



Early changes in the pupal transcriptome of the flesh fly *Sarcophaga crassipalpis* to parasitization by the ectoparasitic wasp, *Nasonia vitripennis*



Ellen L. Danneels^{a,*}, Ellen M. Formesyn^{a,1}, Daniel A. Hahn^b, David L. Denlinger^c, Dries Cardoen^d, Tom Wenseleers^d, Liliane Schoofs^e, Dirk C. de Graaf^a

^a Laboratory of Zoophysiology, Ghent University, Krijgslaan 281 S2, B-9000 Ghent, Belgium

^b Department of Entomology and Nematology, University of Florida, Gainesville, FL 32611-0620, USA

^c Department of Entomology, Ohio State University, Columbus, OH 43210, USA

^d Laboratory of Ecology, Evolution and Biodiversity Conservation, KU Leuven, B-3000 Leuven, Belgium

^e Laboratory of Animal Physiology and Neurobiology, KU Leuven, B-3000 Leuven, Belgium

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ABSTRACT

We investigated changes in the pupal transcriptome of the flesh fly *Sarcophaga crassipalpis*, 3 and 25 h after parasitization by the ectoparasitoid wasp, *Nasonia vitripennis*. These time points are prior to hatching of the wasp eggs, thus the results document host responses to venom injection, rather than feeding by the wasp larvae. Only a single gene appeared to be differentially expressed 3 h after parasitization. However, by 25 h, 128 genes were differentially expressed and expression patterns of a subsample of these genes were verified using RT-qPCR. Among the responsive genes were clusters of genes that altered the fly's metabolism, development, induced immune responses, elicited detoxification responses, and promoted programmed cell death. Envenomation thus clearly alters the metabolic landscape and developmental fate of the fly host prior to subsequent penetration of the pupal cuticle by the wasp larva. Overall, this study provides new insights into the specific action of ectoparasitoid venoms.

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1. Introduction

Endoparasitoids, wasps that deposit their eggs inside the body of their arthropod hosts, have evolved sophisticated mechanisms to disable the host's defensive responses. An array of virulence factors including polydnnaviruses, venoms, virus-like particles, ovarian fluids and teratocytes are used to combat the host's defense system, thus enabling the wasp larvae to freely manipulate and devour the host (Glatz et al., 2004; Labrosse et al., 2003; Pennacchio and Strand, 2006). Ectoparasitoids, wasps that deposit their eggs on the surface of the host, appear to lack many of the virulence factors known from endoparasitoids, yet they too are capable of manipulating the host, mainly through the use of the venom that they inject (Rivers et al., 1999).

Transcriptomic approaches have pinpointed host pathways that are targeted during parasitization, as demonstrated in recent

studies that have probed endoparasitoid-host relationships including work completed on *Drosophila melanogaster*-*Asobara tabida* (Wertheim et al., 2005, 2011), *D. melanogaster*-*Leptopilina* spp. (Schlenke et al., 2007), *Bemisia tabaci*-*Eretmocerus mundus* (Mahadav et al., 2008), *Pieris rapae*-*Pteromalus puparum* (Fang et al., 2010), *Spodoptera frugiperda*-*Hyposoter didymator*, *Ichnovirus/Microplitis demolitor* *Bracovirus* (Provost et al., 2011), *Plutella xylostella*-*Diadegma semiclausum* (Etebari et al., 2011), and *Pseudoplusia includes*-*M. demolitor* (Bitra et al., 2011). In spite of this burst of recent work, none of the above analyses have used a transcriptomic approach to examine similar responses elicited by an ectoparasitoid. In this study we examine the response elicited by an ectoparasitoid, *Nasonia vitripennis*, on one of its favored hosts (Whiting, 1967), the flesh fly *Sarcophaga crassipalpis*.

Although *N. vitripennis* larvae are capable of developing on late larvae or even pharate adults of their hosts, parasitoid survival is highest when parasitizing hosts that have just entered the pupal stage. Thus, *N. vitripennis* is attracted to sites where fly larvae are wandering and preparing for pupariation so that it may parasitize freshly pupated hosts. The wasp inserts its ovipositor through the puparium, envenomating the fly pupa and then depositing its egg on the surface of the pupal body. The egg is lodged within the space

* Corresponding author. Tel.: +32 9264 8734; fax: +32 9264 5242.

