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Lepidopteran transcriptome analysis following infection by phylogenetically unrelated polydnaviruses highlights differential and common responses

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ABSTRACT

The Polydnaviridae is a family of double-stranded DNA viruses that are symbionts of parasitoid wasps. The family is currently divided into two genera, the Ichnovirus (IV) and Bracovirus (BV), which are associated with wasps in the families Ichneumonidae and Braconidae, respectively. IVs and BVs have similar immunosuppressive and developmental effects on parasitized hosts but their encapsidated genomes largely encode different genes. To assess whether IV and BV infection has similar or disparate effects on the transcriptome of shared hosts, we characterized the effects of *Hyposoter didymator* Ichnovirus (HdIV) and *Microplitis demolitor* Bracovirus (MdBV) on the fat body and hemocyte transcriptome of *Spodoptera frugiperda* (Lepidoptera: Noctuidae). Our results indicated that HdIV and MdBV infection alters the abundance of a relatively low proportion of *S. frugiperda* transcripts at 24 h post-infection. A majority of the transcripts affected by infection also differed between MdBV and HdIV. However, we did identify some host transcripts that were similarly affected by both viruses. A majority of these genes were transcribed in the fat body and most belonged to functional classes with roles in immunity, detoxification, or cell structure. Particularly prominent in this suite of transcripts were genes encoding for predicted motor-related and collagen IV-like proteins. Overall, our data suggest that the broadly similar effects that HdIV and MdBV have on host growth and immunity are not due to these viruses inducing profound changes in host gene expression. Given though that IVs and BVs encode few shared genes, the host transcripts that are similarly affected by HdIV and MdBV could indicate convergence by each virus to target a few processes at the level of transcription that are important for successful parasitism of hosts by *H. didymator* and *M. demolitor*.

1. Introduction

Thousands of ichneumonoid, parasitoid wasps carry symbiotic viruses in the family Polydnaviridae (summarized by Strand, 2010). Polydnaviruses (PDVs) persist in wasps as integrated proviruses, which are transmitted vertically to offspring. Replication in contrast is restricted to specialized calyx cells in the ovaries of females where virions accumulate to high densities. Female wasps inject a quantity of these virions, which contain a poly-dispersed dsDNA circular genome, when they oviposit into a host insect. No replication occurs in the wasp's host, but expression of viral genes causes alterations in host immune defenses and development that are essential for the successful development of the wasp's offspring.

The Polydnaviridae is currently divided into two genera, the Ichnovirus (IV) and Bracovirus (BV), which are associated with wasps in the families Ichneumonidae and Braconidae respectively. Recent studies indicate that IVs and BVs differ molecularly and morphologically, and that their association with wasps arose independently (Bezier et al., 2009; Volkoff et al., 2010; Whitfield and Asgari, 2003). However, IV and BV genomes share several organizational features that likely evolved by convergence and their analogous roles in parasitism (Kroemer and Webb, 2004; Webb et al., 2006). One of

Abbreviations: PDV, Polydnavirus; BV, Bracovirus; IV, Ichnovirus; MdBV, *Microplitis demolitor* Bracovirus; HdIV, *Hyposoter didymator* Ichnovirus.

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these shared traits is that viruses in both genera encode several multimember gene families, but with exception of the ankyrin domain containing genes (Anks) most gene families are unique to one genus or the other (Espagne et al., 2004; Lapointe et al., 2007; Tanaka et al., 2007; Webb et al., 2006). Yet, a large literature also indicates IVs and BVs infect the same host tissues and have broadly similar effects on host physiology (Beckage and Kanost, 1993; Kroemer and Webb, 2004; Pennacchio and Strand, 2006; Pruijssers et al., 2009; Rodriguez-Perez and Beckage, 2006). Taken together, these patterns suggest that the different gene products IVs and BVs encode either target the same host molecules or they target different host molecules in a given pathway which results in similar alterations in host development and immune defenses.

As a first step toward addressing such questions, we compared the effects of infection by *Hyposoter didymator* Ichnovirus (HdIV) and *Microplitis demolitor* Bracovirus (MdBV) on the transcriptome of the host *Spodoptera frugiperda* (Lepidoptera: Noctuidae). The partially sequenced HdIV genome (Volkoff et al., unpub.) is very similar to the 250 kbp *Hyposoter fugitivus* IV genome which contains 150 predicted genes including the same gene families (Anks, Repeat element, Viral innexins, Cys-motif proteins, Proline-residue-rich-proteins and N-proteins) found in other campoplegine IVs (Tanaka et al., 2007). The fully sequenced MdBV genome has an aggregate size of 189 kbp and encodes 63 predicted genes that include 13 protein tyrosine phosphatases (PTPs), 12 Anks, and two specific families named the Glc and Egf genes (Webb et al., 2006). We focused our analysis on the host fat body and hemocyte transcriptome as the former is primary metabolic organ of the host and the latter is essential for immune defense against parasitoids including *H. didymator* and *M. demolitor* (Strand, 2010). Our results indicated that HdIV and MdBV primarily affected the abundance of different host genes with a few host genes similarly affected by both.

2. Materials and methods

2.1. Biological materials

2.1.1. Insect rearing and virus isolation

S. frugiperda, *H. didymator*, and *M. demolitor* were reared as described (Strand, 1994; Volkoff et al., 1995), while HdIV and MdBV were isolated as outlined by Volkoff et al. (1999). One wasp equivalent is defined as the amount of virus present in the calyces of a single wasp. *S. frugiperda* larvae (day 1 fifth instars = L5) were injected with 0.5 wasp equivalents of either HdIV or MdBV in a volume of 9 μL using a syringe. Larvae injected with PBS only served as negative controls. In some instances, HdIV and MdBV were inactivated by 10 min exposure to 1000 mJ of UV light at 254 nm using a DNA cross-linker (Spectrolinker XL1500UV). We confirmed by transmission electron microscopy and PCR assays that inactivated virus remained capable of entering host hemocytes but expressed no viral genes (data not presented).

2.1.2. Larval weight and hemocyte analysis

Thirty *S. frugiperda* L5 were weighed before and 24 h after injection with HdIV or MdBV. Total and differential hemocytes counts were performed on the same 30 larvae 24 h after injection. Total Hemocyte Counts (THCs) were determined using a Malassez hemocytometer. Differential hemocyte counts (DHCs) were determined by adding 10 μL of non-diluted hemolymph per larva to a glass slide and counting the number of each major hemocyte type (granulocytes, plasmatocytes, spherule cells) in four randomly selected fields of view using previously described identification criteria (Ribeiro and Brehelin, 2006). THCs and DHCs were expressed as number of cells per larva. Average weights were analyzed by repeated measures ANOVA with time as the repeated factor. For hemocyte counts, which exhibit a Poisson distribution, difference between HdIV- or PBS-injected larvae were compared for each cell type using a Log-linear model (McCullagh and Nelder, 1989). All analyses were performed using SAS (version 9.1.3, SAS Institute Inc., Cary, North Carolina).

