggest a specific target for MdBV in metabolism alteration due to its stark contrast from starvation alone.

Feeding, Growth, and Sugar Concentrations are Dose-Dependent during MdBV Infection

To assist in characterizing metabolism dysregulation during MdBV infection, I attempted to analyze the dose-dependency of these different effects. Physiological abnormalities that materialize due to a specific activity of an agent should show a dose response effect if the relationship is a direct target of the agent's pathogenicity. In order to further elucidate which observations are direct targets of infection from which are less specific, or indirect responses, I created dose response curves. Selected *P. includens* larvae were injected with different doses of MdBV directly after ecdysis into the final instar and observed over the course of 48 h for growth and feeding. All larvae were bled and dissected at the final time point for analysis of hemolymph sugar concentrations and glycogen and triglyceride reserves (Fig 4.4). Both of the rates for growth and feeding, as well as the degree of hypertrehalosemia, were clearly dose-dependent (Fig. 4.4 A, B, C). Glycogen and triglyceride degradation, however, did not follow such a response (Fig. 4.4 D, E). For glycogen, most infected larvae, regardless of dose, were not significantly different from either the feeding or starved controls, but do appear to have retained at least a little of their glycogen reserves compared to the starvation (Fig. 4.4 D). In terms of triglycerides, all infected larvae, regardless of dose, were significantly different from starvation

controls and not significantly different from feeding controls, indicating once again that triglycerides are mobilized less rapidly during infection, but not dose dependently (Fig. 4.4E).

Thus far, I have identified trehalose as the sole sugar involved in hyperglycemia during infection, as well as the only metabolite that responds dose-dependently to MdBV infection. Hypertrehalosemia also appears to be the metabolic change that differs most markedly from starvation, and since trehalose accumulation appears as a dramatic, persistent, and intentional alteration in host metabolism, it may serve a vital role in regulating processes important for parasitoid survival. How or why this shift is taking place remains to be determined. This hypertrehalosemic condition can be derived from one of three scenarios: (1) an overproduction of trehalose, (2) an inability of the host to utilize trehalose, or (3) a combination of the first two. In consideration of the first option, there are only three known sources of trehalose in insects: dietary glucose, glycogen degradation, and gluconeogenesis as reviewed (Thompson, 2003). Because infected larvae cease ingesting completely, and glycogen degradation is slowed compared to starvation alone, it seems unlikely that either of the first two sources could provide sufficient trehalose to induce the observed state. Gluconeogenesis, which is known to serve as a source for trehalose during starvation, also seems an unlikely source for massive overproduction as it is an extremely energetically expensive process that could not be explained in light of the dramatic increase in longevity during infection. But to address trehalose overproduction directly, I needed to assess ability the of healthy *P. includens* larvae to clear high concentrations of trehalose from the hemolymph. The time course experiment taught us that hypertrehalosemia exists beyond 60 h post infection, so if the source of hypertrehalosemia is simply that the rate of trehalose synthesis is much greater than the rate at which an organism can utilize it, then I should

be able to inject infection-level trehalose into the hemolymph of a healthy, feeding larva and observe hypertrehalosemic levels for longer than 60 h post injection.

Injection-induced Hypertrehalosemia is Recoverable in Healthy Larvae

I injected several feeding stage, final instar larvae with a modified Pringles saline that included 1 M trehalose in lieu of the usual 22.2 mM dextrose. I then collected pooled hemolymph samples at different time points post injection and measured for glucose concentrations after complete hydrolysis by trehalase. Glucose was not subtracted from the calculations since levels are so low in comparison under normal conditions, that they do not disrupt the overall trend of trehalose regulation (data not shown).

The injected larvae reduced trehalose from 20+ mg/mL in the first hour post injection to ~7 mg/mL by 12 h and ~4 mg/mL by 48 h (Fig. 4.5). Infection-level trehalose levels very rarely exceeded 15 mg/mL, and normal blood sugar concentrations for feeding stage *P. includens* larvae were between 3 and 5 mg/mL (Fig. 4.2). Because healthy larvae reabsorbed very large concentrations of circulating trehalose at a rate within the time course experiments (Fig. 4.3), trehalose overproduction, at least alone, is not likely the cause of hypertrehalosemia during infection. Therefore, the host organism must experience trouble in utilizing the trehalose.

The inhibition of trehalose utilization could be caused by either (1) a reduction in trehalose uptake into cells involving expression or regulation of the trehalose transporter protein or (2) a reduction in extracellular and/or intracellular trehalase activity for cellular uptake/utilization of glucose (the only usable form of carbohydrate). Recall from chapter two that trehalose undergoes facilitated, passive transport into cells, but the expression of the trehalose transporter in *B. mori*, Tret1, is restricted to certain tissues including fat body and

testes, but mostly muscle cells (Kanamori et al., 2010) . Considering that all cells require energy, especially during starvation, MdBV infection may alter trehalose transport.

Hemolymph Trehalase Activity is Reduced during MdBV Infection

To determine if hypertrehalosemia may, at least in part, be due to a reduction in the abiliyt of infected larvae to hydrolyze trehalose in the hemolymph, I compared extracellular trehalase activity in infected and uninfected *P. includens* larvae at 48 and 72 h post infection (Fig. 4.6). My results indicated that infected larvae exhibited reduced levels of trehalase activity, and declines with time. This supports my hypothesis that trehalose utilization is inhibited during MdBV infection and is caused by the unavailability of glucose for cellular uptake. Without hydrolysis of trehalose in the hemolymph, most cells cannot utilize the carbohydrate for energy because they do not express the trehalose transporter and require carbohydrates in the form of the monosaccharide, glucose. It would be interesting to determine intracellular trehalase activity as well especially in considering that trehalose is transported passively and requires a proper concentration gradient to function. If trehalase activity is inhibited within the cells of tissues that do express a transporter, the inability to hydrolyze trehalose into glucose would quickly set the concentration gradient to zero, preventing further uptake and helping to create a hypertrehalosemic condition at the organismal level.