E-mail addresses: Ellen.Danneels@UGent.be, ellen_danneels@hotmail.com (E. L. Danneels).

¹ Shared first authors.

between the puparium and the pupal cuticle, thus it is encased within the puparium but remains on the external surface of the fly pupa. The wasp's venom arrests development in the host (Rivers and Denlinger, 1994a), alters host metabolism (Rivers and Denlinger, 1994b), and induces paralysis and apoptosis (Rivers et al., 1999), thus ensuring a suitable environment for growth and development of the parasitoid. Although *N. vitripennis* lacks some of the virulence factors commonly documented in endoparasitoids, like teratocytes or symbiotic polydnnaviruses, the venom proteome shows high similarity to the venoms of endoparasitoids (Werren et al., 2010; de Graaf et al., 2010). This means that the early steps of host manipulation are solely the result of the venomous mixture injected by *N. vitripennis*, clearly differentiating this ectoparasitoid from endoparasitoids that employ teratocytes or symbiotic viruses.

The recent EST dataset available for *S. crassipalpis* (Hahn et al., 2009) has enabled us to examine transcriptomic responses of the flesh fly to envenomation by the ectoparasitoid, *N. vitripennis*. Using microarrays we examine transcript expression in the host 3 h and 25 h after envenomation. The sampling points selected are both prior to hatching of the wasp larva, thus the response we document here is the response to injection of the venom rather than later responses associated with feeding by the wasp larvae. We report little response at the transcript level by 3 h after envenomation, but by 25 h after envenomation expression patterns of key immune, developmental, and metabolic pathways have clearly been altered by envenomation. We use these data to build hypotheses for the molecular underpinnings of the *Sarcophaga* host response to envenomation by *N. vitripennis*, and we compare our results from this endoparasitoid with molecular patterns observed in other systems involving host attack by endoparasitoids.

2. Material and methods

2.1. Preparation of parasitized and non-parasitized flesh fly pupae

2.1.1. Insect strains

The laboratory strain *N. vitripennis* Asym C was kindly provided by Prof. Dr. L. W. Beukeboom from Evolutionary Genetics, Centre for Ecological and Evolutionary Studies in The Netherlands. This wild-type line collected in The Netherlands was cured from *Wolbachia* infection and maintained in the laboratory since 1971 (Van den Assem and Jachmann, 1999). Wasps were reared at 25 °C under a 16:8 light:dark cycle. The flesh flies (*S. crassipalpis*) were provided from a culture maintained at the University of Florida, and cultured in the laboratory as described by Denlinger (1972). Larvae were fed on beef liver at 25 °C and exposed to a 16:8 light:dark cycle.

2.1.2. Collection of parasitized and control hosts

Before the start of the experiment, female wasps were exposed to flesh fly puparia for 6 h to condition them to oviposit. The next day, new *S. crassipalpis* puparia (4 days after puparium formation) were placed in a culture tube. Holes the size of a flesh fly puparium were made in the stopper of the culture tube, so only the posterior region of the puparium was accessible to the parasitoids. The experienced *N. vitripennis* females and the pupae, 5 days after pupariation, were placed together in a tube in a 3:1 ratio, to promote parasitization. After 2 h, females of *N. vitripennis* were removed. Flies were sampled 2 or 24 h after parasitoids were removed from the tube, snap-frozen in liquid nitrogen, and then stored at –80 °C until RNA extraction. Because the *N. vitripennis* females had 2 h to parasitize the pupae, the sampling points correspond on average to 3 and 25 h post-parasitization. Control pupae were treated identically except that they were not exposed to the parasitoid.

2.2. Sample preparation for the microarray and validation experiment

2.2.1. RNA isolation and cDNA synthesis

RNA was extracted using the RNeasy Lipid Tissue Mini Kit (Qiagen). From each treatment (3 h control, 3 h parasitized, 25 h control, and 25 h parasitized), 4 replicates consisting of a single fly pupa each were analyzed. Due to the hard puparium, the Precellys® 24 Homogenizer (Bertin Technologies, Montigny le Bretonneux, France) was used after adding one stainless steel bead (2.3 mm mean diameter) and ¼ of a PCR tube of zirconia/silica beads (0.1 mm mean diameter) to an individual pupa. An on-column DNase I treatment with the RNase-free DNase set (Qiagen) was performed. RNA was eluted twice, first with 30 µl RNase free water, then with 20 µl RNase free water, and then stored at –80 °C. Five µg of total RNA from each sample was converted to cDNA using Oligo(dT)₁₈ primers (0.5 µg/µl) and was carried out according to the RevertAid H Minus First strand cDNA Synthesis kit protocol (Fermentas).