2.1.3. RNA sample collection

Host hemocytes and fat body were collected from virus-infected and control larvae 24 h post-injection (Volkoff et al., 1999). Total RNA from each tissue was isolated using the RNeasy Mini Kit (Qiagen) according to the manufacturer's instructions. Samples were DNase treated using the Turbo DNA-*free* kit (Ambion – Applied BioSystem) and RNA concentrations were determined using a NanoDrop ND-1000 spectrophotometer. For microarray experiments, RNA samples were microcapillary electrophoresed using a Bioanalyzer 2100 (Agilent Technologies, Wilmington, DE).

2.1.4. Immunofluorescence assays

Hemolymph was collected from larvae 24 h post-injection with HdIV or PBS in sterile anticoagulant buffer (62 mM NaCl, 100 mM glucose, 10 mM EDTA, 30 mM trisodium citrate, 26 mM citric acid) at 4 °C. After centrifugation (800 × *g* for 5 min), the hemocyte pellet was resuspended in PBS and cells were allowed to adhere to polylysine-coated microscope slides for 20 min. Cells were fixed with 8% paraformaldehyde and 60 mM sucrose (1:1) for 30 min, washed with PBS, and permeabilized with 0.1% Triton X-100 for 20 min. Blocking was performed in PBS containing 3% nonfat milk powder and 0.05% Tween-20 for 1 h. Cells were then incubated with affinity purified rabbit anti-collagen Type IV (1:20) (Rockland) overnight at 4 °C. Control staining was performed by incubating cells in blocking buffer without primary antibody for a similar period. Cells were washed in PBS and incubated with Alexa-Fluor-594 goat anti-rabbit IgG (1:500) (Molecular Probes) for 1 h at room temperature. After washing with PBS, Hoechst 33258 (Sigma) (0.5 μg/mL) was added to cells for 15 min and Prolong Gold antifade reagent (Invitrogen) was used to mount cells followed by examination using a Zeiss Axiovert 200 M inverted microscope.

Fat bodies were dissected and fixed in 4% paraformaldehyde and 0.25% glutaraldehyde for 1 h at 4 °C, washed with PBS, dehydrated and embedded in Unicryl resin (EMS). Semi-thin sections (1 μm) were incubated in 1% BSA for 10 min followed by rabbit anti-collagen Type IV (1:25) (Rockland) for 2 h at room temperature (RT). Sections were incubated with Alexa-Fluor-594 goat anti-rabbit IgG (1:500) for 45 min at RT, with FITC-phalloidin (1:1000) (Sigma) for 30 min at RT room temperature and DAPI (1 μg/mL PBS) (Sigma) for 5 min at RT. After washing with PBS and distilled water, Vectashield (Vector laboratories) was added to slides, and samples examined using a Zeiss Axioimager microscope with an Apotome Imaging System.

2.2. Microarray experiments

2.2.1. Experimental design

A balanced complete block design with dye swaps (Knapen et al., 2009) was performed. The microarray experiments included 3 treatments (e.g. HdIV, MdBV, and PBS) on two host tissues (hemocytes and fat body) collected 24 h post-infection. Three biological replicates were performed injecting 30 *S. frugiperda* fifth instars for each treatment. Each experiment compared the hemocyte and fat body transcriptome of virus-injected larvae to PBS-injected larvae.

2.2.2. Microarray hybridization experiments

The microarray contained 3072 60-mer oligonucleotide (GC content average 46%, Tm average 86.8 °C) sequences designed from the *Spodoptera frugiperda* EST library (Spodobase http://bioweb.

ensam.inra.fr/Spodobase/) plus 11 viral genes (7 from HdIV and 4 from MdBV). All 60-mer oligonucleotides were synthesized by Sigma and can be viewed athttp://www.ncbi.nlm.nih.gov/geo/(GEO Accession number GSE17293).

The DNA chips used for hemocyte studies were manufactured using the Biochips platform of Toulouse-Genopole (http://biopuce.insa-toulouse.fr/Maquette/en/) with spotting information available underhttp://www.ncbi.nlm.nih.gov/geo/index (GEO Accession number GPL8718). The DNA chips used for the fat body studies were manufactured using the transcriptomic platform of NICE – Sophia-Antipolis (http://www.microarray.fr/) with spotting information available underhttp://www.ncbi.nlm.nih.gov/geo/index(GEO Accession number GPL8719).Total RNA (5 or 7 μg) from each host tissue was labeled with either Cy5 dCTP or Cy3 dCTP (Amersham Biosciences) using the ChipShot™ Direct Labelling System (Promega). Dye incorporation was quantified using a nanodrop spectrophotometer (Labtech – France). Fifty pmol of each labeled cDNA pool was dried in a speed-vacuum concentrator and resuspended in 20 μL of DNase free water or 10 μL of Corning hybridization buffer. For hemocyte samples, hybridizations were performed in an automatic hybridization chamber (Discovery, Ventana Medical system) where microarray slides were prehybridized with a solution of 2X SSC, 0.2% SDS, 1% BSA at 42 °C for 30 min. Slides were hybridized with 200 μL of the hybridization solution, containing 20 μL of each labeled cDNA (50 pmol of Cy3 and 50 pmol of Cy5) and 180 μL of hybridization buffer (Chyp Hyb buffer, Ventana Medical System). After 8 h of hybridization at 42 °C, slides were washed following the manufacturer's recommendations (Corning). For fat body experiments, hybridizations were carried out manually using Corning Hybridization Chambers. Slides were hybridized with 30 μL of labeled cDNA mix, containing 10 μL of each labeled cDNA (50 pmol of Cy3 and 50 pmol of Cy5) and 10 μL of Corning Hybridization Buffer. After 14 h of hybridization at 42 °C, slides were washed following the manufacturer's recommendations (Corning). After hybridization, slides were scanned with a 2 laser scanner (GenePix 4000A or B Scanner-Axon Instruments). The Photo-Multiplier Tubes were adjusted to overlay intensity curves of both wavelengths and to avoid saturation. Images were analyzed, spots delimitated, and hybridization signals were quantified and transformed into numerical values using GenePixPro (Axon) software (version 3.01 or 4.01). After Genepix image analysis, non-exploitable spots were eliminated. A total of 3067 and 1328 spots were analyzed for fat body and hemocyte samples from larvae infected with HdIV, while 3061 and 1714 spots were analyzed for fat body and hemocyte samples from larvae infected with MdBV.