In conclusion, it appears likely that MdBV directly effects trehalase or trehalase regulatory mechanisms in order to create an altered metabolic state that somehow benefits the developing parasitoid. What still remains unclear about the physiological effects of MdBV infection, is the true cause of developmental arrest. While hypertrehalosemia is interesting, and likely very important to pathogenicity, it cannot account for the cessation of feeding, growth, and pupation.

Trypsin Activity in the Gut is not Altered during MdBV Infection

Lastly addressed, but perhaps most importantly, the clearest and most obvious pathology associated with MdBV infection is the induced anorexia that leads to the eventual wasting and death of the host organism. Infections with other pathogens have largely been recognized to suppress or alter appetite, but rarely ever to the point where the host starves to death. This level of appetite suppression may indeed be the most important physiological regulator the MdBV/parasitoid team has in terms of inducing developmental arrest upon its host. Much remains to be elucidated in terms of the interruption of feeding behavior, but I did begin the investigation by testing a single hypothesis: feeding is inhibited by a reduction in gut trypsin activity, preventing the processing of previously ingested food to disallow the intake of more diet. I subjected feeding stage, final instar larvae to any of the four treatments: starvation, neck ligation, infection, or neck ligation + infection for 24 and 72 h. I collected guts samples from individual larvae and were tested for trypsin activity using a fluorescent substrate (Fig. 4.7). Trypsin activity was decreased in all treatments compared to a feeding control group at 24 h post treatment (Fig. 4.7 A) and even more-so at 72 h post treatment (Fig. 4.7 B). This indicated that digestion is slowed during MdBV infection; however, it is not different than starving or ligation. The samples with the highest activities were those that still contained some diet in the midgut, while the samples with the lower activities were from guts that were mostly, if not completely, voided. Together, these data suggest that digestion is slower during infection, but is most likely caused entirely by the lack of food entering the gut for processing, and not specific viral effectors. Thus far it remains likely, if not probable, that developmental arrest may be the direct result of appetite suppression.

Discussion

In consideration of the experimental evidence accrued by this study in addition to Pruijssers et al. (2009), it is still quite plausible, if not probable, that the most important factor involved in the developmental arrest of pre-critical weight *P. includens* larvae during MdBV infection is the unrecoverable suppression of feeding behavior. Holometabolous insects only grow during the larval period of their life cycle, and require a species-specific weight threshold in order to undergo metamorphosis. The complete cessation of nutrient acquisition during this critical feeding period inhibits the attainment of such a weight, and consequently halts further development. Although many studies have investigated the direct effects PDVs have on prothoracic glands and the endocrine elements involved in signaling this developmental transition (Falabella et al., 2003; Pennacchio et al., 1998a; Pennacchio et al., 1998b; Pennacchio et al., 2001), it has remained unclear whether dysregulation at this site is beyond what is necessary to halt the progression. The purpose of this study was to investigate the metabolic pathology that ensues due specifically to MdBV infection beyond the effects that occur from starvation alone. Even though it is widely recognized that PDVs cause a dramatic and permanent suppression of appetite in most host insects, many of the previous studies on PDV-induced developmental arrest have only compared infected larvae to control larvae that were fed *ad libitum*, which identifies metabolic alterations due to both infection and starvation, without allowing the distinction between the two. To my knowledge, Pruijssers et al. (2009) was the first group to use starvation controls when comparing the metabolic changes in larvae infected with virus, and the current study was a continuation on their hypothesis that developmental arrest may simply be the direct result of a failure to attain critical weight.

This study focused on the patterns and rates of metabolite utilization during starvation in both control and infected, as well as in neck-ligated larvae; but before any valid comparisons could be made, it was firstly important to understand the normal conditions at which these metabolites (protein, sugar, glycogen, and triglycerides) existed among different weights of healthy larvae within a developmental stage. In designing experiments with mass-reared insects, there was usually a notable variance in growth progression. Several larvae that were at the same physiological stage had a sizeable range of weights, and these differences proved to be important not only for metabolite measurements themselves, but also for the relative effects of treatments. In the current study, only final instars were used, and for most experiments, treatments occurred at 18 hours post eclosion; however, distinction between fifth and sixth instars was not controlled. For experiments designed to determine critical weights, controlling for this is important, but for starvation experiments that rely almost entirely on total weight, it seemed insignificant.

The normalization curves did prove important for making effective comparisons among individuals, as none of the relationships for hemolymph protein, glycogen nor triglycerides exhibited a linear relationship with wet larval weight. This indicated, especially for glycogen measurements, that simply reporting calculated metabolite concentration or mass over total larval weight is not a true comparison among individuals, especially when there is significant variance among larval weights between treatment groups and/or time points. Yet another consideration for experiments comparing metabolite measurements is the effect of treatment in relation to total weight. Secondary reflection on the dataset collected for the experiments in this study suggested that oftentimes the larger larvae suffered a more dramatic loss of glycogen and triglycerides during the time courses than smaller larvae, but had little to no effect on protein concentrations. This is both informative in itself, as well as important when considering how to interpret data

that compares larvae within a large range of weights. This observation could be attributed to a reduction in MdBV titer due to the increase in volume of circulating hemolymph, or could also be an indicator of decreasing viral susceptibility with associated growth. Pre-critical weight larvae all suffer developmental arrest and fail to pupate successfully when injected with an infective dose of MdBV (Pruijssers et al., 2009); however, the degree to which infection affects metabolism at a given dose could have a linear response as a function of weight increase associated with a decrease in susceptibility. Preliminary data specifically targeting this question used larvae with differing weights for the same treatment, and indicated that larger larvae are more similar to starvation controls than smaller larvae when both groups are infected with an equal dose of MdBV (data not presented).