2.2.2. Checking parasitized pupae for the nanos gene

To ensure that the pupae were parasitized, samples were checked for the presence of *N. vitripennis nanos* (nos) (NM_001134922.1) (Olesnick and Desplan, 2007). This early embryonic protein is present in eggs of *N. vitripennis* but not in pupae of *S. crassipalpis*, thus enabling us to distinguish parasitized from non-parasitized fly pupae. The following primer set was used for reverse transcriptase PCR: 5'-TGGCAGATTCTTGTCTAT-3' and 3'-AGAAACAGGTAACTGTCCGC-5'. The obtained amplicon has a length of 264 basepairs and was loaded on a 0.8% agarose gel and visualized by ethidium bromide staining.

2.3. Microarray study of *S. crassipalpis* pupae transcriptional response to parasitization by *N. vitripennis*

2.3.1. Selection of the EST dataset

An EST dataset for *S. crassipalpis* (whole bodies of different life stages including pupae as well as protein-fed and protein-starved males and females) became available in 2009. It was produced by parallel pyrosequencing on the Roche 454-FLX platform and identified approximately 11,000 independent transcripts that are a representative sample of roughly 75% of the expected transcriptome (Hahn et al., 2009). A sub-set of these sequence data was made by blasting the sequences against the protein sequences of *D. melanogaster*. The sequences that showed the best homology to known genes were retained, resulting in a dataset of 10,129 EST sequences. Probes were designed and spotted on a custom 8 × 15k Agilent array developed with Agilent eArray software.

2.3.2. Microarray experimental procedures

RNA concentration and purity were determined using a Nano-drop ND-1000 spectrophotometer (Nanodrop Technologies) and RNA integrity was assessed using a Bioanalyser 2100 (Agilent). RNA concentrations varied between 1.9 and 8.6 µg/µl, and the RNA Integrity Numbers and the RNA ratios indicated high quality of the RNA samples (Table S1). Per sample, 100 ng of total RNA spiked with 10 viral polyA transcript controls (Agilent) was converted to double stranded cDNA in a reverse transcription reaction. Subsequently the sample was converted to antisense cRNA, amplified and labeled with Cyanine 3-CTP (Cy3) or Cyanine 5-CTP (Cy5) in an *in vitro* transcription reaction according to the manufacturer's protocol (Agilent). A mixture of purified and labeled cRNA (Cy3 label: 300 ng; Cy5 label: 300 ng) was hybridized on the custom Agilent array followed by (manual) washing, according to the manufacturer's procedures. To assess the raw probe signal intensities, arrays

were scanned using the Agilent DNA MicroArray Scanner with surescan High-Resolution Technology, and probe signals were quantified using Agilent's Feature Extraction software (version 10.7.3.1). The microarray data were deposited in the NCBI Gene Expression Omnibus (GEO) database (<http://www.ncbi.nlm.nih.gov/geo/>) under accession numbers GPL15391 (microarray including detailed annotation) and GSE36996.

2.3.3. Microarray data quality control and statistical analysis

Statistical data analysis was performed on the processed Cy3 and Cy5 intensities, as provided by the Feature Extraction Software version 10.7. Further analysis was performed in an R programming environment using a series of Bioconductor packages (<http://www.bioconductor.org>; Gentleman et al., 2004). Differential expression between the parasitized and non-parasitized hosts, as well as time comparisons within each group was assessed via the moderated t-statistic, described in Smyth (2005) and implemented in the limma package of Bioconductor. This moderated t-statistic applies an empirical Bayesian strategy to compute the gene-wise residual standard deviations and thereby increases the power of the test, which is especially suitable for smaller data sets. To control the false discovery rate (FDR), multiple testing correction was performed (Benjamini and Hochberg, 1995) and probes were called differentially expressed in case of a corrected *p*-value below 0.05 and an absolute fold change larger than 1.25.