2.2.3. Data analyses

Numerical data from the microarray studies were analyzed using SAS and R analyses. For SAS analysis, numerical data were normalized using the TIGR Microarray Data Analysis System following 3 steps: (a) a low-intensity filter to remove spots with low signal intensity; (b) global normalization; and (c) a flip dye consistency check to account for differences in labeling efficiency. Intensities were then log-transformed, centered by their average value and reduced by their standard deviation (Le Goff et al., 2006). The resulting data for each gene were analyzed by type III two-way analysis of variance (ANOVA) taking into account the presence of missing values. A gene was considered as differentially expressed if 50% or more of its variance was explained by the treatment effect with a corresponding *P*-value lower than 0.05. The second criterion used to consider data as statistically significant was a variance explained by the biological replicate lower than 0.5 and a variance explained by the technical replicate lower than 0.407. After running GeneANOVA (Didier et al., 2002) on the raw data to verify grid alignments, data were analyzed using R (R Development Core Team, 2008) and the limmaGUI package (Wettenhall and Smyth, 2004). Data were used without background correction but with spot quality weighting. Print tip loss normalizations were used for within arrays and to scale between array normalizations. To fit the linear model we used the least squares method. *P*-values were adjusted with the Benjamini and Hochberg method. Genes differentially expressed were those with an adjusted *P*-value lower or equal to 0.05. Only data supported by both analyses and with an expression ratio between virus and PBS controls with values higher than 1.5 or lower than 0.66 were used for final results (Yang et al., 2002).

2.3. Relative quantitative PCR experiments (rqRT-PCR) analysis

Transcript abundance of selected genes were analyzed by rqRT-PCR using total RNA reverse transcribed with SuperScript™ III Reverse Transcriptase (Invitrogen) as template. PCR reactions were performed in 25 μL with 0.75 u of the Platinum® SYBR® Green qPCR SuperMix-UDG with ROX (Invitrogen). Each reaction contained cDNA from 20 ng of total RNA, 200 μM dNTP mix and 0.4 μM of each gene specific primer (Supp. Table 1). Reactions were run using the ABI Prism 7000 Sequence Detection System under the following conditions: 50 °C 2 min; 95 °C, 2 min; 95 °C 15 s, 60 °C 1 min, 40 cycles. Each sample was run in triplicate with results analyzed using LinReg PCR program (Ramakers et al., 2003) that calculates the initial molecule number (N0). Normalization was performed with 3 reference genes, ATP synthase, RNA polymerase II and Ubiquitin E2 (Barat-Houari et al., 2006).

3. Results and discussion

3.1. HdIV and MdBV similarly repress growth and hemocyte abundance in S. frugiperda

HdIV and MdBV both infect *S. frugiperda* larvae with viral transcription for each beginning within 2 h of infection and continuing

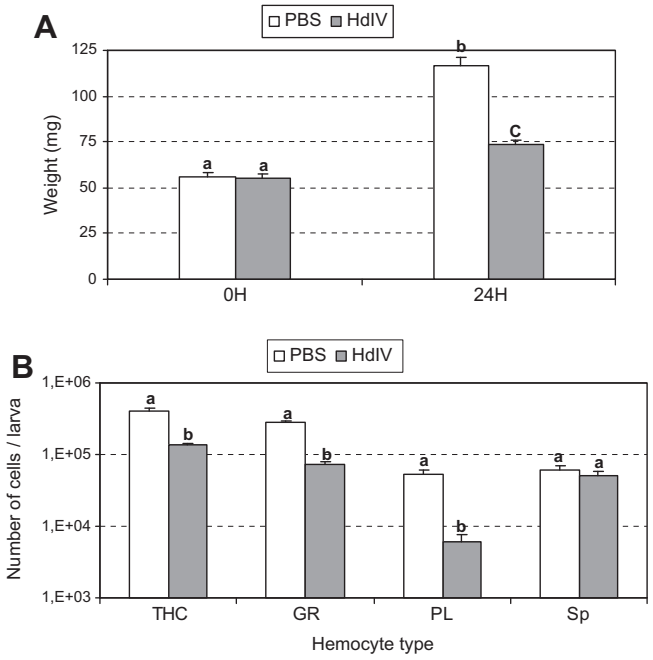

Fig. 1. Effects of HdIV infection on weight gain and hemocyte abundance in *S. frugiperda* fifth instars. (A) Larval weight before (0 H) and 24 h (24 H) after injection of 0.5 wasp equivalents of HdIV or PBS. For each, standard errors are indicated. (B) Total (THC) and differential hemocyte counts per larva 24 h after injection of HdIV or PBS. GR: granulocytes, PL: plasmatocytes, Sp: spherule cell. Number of cells ± SE is presented in log scale. Different letters indicate treatments that statistically differ.

for multiple days thereafter (Galibert et al., 2006, 2003; Strand et al., 1997; Trudeau et al., 2000; Volkoff et al., 1999, 2001). Prior results further indicated that MdBV infection significantly reduces weight gain by *S. frugiperda* and disables adhesion of hemocytes to foreign surfaces including parasitoid eggs (Strand and Trudeau, 1998). In the current study, we determined that HdIV had a similar effect. As shown in Fig. 1, HdIV infection significantly reduced larval weight gain (time × treatment: $F_{1,\ 52} = 83.80$, $P < 0.001$) and total hemocyte abundance ($X^2 = 58.12$, $df = 1$, $p < 0.0001$) relative to saline-injected controls. The relative abundance of granulocytes ($X^2 = 63.82$, $df = 1$, $p < 0.0001$) and plasmatocytes ($X^2 = 33.14$, $df = 1$, $p < 0.0001$) was also affected by HdIV infection (Fig. 1). Granulocytes are the primary phagocytic hemocyte type in Lepidoptera and are also required for encapsulation of certain foreign targets, whereas plasmatocytes are the main capsule-forming hemocyte (Lavine and Strand, 2002; Suderman et al., 2008).