It was previously reported by Pruijssers et al. (2009), that MdBV infection induces wasting in host larvae by rapidly degrading energy reserves in the form of glycogen and triglycerides beyond that observed in starvation controls. In an attempt to replicate this effect, I observed a completely different trend. As presented in the time course figure (Fig. 4.3), glycogen degradation appeared to be slowed during the first 12 hours, and was not different from starving or ligated treatments by 24 hours and beyond. Triglyceride mobilization and utilization, on the other hand, was clearly slowed throughout the four day period. Starvation resulted in almost complete loss of all glycogen and triglycerides by 96 hours, whereas ligation and infection treatments maintained significant triglyceride storage throughout. The MdBV dose response curves for glycogen (Fig. 4.4D) and triglycerides (Fig. 4.4E) also demonstrated that at 48 hours post infection, glycogen mass was similar to starvation, while triglycerides were largely unchanged regardless of dose. Together these data support that all glycogen degradation observed during infection is a condition derived strictly from starvation, and that in fact,

triglyceride mobilization (and likely early glycogen utilization) is actually slowed significantly during infection. Overall metabolism rates have been reportedly slowed during periods of starvation in other larval Lepidoptera (Ziegler, 1985), but it seems possible that infection causes an even slower rate of storage energy utilization. This would also be a logical explanation for the consistent observation that larvae infected with MdBV survive much longer than larvae that are merely removed from diet (data not presented). Very rarely do starving final instars of *P. inludens* live past five days on nutrient-free agar, whereas most infected larvae live beyond a week, and sometimes over 15 days. Increases in longevity have been connected to slowed metabolism repeatedly in the literature for both invertebrates and vertebrates (Harshman and Schmid, 1998; Rogina et al., 2000; Slack et al., 2010; Voorhies, 2002; Voorhies and Ward, 1999). Neck ligation can also result in increased longevity, but not as consistently or substantially. It would be a simple and interesting experiment to measure total oxygen consumption of larvae during infection to determine if this reduction in metabolism rate does in fact occur as a direct effect of MdBV physiological interference. This pathology of infection may very well be a pivotal target of viral gene activity, as it assists its symbiotic parasitoid greatly. An *M. demolitor* larva needs a minimum of seven days to complete its own development before emergence from the host coelom for pupation; simple starvation would leave the larval wasp in a decaying carcass before it would be ready for emergence.

Another important difference identified between simple starvation and infection-induced starvation is in sugar metabolism, specifically an observed increase in hemolymph trehalose concentrations (Fig. 4.3 C). This hypertrehalosemic response has been reported numerous times as a consequence of PDV infection in larval Lepidoptera (Dahlman, 1975; Dahlman and Vinson, 1975, 1980; Thompson, 1982), but separation of trehalose and glucose concentrations was not

previously reported for MdBV infection in *P. includens.* Although glucose concentrations usually exist greater than an order of magnitude below that of trehalose, it still serves an important regulatory role as the substrate for the prominent catabolic pathway, glycolysis, and cannot be ignored. Other studies have implicated roles for glucose in sensing nutritional status as well as serving as regulatory metabolites for the complex interactions of metabolic pathways (Gies et al., 1988; Thompson, 2003). Glucose concentrations, however, dropped as readily during MdBV infection as it did for starvation and both ligation treatments (Fig 4.3 B) and does not appear to play a unique role in metabolism dysregulation beyond the inherent effects of starvation.

What was most interesting about trehalose, however, was the sharp and persistent hypertrehalosemic response to both ligation treatments that were even more dramatic than the response observed during infection. The metabolic effect of infection lies somewhere between ligation and starvation, and may indicate a role for interference of endocrine signaling involving hormones or neuropeptides secreted from the head. This further supports the hypothesis proposed by Pruijssers et al. (2009) for an MdBV viral effector either inhibiting the synthesis and/or release of a hypoglycemic hormone, or the sensitivity of target tissues to the respective signal. There is also some evidence that soluble trehalase activity is regulated by endocrine signals, specifically hypoglycemic hormones, which encourage the hydrolysis of trehalose for the cellular uptake of glucose used in energy metabolism (Tatun et al., 2008). The preliminary experiments presented in this study demonstrated a reduction in total hemolymph trehalase activity during infection (Fig 4.6), suggesting that MdBV may target the regulation of this enzyme to promote dramatic increases in circulating trehalose by inhibiting cellular uptake. Trehalase activity was also reduced in starving larvae; however, the unique difference between

starvation and infection is hypotrehalosemia versus hypertrehalosemia, respectively. Trehalase in starving larvae that were later injected with large concentrations of trehalose did show some increase in activity. This could indicate at least some role for trehalose concentrations in the regulation of this enzyme under normal conditions. Whether this reduction in enzyme activity is due to suppressed expression of trehalase genes or involves post-translational regulatory mechanisms in unknown. Both proteinaceous and non-proteinaceous inhibitors of trehalase activity have been characterized in *B. mori* (Tatun et al., 2009), although not much is known about the regulation of trehalose hydrolysis or cellular uptake.