2.4. Validation experiment

2.4.1. Reference gene selection and primer design

Because no reference genes for RT-qPCR were available for *S. crassipalpis*, candidate reference genes were chosen due to their stable expression in other studies that had exposed either *D. melanogaster* or *Apis mellifera* to a bacterial challenge (Ling and Salvaterra, 2011; Scharlaken et al., 2008). Primers with product size-ranges of 80–150 bp, were designed with Primer3Plus (Untergasser et al., 2007), using the default settings (Table S5).

2.4.2. RT-qPCR reaction mixture and cycling program

RT-qPCR was executed in opaque white 96 well microtiter plates (Hard-Shell 96 Well PCR plates, Bio-Rad), sealed with Microseal 'B' seals (Bio-Rad), using the CFX96 Real-Time PCR Detection System (Bio-Rad). Each 15 µl reaction consisted of 7.5 µl 2× Platinum SYBR Green qPCR SuperMix-UDG (Invitrogen), 0.2 µM forward and 0.2 µM reverse primers (Integrated DNA Technologies), 6.5 µl Milli-Q and 1 µl cDNA template.

Each sample was run in triplicate using a PCR program with the following conditions: 50 °C 2 min; 95 °C 2 min; and 40 cycles of a combined denaturation (95 °C 20 s) and annealing (60 °C 40 s) step.

Fluorescence was measured after each cycle. At the end of the program, a melt curve was generated by measuring fluorescence after each temperature increase of 0.5 °C for 5 s over a range from 65 °C to 95 °C.

2.4.3. Computational selection of reference genes

Primer efficiencies, R^2 values and melt curves were calculated with CFX Manager Software (Bio-Rad). Reference gene stability was analyzed using the geNorm^{PLUS} algorithm within the qBase^{PLUS} environment (Biogazelle NV). Default settings were kept, except that target specific amplification efficiencies were used.

2.5. Microarray data analysis

S. crassipalpis genes that were differentially expressed across our treatments were functionally annotated with Blast2GO (Conesa et al., 2005; Gotz et al., 2008). After GO term annotation, an enrichment analysis (two-tailed Fisher's exact test with default settings) within the Blast2GO environment was undertaken (Table 1). Clusters of Orthologous Groups (COG) functional categories were assigned with COGNITOR and stand-alone PSI-BLAST using the Eukaryotic Orthologous Groups (KOG) database (Tatusov et al., 2000). Further, genes were manually clustered by searching for groups of genes with the same GO-terms, or by putative functions of homologue genes in other species.

3. Results and discussion

3.1. Verifying parasitization

Before conducting the microarray experiment, we verified whether individual flesh flies were parasitized by screening for transcripts of *nanos* (*nos*), a developmental patterning gene that should be abundant in early embryos of *N. vitripennis*, but not in pupae of *S. crassipalpis*. Only individual RNA samples that showed a clear band at 264 nucleotides indicating *N. vitripennis nos* expression were used for further experiments (Fig. 1).

3.2. Overview of differential gene expression

In our analyses, we considered a transcript to be differentially expressed between envenomated and non-parasitized control samples if the FDR-adjusted *p*-value was <0.05 and the fold change was >1.25. When comparing all four treatments to each other (Control 3 h, Parasitized 3 h, Control 25 h, and Parasitized 25 h) hundreds of genes were differentially expressed between the 3 h and 25 h samples in both control and parasitized samples (Fig. 2). The large changes in transcript profiles observed through time do

Table 1

Selected biological process GO-terms which were enriched among the EST's that were differentially expressed in control and parasitized pupae, 3 h and 25 h after parasitization.