3.2. HdIV and MdBV alter transcript abundance of selected *S. frugiperda* genes

We used previously designed microarrays to assess how HdIV and MdBV infection affected the fat body and hemocyte transcriptomes of *S. frugiperda* at 24 h post-infection. Our results identified 342 transcripts whose abundance was significantly affected by HdIV (274), MdBV (199) or both (131) (Fig. 2, Table 1, Supp. Table 2). However, the total number of affected transcripts accounted for only 1–10% of the total sequences from host hemocytes and fat body we screened (Table 1). This relatively small proportion of affected transcripts could in part be explained by our

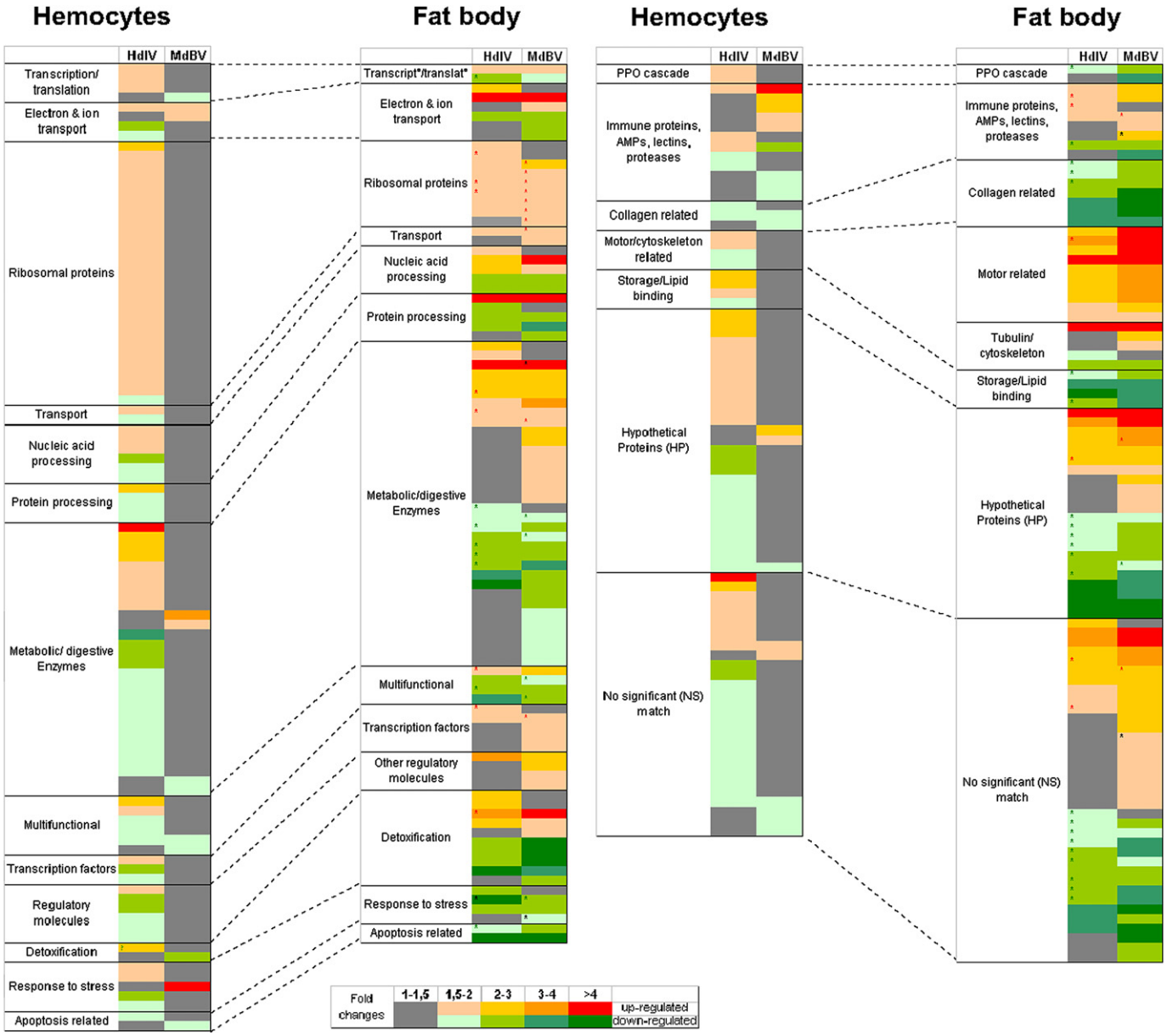

Fig. 2. Expression levels for genes differentially expressed in hemocytes or the fat body after 24 h post-infection by HdIV or MdBV. The transcripts are classified according to their predicted function (see the list in Supp. Table 1). Each lane corresponds to one microarray oligonucleotide (spot). Color intensities indicate the fold change range for each spot while heights of each category reflect the relative number of genes within a given functional category. Spots with an asterisk are those for which the fold change was statistically significant with only one of the R or SAS tests; the others are spots for which fold change was statistically significant using both tests.

Table 1
S. frugiperda hemocyte and fat body transcripts that are significantly higher or lower 24 h after infection by HdIV or MdBV. The total number of *S. frugiperda* transcripts (spots) analyzed for each treatment is shown to the left while the number of significantly up and down-regulated transcripts are shown to the right.

PDV	Number of spots analyzed	Number of affected sequences FC > 1.5 significant with SAS OR R		
		Total	Down	Up
Hemocytes				
HdIV	3067	156 (5.09%)	73 (47%)	83 (53%)
MdBV	3061	32 (1.05%)	18 (56%)	14 (44%)
Fat body				
HdIV	1328	136 (10.24%)	69 (51%)	67 (49%)
MdBV	1714	175 (10.21%)	84 (48%)	91 (52%)

conservative scoring criteria, which required that a transcript must differ from our control using 2 statistical tests to be classified as significantly affected by infection. The proportion of significantly up or down-regulated sequences ranged from 44 to 56% in the two host tissues (Table 1). However, the specific transcripts affected by HdIV and MdBV in each tissue largely differed indicating that each PDV affected the fat body and hemocyte transcriptome of *S. frugiperda* differently (Table 2). We further noted that the two PDVs affected more sequences in the host fat body ($n = 311$) than in hemocytes ($n = 188$), and that a larger proportion of fat body than hemocyte transcripts exhibited a >2-fold change after infection (Fig. 3). This was particularly evident for MdBV which affected relatively few hemocyte sequences (Tables 1 and 2). Overall then, our results showed that HdIV affected the transcriptome of both host tissues, while MdBV primarily affected the host fat body (Fig. 2). One consequence of this bias was that a large majority of the 131 host sequences affected by both viruses derived from the fat body ($n = 104$, 79.4%). Another was that a majority of the 143 genes specifically affected by HdIV derived from hemocytes ($n = 128$, 86.5%) while most of the 88 genes specifically affected by MdBV derived from the fat body ($n = 48$, 54.5%).

We validated the results of our microarray experiments by selecting 13 transcripts affected by both viruses and conducting rqRT-PCR. These genes either belonged to the same molecular function family (Collagen), shared involvement in the same biological process (Myosin, Tropomyosin, Troponin), or exhibited opposite responses to each virus (Hemolymph Protease 3). We also verified that altered expression of these host genes was due to HdIV or MdBV transcription by conducting rqRT-PCR assays with samples prepared from hosts injected with UV-inactivated virus. Overall, our qRT-PCR results were congruent with our microarray data (Table 3). We also detected no differences among these 13 target genes between host larvae injected with each UV-inactivated virus and PBS (Table 3).