Thus far, the most appropriate theory for the observed differences between simple starvation and MdBV-induced starvation involve hypoglycemic endocrinology. Reduction in insulin-like signaling would explain the hypertrehalosemia and trehalase inhibition, and would also explain the markedly enhanced longevity in light of the complete cessation of feeding. Additionally, both hypertrehalosemia and increased longevity can be produced simply by neck ligation. High trehalose levels have also been indicated to play a role in inhibiting lipid metabolism by interfering with the loading of the triglycerides onto apolipophorin molecules in the hemolymph (Lum and Chino, 1990). Fatty acid oxidation has also been described as the energy source used for trehalose biosynthesis in the fat body, indicating a tight link between these two metabolites (Arrese and Soulages, 2010; Thompson, 2003). These complex interactions would explain the maintenance of triglycerides, at least in part, under hypertrehalosemic conditions. Insulin signaling has also been linked to reduction in net rates in metabolism, which is also a heavily supported theory given the accrued lines of evidence.

Aside from these theories, it still remains probable that the most important metabolic malfunction during MdBV infection is the severe and permanent appetite suppression.

Unfortunately, aside of choice experiments, very little is known about feeding mechanisms in insects. There is some evidence that an abnormally high hemolymph trehalose concentration can sometimes act as an antifeedant in food choice experiments (Thompson, 2003); although, in considering the rate of *in vivo* trehalose clearance alongside the rate at which the trehalose-injected individuals restart feeding, it is a very low possibility that trehalose, at least alone, is the cause of this feeding inhibition. Another hypothesis investigated in this study was that digestion is inhibited by a reduction in trypsin activity. The results presented here, however, suggested that this was also not likely to be the case. Trypsin activity was indeed reduced during infection, but was not different from ligation or starvation. The rate of trypsin digestion appeared to be regulated by the amount of food present in the gut cavity, and that the reduction observed during all treatments was due to the fact that no new food was entering the gut to be processed. The gut samples that demonstrated some activity were also the guts that had residual food remaining in them, while the guts that had been completely voided had the least activity.

Also, it seemed that stretch receptors must be the main regulators for ingestion and peristalsis in the gut because starving larvae were maintained on nutrient-free agar, but continued to consume the agar for days even though they only produced pure agar frass. There is a long list of literature indicating the gustatory system's involvement in ingestion (Bernays and Simpson, 1982; Morita and Shiraishi, 1968; Schiff et al., 1989; Shiraishi and Yano, 1984; Simpson and Raubenheimer, 1996); however, it does not explain why a larva would continuously feed on a nutrient-free substance. It is also uncertain, but suggested by observation, that muscle contractions independent of the neuroendocrine system are also not directly affected by viral activity because starving larvae took just as long to void their guts when the agar was wrapped in cheese cloth to prevent ingestion as larvae that were infected. The ligated larvae did take

somewhat longer, though, indicating some involvement of hormone or neural signals from the brain. None-the-less, this is not a viral mechanism for the cessation of feeding during infection. It also remains unclear whether appetite suppression is even a viral effect at all. Reasons against an illness-induced anorexia are discussed in detail in Pruijssers et al. (2009).

Lastly, neck-ligation in other Lepidoptera, specifically *Manduca sexta* (Siegert, 1987; Siegert et al., 1993) and *Bombyx mori* (Satake et al., 2000), reportedly induced a decrease in trehalose, rather than an increase, and has been traditionally considered strong evidence in support of hormonal control over glycogen degradation. It remains unclear as to why neck ligation in *P. includens* did not reduce the rate of glycogen mobilization due to the restraint of adipokinetic hormones from the corpora cardiaca, however, octopamine has many similar effects and is stored elsewhere in the body and may account for these observations (Davenport and Evans, 1984). Also, there was one other report of an increase in trehalose concentration upon neck ligation (Dahlman, 1973), but was later criticized by Gies et al. **(**1988) for using glucose oxidase for their sugar measurements, as previous studies had apparently demonstrated hemolymph to contain factors that interfere with the reaction (Matthews et al., 1973). I used glucose oxidase to determine sugar concentrations as well; therefore, precautions should be taken before continuing these studies.