| GO-nr | GO biological process term | Control 3 h vs. 25 h | | | | Parasitized 3 h vs. 25 h | | | |
|------------|--|----------------------|-----------------|-----------------|-----------------|--------------------------|-----------------|-----------------|-----------------|
| | | FDR ^a | <i>p</i> -value | NS ^b | FE ^c | FDR ^a | <i>p</i> -value | NS ^b | FE ^c |
| GO:0006119 | Oxidative phosphorylation | 4.32E-05 | 4.78E-08 | 14 | 7.53 | 5.09E-07 | 5.54E-10 | 20 | 6.87 |
| GO:0022900 | Electron transport chain | 6.53E-05 | 8.83E-08 | 15 | 6.53 | 4.79E-05 | 1.06E-07 | 19 | 6.70 |
| GO:0042773 | ATP synthesis coupled electron transport | 8.21E-05 | 1.21E-07 | 13 | 7.64 | 3.46E-06 | 5.37E-09 | 18 | 5.04 |
| GO:0022904 | Respiratory electron transport chain | 8.71E-05 | 1.39E-07 | 14 | 6.83 | 4.41E-05 | 9.20E-08 | 18 | 6.71 |
| GO:0045333 | Cellular respiration | 1.29E-04 | 2.21E-07 | 16 | 5.61 | 3.46E-06 | 5.53E-09 | 23 | 4.31 |
| GO:0006091 | Generation of precursor metabolites and energy | 2.24E-04 | 4.42E-07 | 19 | 4.48 | 5.78E-05 | 1.38E-07 | 26 | 5.39 |
| GO:0042775 | Mitochondrial ATP synthesis coupled electron transport | 2.24E-04 | 4.68E-07 | 12 | 7.43 | 7.41E-06 | 1.37E-08 | 17 | 5.04 |
| GO:0015980 | Energy derivation by oxidation of organic compounds | 4.40E-04 | 1.30E-06 | 16 | 4.84 | 3.47E-05 | 6.83E-08 | 23 | 3.73 |

^a False discovery rate.

^b Number of genes associated with GO-term which were differentially expressed.

^c Fold enrichment.

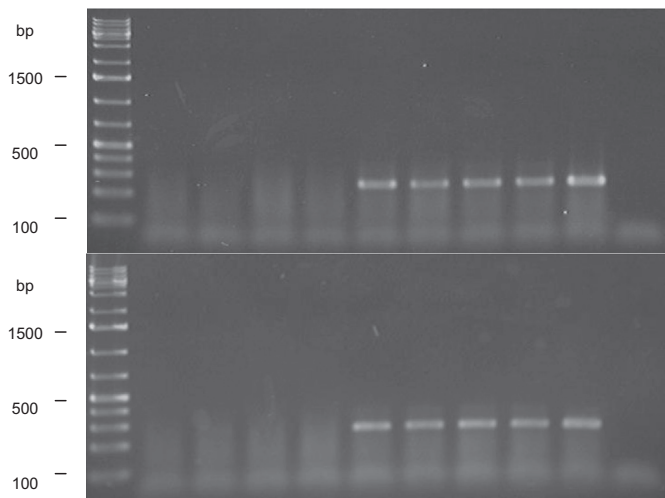


Fig. 1. Gel electrophoresis analysis for the presence of *nos* in test samples for microarray and validation experiment. Top: samples at 3 h parasitization, Bottom: samples at 25 h parasitization, from left to right: 1 kb ladder (Fermentas), 4 control samples, 4 parasitized samples, positive control (abdomen from *N. vitripennis* female), negative control.

not reflect the effects of envenomation on the transcriptome, but rather reflect the dynamic nature of development as *S. crassipalpis* pupae undergo metamorphosis and transition into pharate adult development (Denlinger and Zdarek, 1994; Ragland et al., 2010). Reinforcing the effect that envenomation has on host physiology, the numbers of differentially expressed transcripts between the 3 and 25 h parasitized pupae were greater than the numbers of differentially expressed transcripts between the 3 and 25 h control pupae. When comparing solely the time-matched parasitized and

control samples, only 1 transcript was differentially expressed 3 h after envenomation and 128 transcripts were differentially expressed 25 h after envenomation. The 1 differentially regulated transcript at 3 h after envenomation was a leucine rich repeat protein that was slightly down-regulated (–0.74 fold change). By 25 h after envenomation, 19 transcripts were down-regulated and 109 were up-regulated (Fig. 2). We found fewer transcripts differentially regulated than several other studies of parasitic wasp–host interactions (Wertheim et al., 2005; Fang et al., 2010; Bitra et al., 2011; Etebari et al., 2011; Provost et al., 2011; Wertheim et al., 2011). Unlike the previous studies that focused on endoparasitoids, *N. vitripennis* is an ectoparasitoid and we sampled fairly early during the interaction, before the developing parasitoid larvae began to interact with the host. Thus, our results represent only the earliest stages of the host response to envenomation.