3.3. PDV infection affects the abundance of host transcripts in several functional classes

Of the 342 host transcripts affected by HdIV or MdBV, 70% shared significant homology to genes of known function, 13% shared homology to hypothetical proteins (HP) from other organisms, and 17% shared no significant similarity (NS) with sequences in current databases (Fig. 2, Supp. Table 2). Among the different functional classes predicted by Gene Ontology, 31% of affected genes were associated with general cell maintenance, which included the "transcription/translation" and "multifunctional" classes (Fig. 2, Supp. Table 2). Among genes with roles in metabolism (e.g. ribosomal proteins, metabolic/digestive enzymes), the most striking result we identified was the upregulation of 26 ribosomal genes in hemocytes by HdIV (Fig. 2). This result suggested that HdIV but not MdBV may affect host translation. In contrast, both viruses affected selected other metabolic transcripts including a riboflavin kinase (Sf1P10253-5-1) that was 16–17 fold more abundant in the fat body. Riboflavin kinases (RFK) catalyze the phosphorylation of riboflavin (vitamin B2), and in mammals are also involved in production of reactive oxygen species (ROS) via bridging of TNF-receptor-1 and NADPH oxidase 1 (Yazdanpanah et al., 2009). Given the diverse roles of ROS in immune defense and signaling, upregulation of RFK by PDVs could reflect either a response to virus infection or an alteration that adversely affects metabolism. We also observed that infection reduced the abundance of an alpha-amylase (Sf1M02253-5-1), which in insects and other organisms regulates starch digestion (Abraham et al., 1992). Reduced abundance of alpha-amylase could thus contribute to the delays in growth that occur after infection by HdIV and MdBV.

Table 2
Number of *S. frugiperda* transcript sequences affected by HdIV, MdBV, or both. The total number of *S. frugiperda* transcripts (spots) analyzed for each treatment is shown to the left while the number of significantly up and down-regulated transcripts are shown to the right.

Total	HdIV		MdBV		Down	Up	Notes
	Hemocytes	Fat body	Hemocytes	Fat body			
Sequences affected by both viruses ($n = 131$)							
104	–	104	–	104	57	47	
12	12	12	–	12	3	9	1 down in F, up in H
5	5	–	5	–	2 IV/3 BV	3 IV/2 BV	1 down in BV, up in IV
3	3	–	–	3	2 IV/1 BV	1 IV/2 BV	1 down in IV, up in BV
3	–	3	3	3	3	0	1 down in F, up in H
2	2	–	2	2	1	1	
1	1	1	1	1	1	0	
1	–	1	1	–	0	1	
Sequences affected by HdIV only ($n = 143$)							
128	128	–	–	–	62	66	
10	–	10	–	–	2	8	
5	5	5	–	–	2	3	
Sequences affected by MdBV only ($n = 68$)							
48	–	–	–	48	16	32	
18	–	–	18	–	10	8	
2	–	–	2	2	1	1	
342							

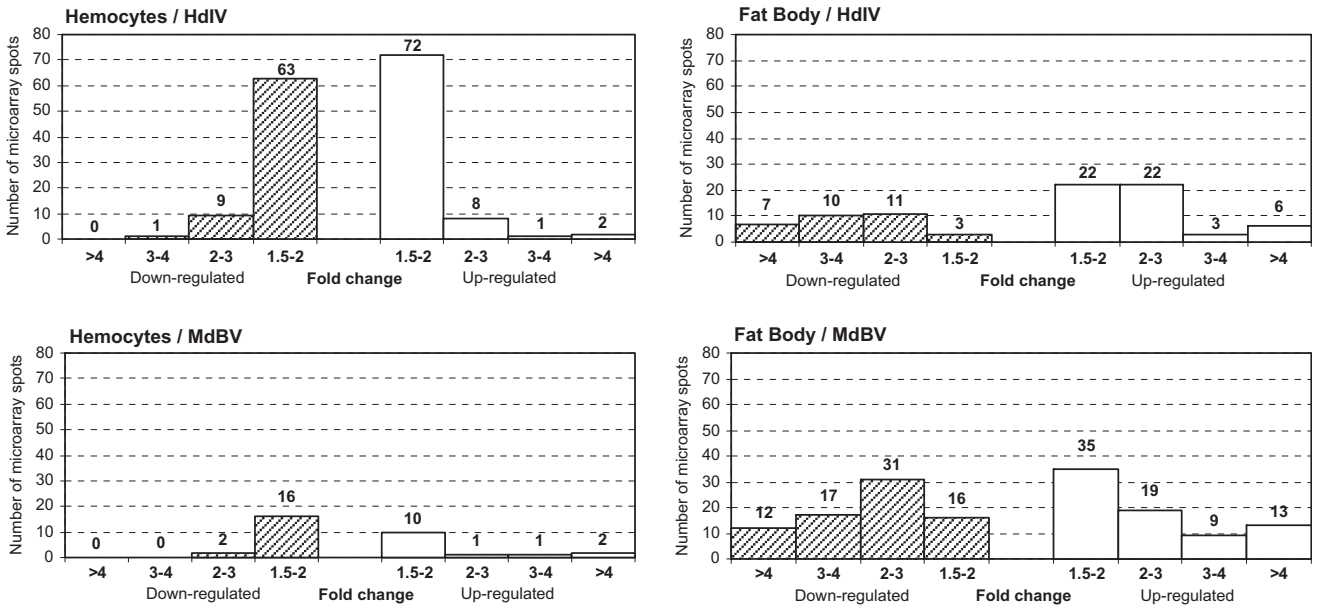

Fig. 3. Distribution of fold changes of *S. frugiperda* genes 24 h post-infection by HdIV and MdBV. For each treatment, the number of spots for the different classes and corresponding fold changes (fold change = 1.5–2, 2–3, 3–4, >4) are given. Values are given only for spots in which fold changes are statistically significant using R and SAS tests. Spots corresponding to down-regulated genes (hatched bars) are shown to the left while spots for upregulated genes (white bars) are shown to the right.

Most host genes in the categories of transcription/translation and regulatory molecules that were affected by infection derived from the fat body. These included a predicted enhancer of transcription factor (SF9L03645) and a GTP-binding protein (Sf2M00383-5-1) that exhibited a 2-fold increase in the fat body after infection by MdBV. Conversely HdIV reduced the abundance of a putative bhlhzip transcription factor (Sf2H02861-5-1) and a probable rho GTPase Guanine Nucleotide Exchange Factor (SF9L02257) in hemocytes.Transcript abundance of only one regulatory gene, a predicted cyclin C (Hoeppner et al., 2005; Ren and Rollins, 2004), was significantly affected by both viruses. Genes with predicted functions in detoxification affected by infection included one glutathione-S-transferase (GST) (Sf1P19613-5-1) that was strongly upregulated in the fat body by MdBV (9 fold increase) and HdIV (3.5 fold increase), and a second GST (Sf1H09713-5-1) that was down-regulated by both viruses (2.5–5

Table 3
Relative quantitative RT-PCR analysis of selected hemocyte and fat body transcripts compared to microarray data. Fold changes for a given transcript following HdIV or MdBV infection and following UV-inactivated HdIV or MdBV injection were compared to larvae injected with PBS. Significant fold changes >1.5 are in bold.