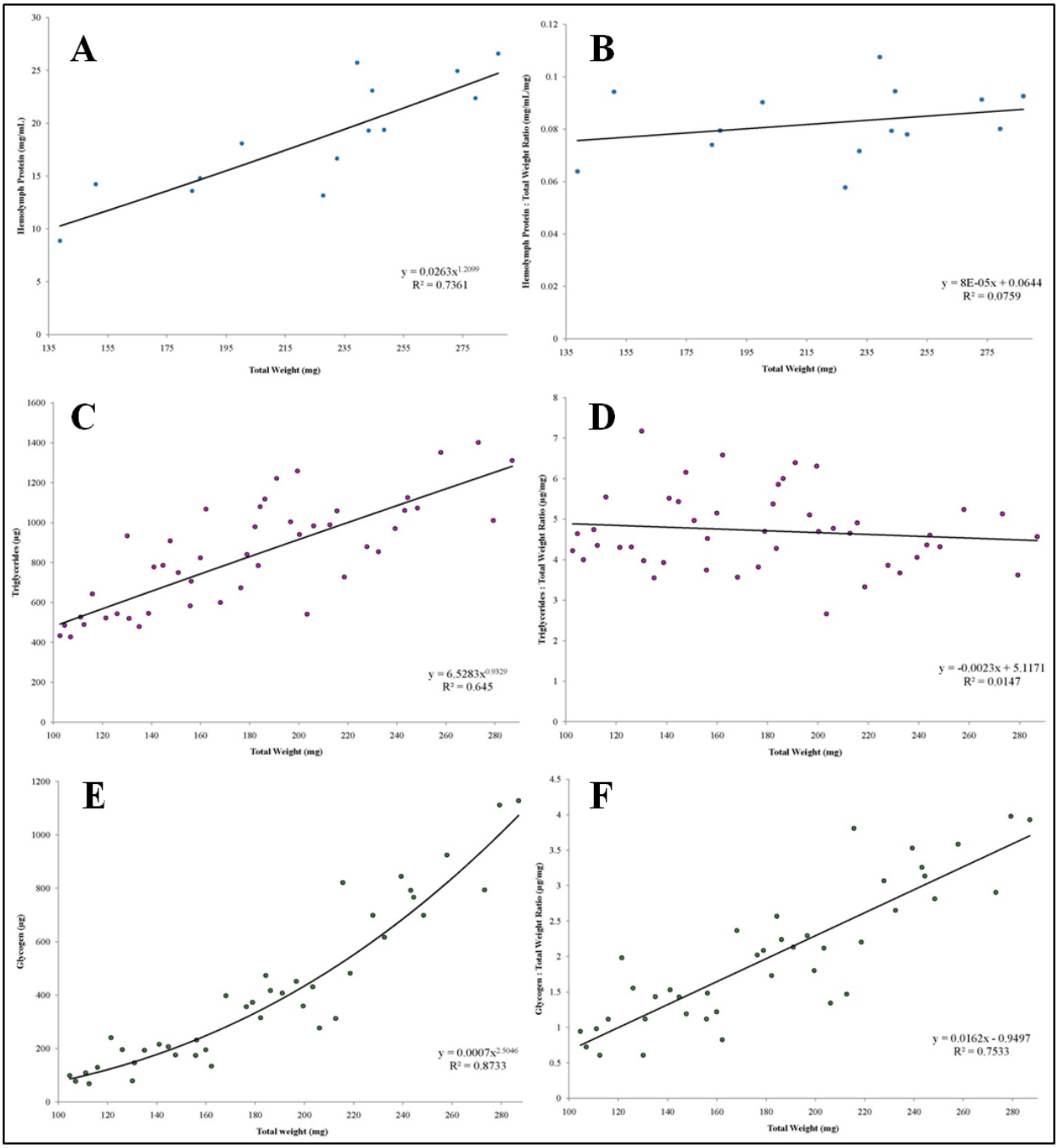

Figure 4.1 Metabolite normalization curves for hemolymph protein (A, B), triglycerides (C, D), and glycogen (E, F). Metabolite samples were collected from several 12-24 h final instar larvae of weights ranging from 100 – 280 mg to determine relationships between metabolite concentrations/masses and total larval weight. Data are fitted to power curves to relating metabolite concentration/mass directly to total weight (A, C, E). Secondarily, data are fitted to

linear curves relating metabolite concentration/mass: total weight proportions to total larval weight to determine relative rates during growth (B, D, F).

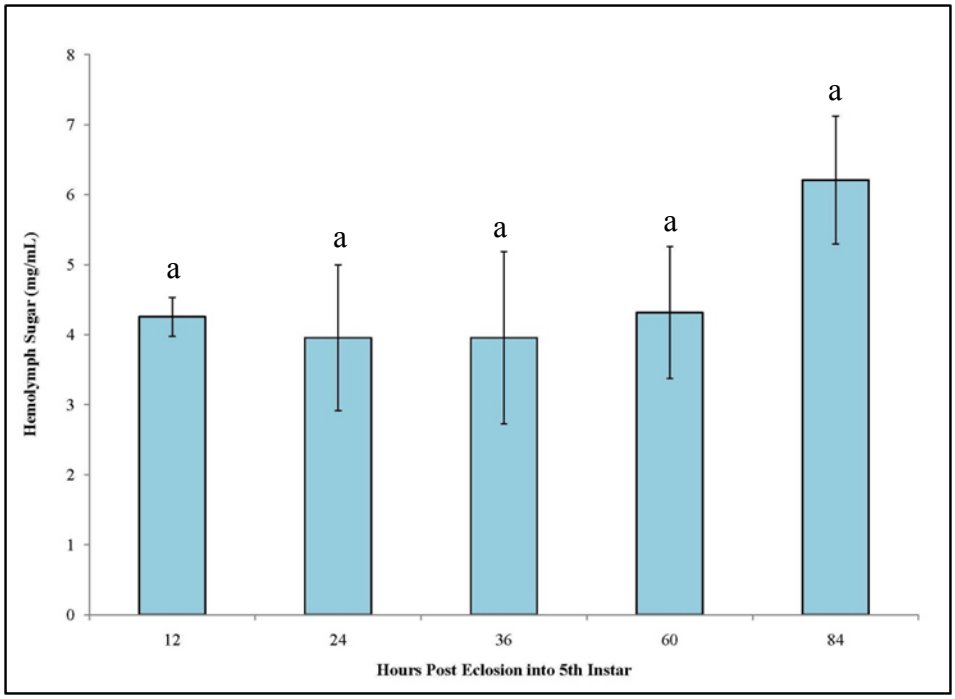

Figure 4.2 Hemolymph sugar concentrations throughout final instar development. Hemolymph was collected from larvae selected based on ages estimated using developmental markers outlined by Strand (1990) and measured for sugar concentrations using a glucose oxidase assay. Bars = 1 SD; ($F_{4, 23} = 0.946$; $p = 0.456$).