Gene name	Oligo ID	HdIV			MdBV		
		Micro-arrays	RT qPCR		Micro-arrays	RT qPCR	
			Normal	UV-inactivated		Normal	UV-inactivated
Hemocytes							
Immune related proteins							
Galectin 4	DY782958	1.58	**1.82**	0.98	**7.83**	5.75	1.06
Hemocyte Protease 3	DY785990	1.87	**2.74**	1.05	**0.47**	0.52	1.09
Collagens							
Collagen IV-1	DY781936	0.75	**0.69**	0.99	**0.67**	0.63	1.04
Collagen IV-2	DY784506	0.74	**0.78**	nd	**0.85**	0.80	nd
Collagen IV-3	DY785223	0.54	**0.62**	0.96	**0.61**	0.74	1.01
Collagen IV-4	DY900227	1.19	1.33	nd	0.96	1.05	nd
Fat body							
Collagens							
Collagen IV-1	DY781936	0.47	**0.37**	1.09	**0.35**	0.29	0.97
Collagen IV-2	DY784506	0.35	**0.27**	1.04	**0.20**	0.21	0.98
Collagen IV-3	DY785223	0.33	**0.34**	1.04	**0.22**	0.29	0.97
Collagen IV-4	DY900227	0.29	**0.19**	0.99	**0.19**	0.19	1.08
Actin related proteins							
Myosin-2	DV075968	2.82	**2.46**	0.98	**5.50**	4.01	1.03
Myosin	DV077070	1.83	**1.98**	1.01	**1.96**	4.33	1.00
Troponin C2	DY777011	1.90	**2.96**	0.97	**1.70**	7.59	1.12
Troponin I1	DV076558	2.41	**2.00**	0.98	**6.03**	6.70	1.09
Tropomyosin	DY792034	2.69	**2.35**	1.04	**3.20**	6.24	1.09
Detox proteins							
GST-1	DY773790	0.41	**0.43**	1.09	**0.21**	0.17	0.94
GST-2	DY774771	0.35	**0.23**	1.05	**0.25**	0.12	1.06

fold). Interestingly, a third GST (Sf1F00968-3-1) was also one of two host transcripts we identified that was differentially affected by infection; increasing in response to HdIV and decreasing in response to MdBV (Table 3, Supp. Table 2).

Among the other functional categories of genes, HdIV and/or MdBV affected several motor/cytoskeleton related transcripts in the fat body including multiple myosins or troponins. A sequence encoding a dynein light chain was 5 fold more abundant in the fat body of hosts infected by HdIV and MdBV, while a beta-tubulin was 2.5 fold less abundant. Alterations in host storage protein levels are also known to occur following parasitism by other PDV-carrying wasps (Beckage and Kanost, 1993; Dong et al., 1996; Shelby and Webb, 1994). The main targets of HdIV and MdBV were apolipophorin and arylphorin which decreased up to 3.6 times in the fat body.

3.4. HdIV and MdBV affect the abundance of selected immune transcripts

The two main alterations to the host immune system observed after PDV infection are the inhibition of hemolymph melanization, and disabled adhesion and spreading of hemocytes that mediate capsule formation (Strand, 2010). Our results indicated that HdIV and MdBV affected the abundance of several transcripts associated with both the phenoloxidase (PO) cascade and cellular immunity (Fig. 2, Supp. Table 2). HdIV increased the abundance of proPO2 (1.7 fold) in hemocytes; a finding that differs from studies of *Choristoneura fumiferana* and the ichneumonid *Tranosema rostrale* where proPO transcript abundance was unaffected by parasitism and transiently decreased after injection of TrIV (Doucet et al., 2008). Upregulation of proPO was previously observed in *S. frugiperda* after HdIV infection but a concomitant decrease in the serine protease that activates proPO was hypothesized to counteract this effect (Barat-Houari et al., 2006). In contrast, MdBV infection reduced the abundance of two serine protease inhibitors (3.7 and 2.4 fold) and a chymotrypsin-like serine protease (Sf2M01928-5-1) in the fat body. Immune-responsive genes differentially affected by both viruses included a sequence with similarity to hemocyte protein 3 (HP3, Sf1H02799-5-1) from *Manduca sexta*, which is down-regulated following immune challenge by *E. coli* (Jiang et al., 1999, 2005). HP3 transcript abundance similarly decreased after infection by MdBV but increased 3-fold after infection by HdIV (Table 3). Two other sequences, Spodo-11-tox (Sf1H04942-5-1) and an Hdd23 homolog (Sf1H06034-3-1), induced after an immune challenge by bacteria (Girard et al., 2008; Shin et al., 1998), were also upregulated in hemocytes after infection by MdBV.

Targets affected by PDV infection with predicted roles in cellular immunity included a transcript with similarity to a hemocyte-specific membrane protein from sarcophagids with multiple EGF-like domains. This sequence increased (1.9 fold) after HdIV infection and decreased (1.5 fold) after MdBV infection.A macrophage migration inhibitory factor (MIF) in contrast declined 5 fold in the

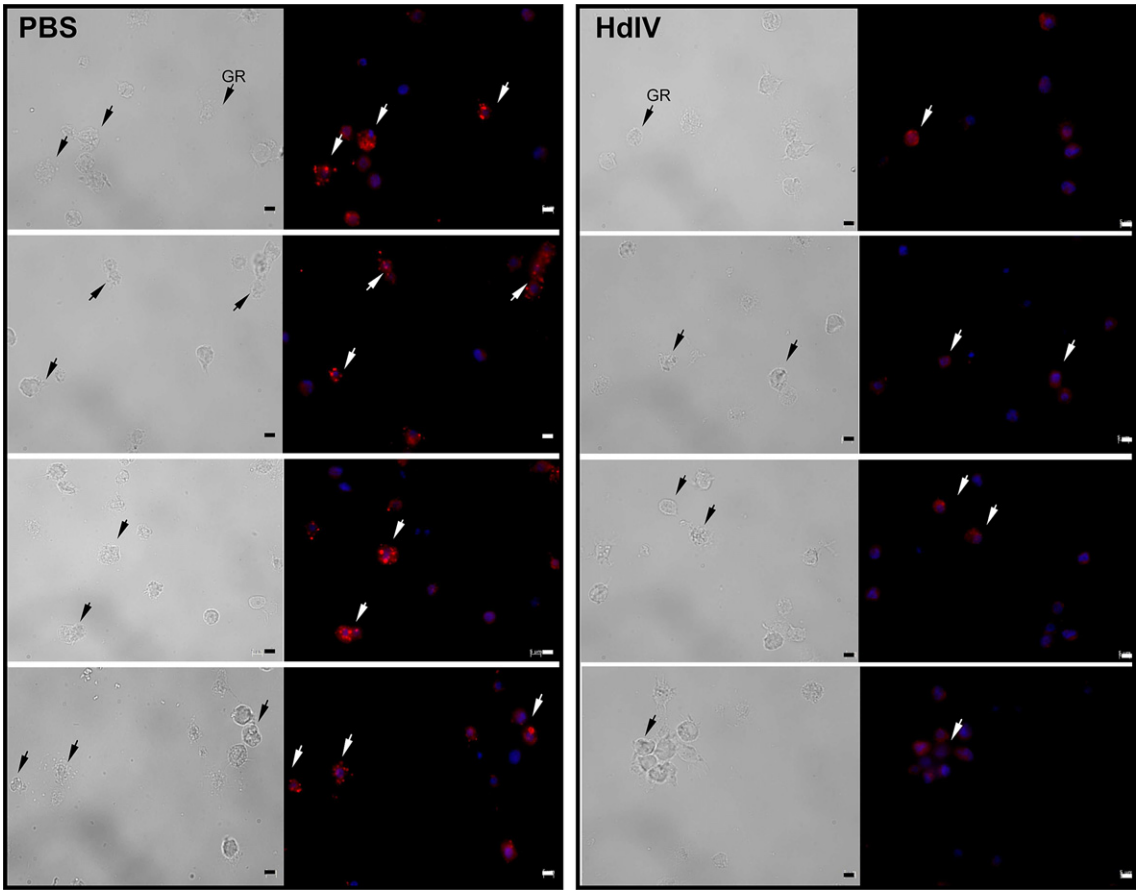

Fig. 4. Immunolocalization of collagen IV in hemocytes from *S. frugiperda* larvae injected with HdIV or PBS. Hemocytes from 4 PBS-treated (left panel) and 4 HdIV-infected (right panel) larvae were stained with an anti-collagen type IV antibody (red) while nuclei were stained with Hoechst 33258 (shown in blue). For each panel, phase contrast images are presented to the left and corresponding epifluorescent images are shown to the right. Type IV collagen localizes to granules of granulocytes (GR, black arrows) of PBS-injected larvae. The fluorescence signal is clearly reduced in hemocytes of HdIV-injected larvae (white arrows). Scale bar: 5 μm.

fat body after infection by both viruses. In mammals, MIF is an important cytokine regulator of innate and adaptive immune responses (reviewed in Calandra and Roger, 2003) but its function in insects is unknown. Our results also indicated that a galactose-binding protein (Galectin) was strongly upregulated after infection by MdBV (7.8 fold) and modestly upregulated (1.6 fold) after infection by HdIV. Galectins are thought to have functions in development and immunity related to adhesion, migration, and growth (Cooper and Barondes, 1999; Pace and Baum, 2004). Both viruses also upregulated several genes in the fat body encoding predicted extracellular matrix proteins. For instance, an integrin encoding sequence was down-regulated (2.5 fold) by HdIV and MdBV. Integrins are cell adhesion molecules that also play important roles in regulating hemocyte adhesion (Lavine and Strand, 2003). Several collagen sequences, which can serve as ligands for integrins (Heino, 2007) were also reduced in the fat body and hemocytes by both viruses.

3.5. Two functional classes of S. frugiperda genes are targeted by HdIV and MdBV

Among the genes that were similarly up- or down-regulated after (see above), two functional classes were notably over-represented: genes encoding predicted motor proteins whose transcripts increased and collagen IV-like proteins whose transcripts declined (Fig. 2, Table 3). Among the 11 *S. frugiperda* transcripts encoding for motor-related proteins (actin, myosin, tropmyosin), 10 were much more abundant in the fat body after infection (Fig. 2, Supp. Table 2). Interactions of actin and myosin, and eventually tropomyosin, are responsible for a variety of processes including cell division and morphogenesis (Halsell and Kiehart, 1998). Myosins also belong to a class of the actin binding proteins with roles in phagocytosis that include phagosome formation, transport of adhesion molecules, and pseudopod extension (Castellano et al., 2001). PDV-associated upregulation in the fat body but not hemocytes suggests these