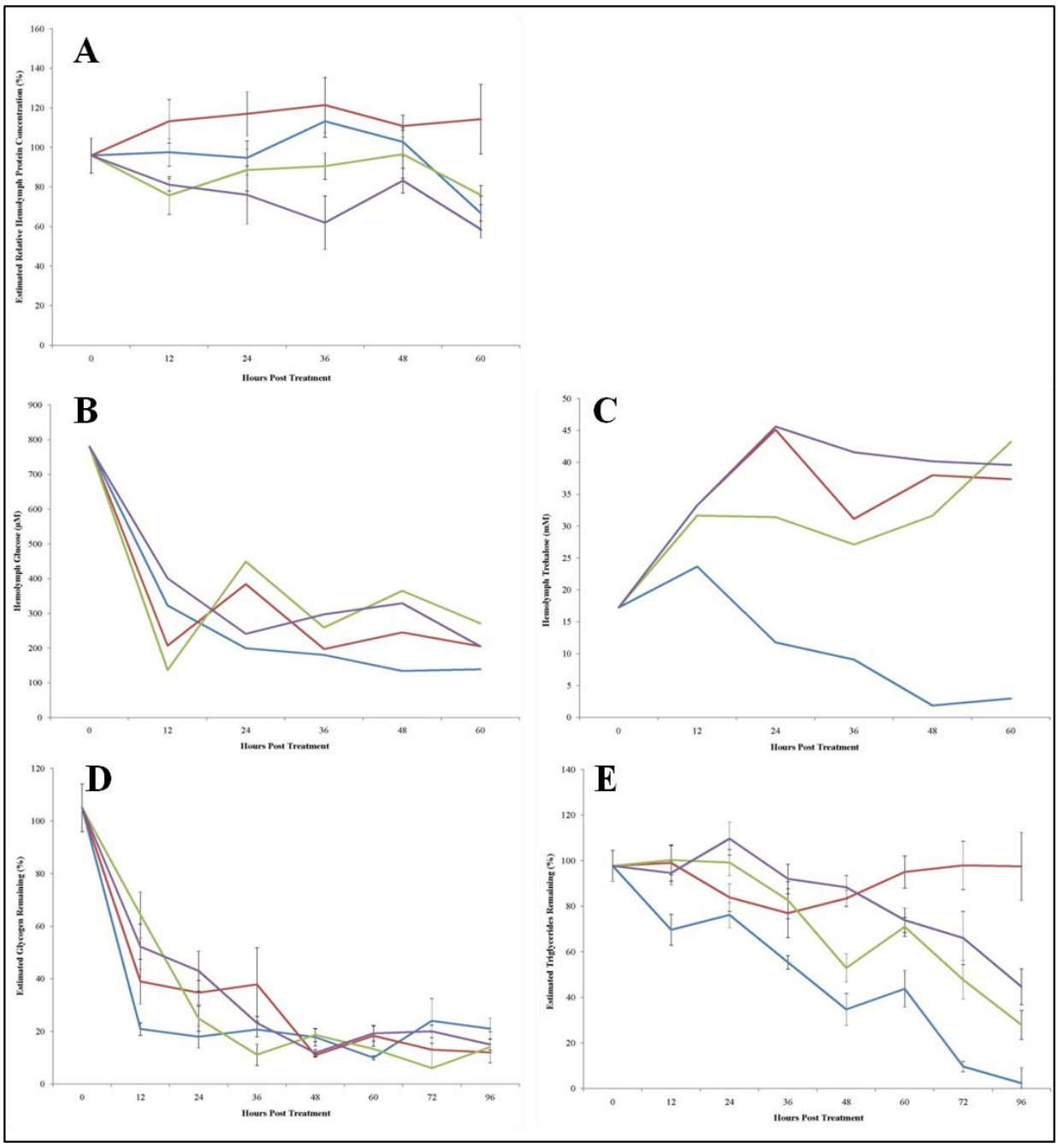

Figure 4.3 Effects of starvation (blue), neck-ligation (red), MdBV infection (green), and neck ligation + MdBV infection (purple) on hemolymph protein (A), hemolymph glucose (B), hemolymph trehalose (C), glycogen (D), and triglycerides (E) over the course of four days. *P. includens* larval weights were measured at 18 h post eclosion into the final instar, and treated with any of the four treatments. Five larvae were bled and dissected at every 12 h for

four days for metabolite measurements for each treatment group. Metabolite values were calculated per individual and reported as an estimated percentage remaining based on larval weights prior to treatment using the normalization curve equations in Figure 4.1 (A, C, E) for protein (A), glycogen (D), and triglycerides (E). Bars = 1 SD. Hemolymph sugar concentrations were measured with pooled hemolymph, each from five larvae; therefore each measurement reflects the average of five larvae with no error bars for glucose (B) nor trehalose (C).