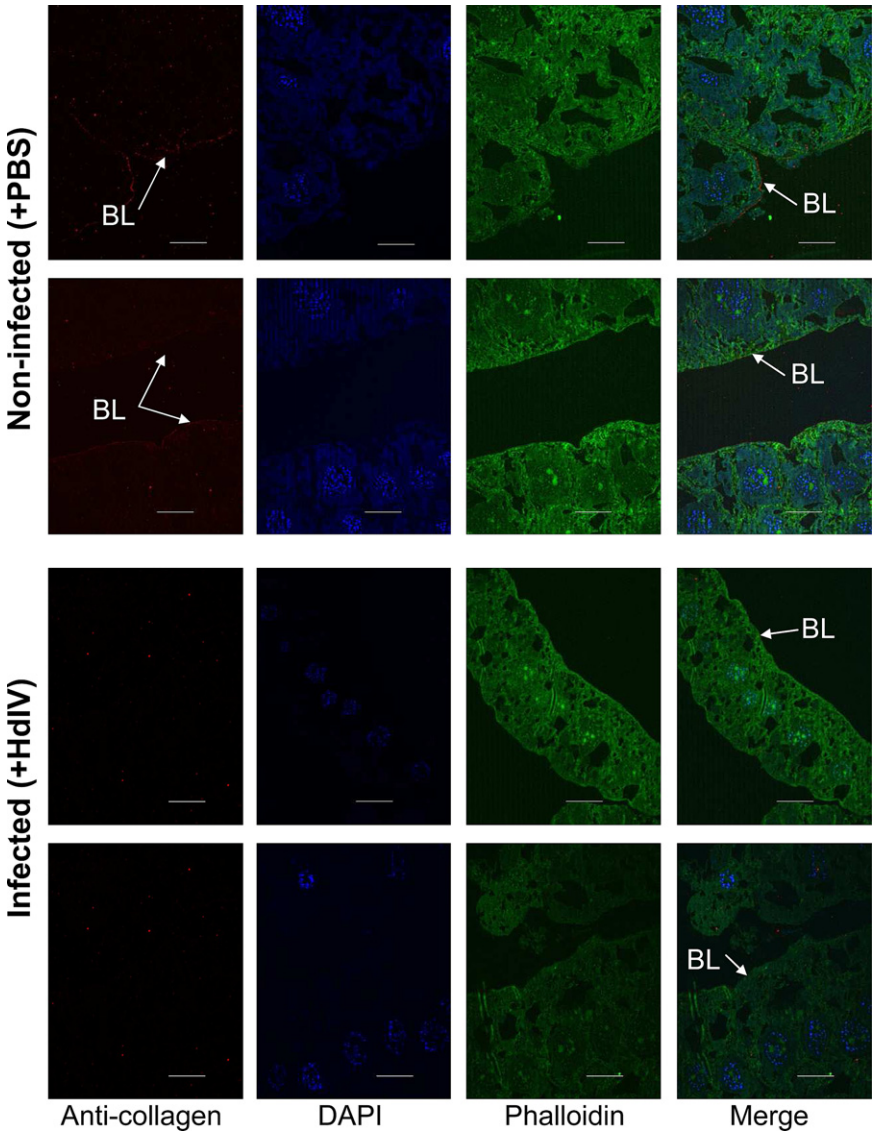

Fig. 5. Immunolocalization of collagen IV in fat body from *S. frugiperda* larvae injected with HdIV or PBS. Fat bodies from 2 PBS-treated (upper panel) and 2 HdIV-infected (lower panel) larvae were stained with an anti-collagen type IV antibody (red) while nuclei were stained with DAPI (shown in blue) and actin with FITC-phalloidin (shown in green). For each panel, merged images are shown to the right. Type IV collagen localizes to the basal lamina (BL, arrows) of control PBS-injected larvae. The fluorescence signal is clearly reduced in basal lamina of HdIV-injected larvae. Scale bar: 20 μm.

alterations do not involve alterations in phagocytosis or other hemocyte-mediated defense responses. However, changes in transcript levels in the fat body could affect cell shape or tissue integrity that impact molting, pupation or immune functions.

Class IV collagens are multimeric proteins and major components of basement membranes (BM). In insects, they are also synthesized and secreted by both hemocytes and the fat body (Natzle et al., 1982; Nardi and Miklasz, 1989; Yurchenco and O'Rear, 1994; Adachi et al., 2005). In *Bombyx mori*, immunolocalization experiments show that granulocytes discharge collagen, which attaches to foreign entities and adjoining hemocytes to facilitate binding in capsules (Adachi et al., 2005). Fragments of collagen IV generated by the activity of microbial metalloproteinases may also play a role in activating hemocytes (Altincicek et al., 2009). In *S. frugiperda*, 4 different transcripts encode predicted collagen IV α chains with HdIV and MdBV reducing the abundance of each (Table 3). To assess whether this decrease in transcript abundance also reduced protein abundance, we used an anti-collagen IV antibody to compare labeling intensity of *S. frugiperda* hemocytes from HdIV-infected and non-infected control larvae. Our results revealed weaker staining of hemocytes from infected larvae than hemocytes from PBS-injected control larvae (Fig. 4).

Collagen synthesis is catalyzed by collagen prolyl 4-hydroxylase (C-P4H) with protein disulfide isomerase (PDI) functioning as a β-subunit of C-P4H (Myllyharju, 2003). Importantly, we identified 2 PDI-like genes that were down-regulated in the fat body after infection by HdIV or MdBV. In addition, MdBV infection reduced the abundance of a proteoglycan precursor, HdIV reduced calreticulin, and both viruses increased the abundance of transcripts for predicted heat shock proteins. Since HSP47 and calreticulin are both molecular chaperones involved in biosynthesis of fibril-forming procollagen (Koide et al., 2006; Nagata, 2003), these observations suggest both PDVs affect host BM formation and function. To assess this possibility more closely, we used an anti-collagen IV antibody to compare labeling intensity of *S. frugiperda* fat bodies from HdIV-infected and non-infected control larvae. Again, our results revealed weaker staining of the basal lamina surrounding fat body cells in infected larvae compared to non-infected control larvae (Fig. 5). Since BMs function as barriers to infection by baculoviruses (Li et al., 2007; Romoser et al., 2005), we speculate they could also affect entry of PDVs into some tissues. EM observations similarly show that PDV particles associate with the BMs surrounding various host tissues (Stoltz and Vinson, 1977, 1979; Stoltz et al., 2007). Thus, a reduction in collagen production and BM formation may facilitate cell infection. Altered BM formation could also well affect other physiological processes and immune defense.

3.6. Conclusions

In summary, we show that HdIV and MdBV alter the abundance of a relatively low proportion of *S. frugiperda* transcripts in hemocytes and the fat body at 24 h post-infection. A majority of the transcripts affected by infection also differ between MdBV and HdIV, although we do identify some host genes that are similarly affected by both viruses. Most of these similarly affected genes are transcribed in the fat body and most also have predicted roles in immunity, detoxification, or maintenance of cell structure. Given that IVs and BVs encode few shared genes, the host transcripts that are similarly affected by HdIV and MdBV infection could indicate functional convergence by each virus to target a few host processes at the transcriptional level that are important to successful parasitism of both *H. didymater* and *M. demolitor*. That the abundance of most transcripts affected by PDV infection varies from 1 to 3-fold relative to our control, further suggests each PDV tends to modulate host gene activity rather than strongly inducing or suppressing expression.

At the tissue level, our results identify stark differences in the effects of HdIV and MdBV on the hemocyte transcriptome with the former affecting the abundance of several transcripts and the latter affecting the abundance of relatively few. Since we examined only one time point post-infection (24 h), it is possible this difference reflects a temporal bias and that analysis of other time points would identify a larger suite of hemocyte-associated genes affected by MdBV infection. Overall though, our data suggest that the broadly similar effects that HdIV and MdBV have on host growth and immunity are not due to profound changes in host gene expression. Our results instead suggest that HdIV and MdBV gene products affect the growth and immune state of hosts through interactions at the protein level. Indeed, functional studies with selected members of the gene families encoded by MdBV show that suppression of melanization, encapsulation and other immune defenses are due to viral proteins interacting with specific host proteins (summarized by Strand, 2010). In contrast, the low and pleiotropic effects on host transcripts observed during the current study could reflect either protein–protein interactions or other processes such as epigenetic regulation. Thus, in addition to the multimember gene families PDVs encode, these viruses may also produce small non-coding RNAs that modulate host gene transcription.

Database linking and accession numbers

Data are deposited at GEO (http://www.ncbi.nlm.nih.gov/geo/) under number GSE17293.

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Appendix. Supplementary data

Supplementary data associated with the article can be found in online version, at doi:10.1016/j.ibmb.2011.03.010.

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