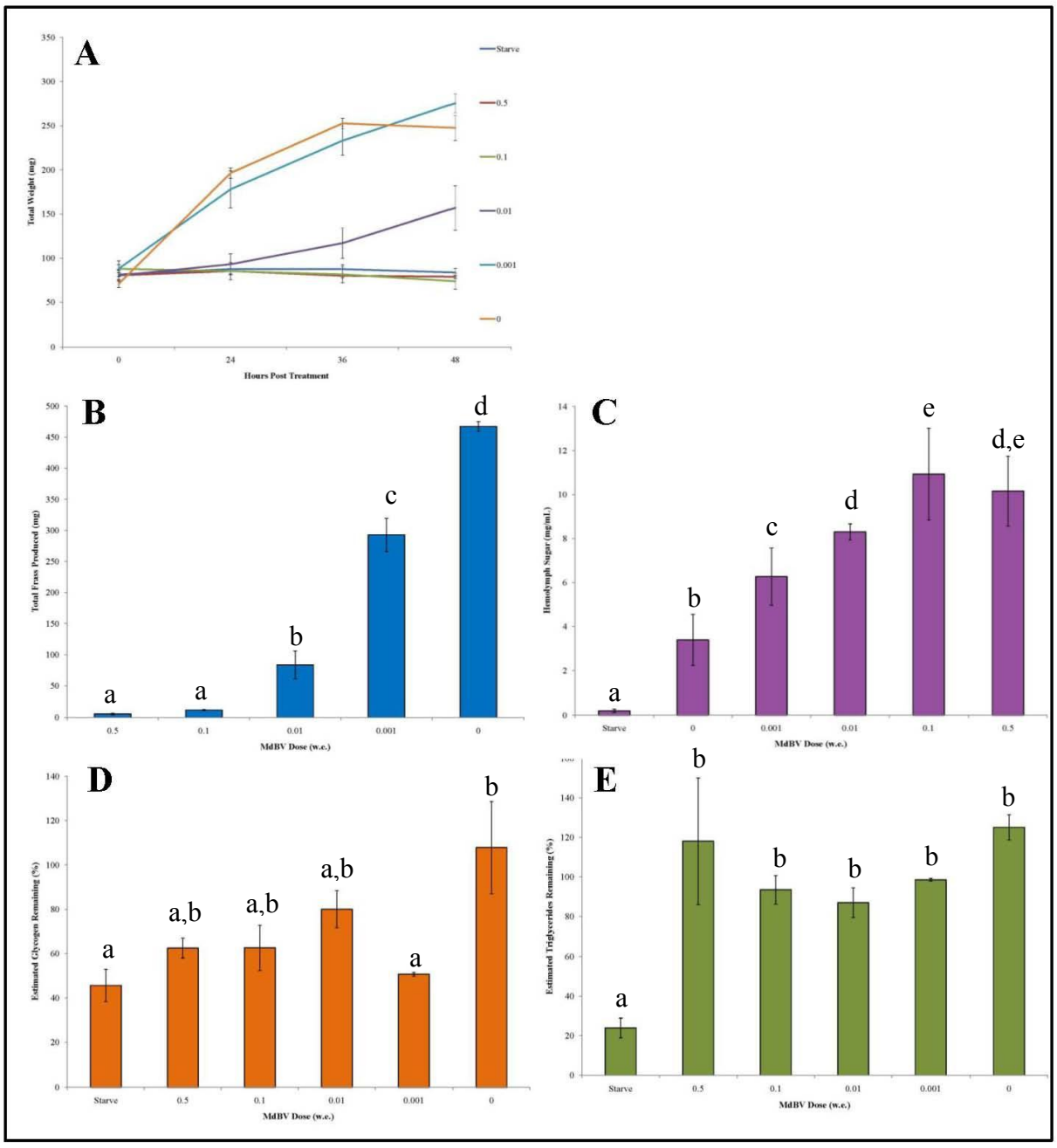

Figure 4.4 MdBV dose response curves for growth (A), feeding (B), hemolymph sugar (C), glycogen (D), and triglycerides (E). *P. includens* larvae were observed during apolysis of the penultimate instar and injected with 2 µL of any MdBV dose (0, 0.001, 0.01, 0.1, or 0.5 w.e.) immediately after ecdysis. All larvae were maintained on diet, except starvation controls which were maintained on nutrient-free agar, and measured at 24, 36, and 48 h post injection for total

wet mass (A) and wet frass mass (B). All larvae were bled and dissected at the final time point for hemolymph sugar (C), glycogen (D), and triglyceride (E) measurements. Bars = 1 SD.

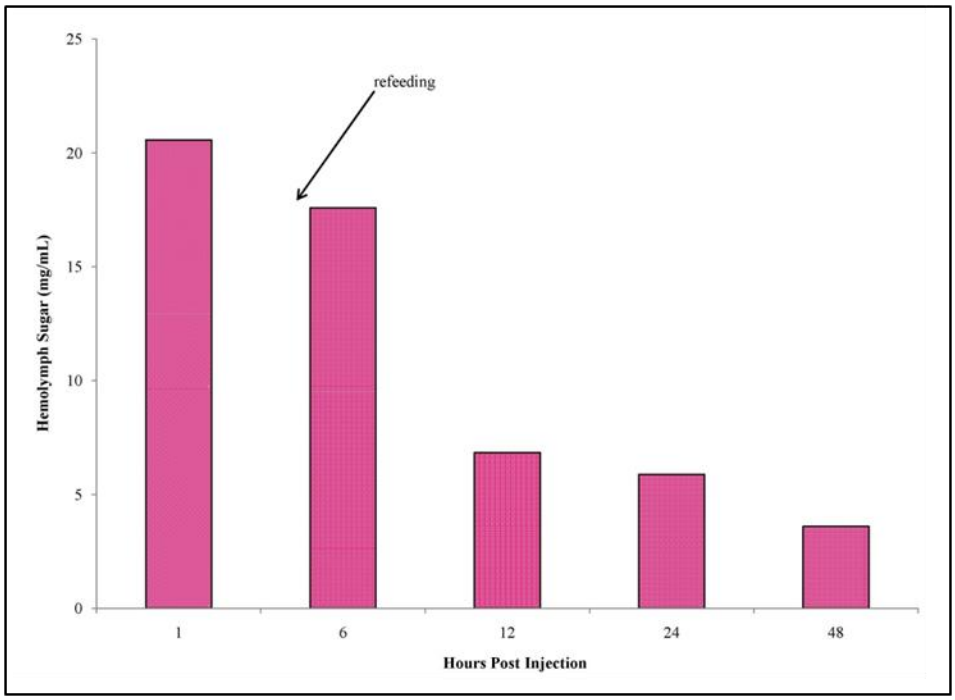

Figure 4.5 Hemolymph trehalose clearance in healthy, feeding *P. includens* larvae. Eighteen final instar larvae between 12 and 24 h post eclosion were injected with Pringles saline (1 M trehalose in lieu of 22.2 mM dextrose). Hemolymph was collected and pooled for measurement from 3 larvae per time point at 1, 6, 12, 24, and 48 h post eclosion. Three control larvae were injected with an equal volume of sugarless Pringles saline and observed with all other larvae for the beginning of re-feeding. Both control and hypertrehalosemic larvae began feeding between 4 and 6 h post injection.

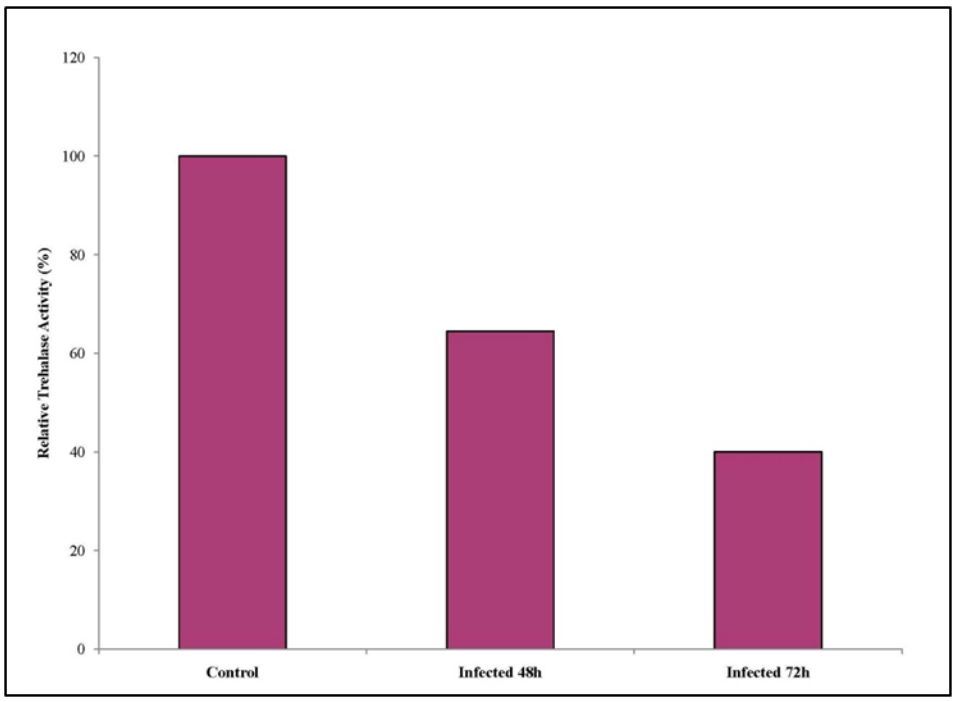

Figure 4.6 Hemolymph trehalase activity during MdBV infection. Six 12 -24 h final instar *P. includens* larvae were injected with 0.1 w.e. MdBV and maintained on diet for 48 and 72 h post injection. The collected hemolymph from three larvae were pooled and diluted in PBS before centrifugation in a 0.5 mL 30 kDa filter to remove trehalose substrate at both 24 and 72 h. A controlled amount of trehalose substrate was added to cleared hemolymph samples and incubated for 1 hr at 37 °C before boiling. Three feeding control larvae were selected at the time of hemolymph collection for each time point and activity is reported relative to the trehalase activity observed in control larvae.

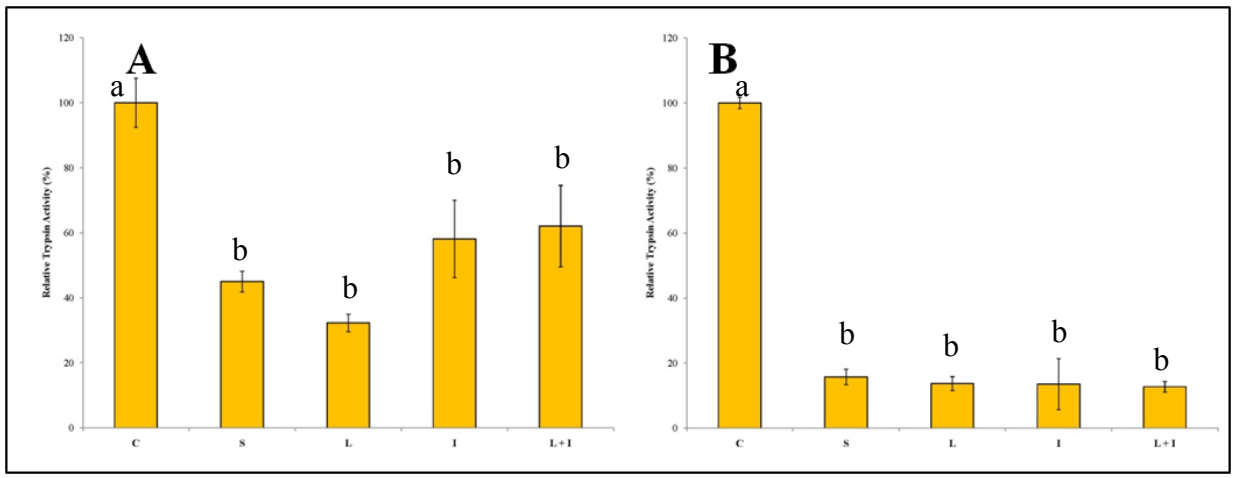

Figure 4.7 Trypsin activity in the gut during feeding (C), starvation (S), neck-ligation (L), MdBV infection (I), and neck-ligation + MdBV infection (L + I). Three 12 -24 h final instar *P. includens* larvae were subject to each treatment group and maintained on diet or nutrient-free agar (S) for 24 or 72 h. Feeding control larvae were selected as 12 -24 h final instars at the time of dissection to avoid complications due to premetamorphic metabolism alterations. All larvae were dissected in PBS to remove guts for homogenization and enzyme activity measurements. Trypsin activity as measured and statistically analyzed based on values derived from standard curves. Values are reported relative to control trypsin activity levels to compensate for incubation time discrepancy between 24 and 72 h time points. Bars = 1 SD.

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