3.3. Validation by RT-qPCR

A sample of 20 ESTs were selected for validation with RT-qPCR, including the single transcript found in the 3 h group (Irr-pr) (Table S4). Ten candidate reference genes were tested for their stable expression levels across all conditions (3 and 25 h, control and parasitized), resulting in two selected reference genes (Table S5, Fig. S1). EIF or eukaryotic translation initiation factor 1 has 97.15% homology to eIF-1A that has been used as a reference target for expression profiles of *D. melanogaster*. Ubq or ubiquitin-conjugating enzyme displays 92.8% homology to Ubi from *D. melanogaster*, which has a function in protein degradation. In all except two cases, RT-qPCR revealed the same log ratio trends as found in the microarray study. Nine of them gave significant differences in expression pattern including 5 that were up-regulated and 4 that were down-regulated (Fig. 5).

3.4. Gene-Ontology analysis of microarray data

Using Blast2GO, we assigned Gene Ontology (GO) terms to the *S. crassipalpis* EST sequences that were used for the microarray analysis, successfully annotating 8618 out of the 10,129 ESTs (85%). We then performed GO term enrichment analyses on 4 sets of data Parasitized 3 h vs. Parasitized 25 h, Control 3 h vs. Control 25 h, Control 3 h vs. Parasitized 3 h and Control 25 h vs. Parasitized 25 h. GO term enrichment analyses of the two sets that compared 3 h vs. 25 h samples showed enrichment of GO terms associated with energy metabolism (Table 1). As with the time comparisons in our transcript-by-transcript level analyses above, the enrichment in energy metabolism categories likely reflects the metabolic demands that have been documented to occur at the pupal–pharate adult metamorphic molt (Denlinger and Zdarek, 1994; Ragland et al., 2010).

To further explore the effects of envenomation at levels above single ESTs we performed a second set of analyses where we tested for enrichment across categories in the COG (Clusters of Orthologous Groups from eukaryotic genomes) functional classification using Blast2GO (Fig. 3). We observed enrichment that suggested differential regulation in categories associated with growth (including replication, transcription, and translation), cell signaling, intermediary metabolism, and defensive mechanisms. In a complementary analysis, we also manually clustered 103 differentially expressed ESTs in control vs. envenomated pupae at 25 h into 8 GO clusters (25 ESTs out of 128 could not be annotated by Blast2GO) representing genetic information processing, metabolism, development, programmed cell death, detoxification, immune system, sensory system and transporters (Table S2 and Fig. 4).

One challenge for transcriptomic studies like ours that use either whole bodies or complex tissues (e.g., hemocytes, brain, or

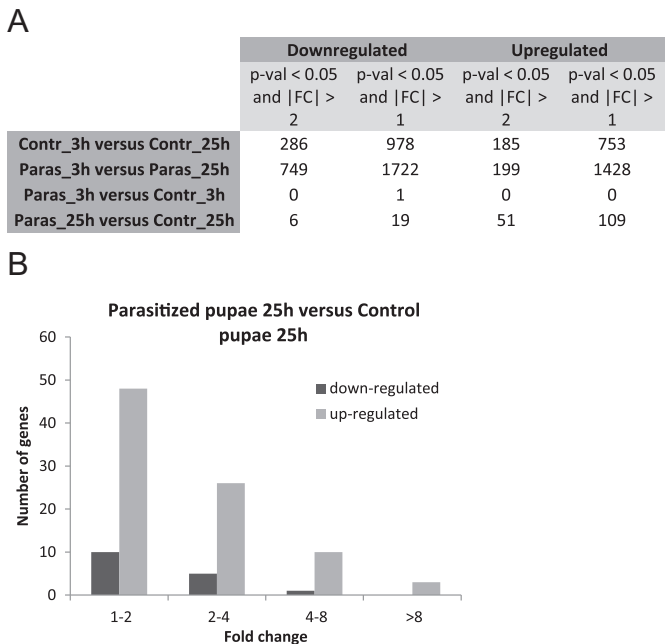


Fig. 2. General statistics on the differentially regulated genes in response to parasitization. (A) The four comparisons with their respective amounts of up- and down-regulated genes that were identified by microarray hybridization according to the following selection criteria: *p*-value < 0.05 and fold change ≥2, or *p*-value < 0.05 and fold change ≥1. (B) Distribution of induced (gray bars) and repressed (dark bars) genes based on their fold change for the comparison of control and parasitized pupae after 25 h.

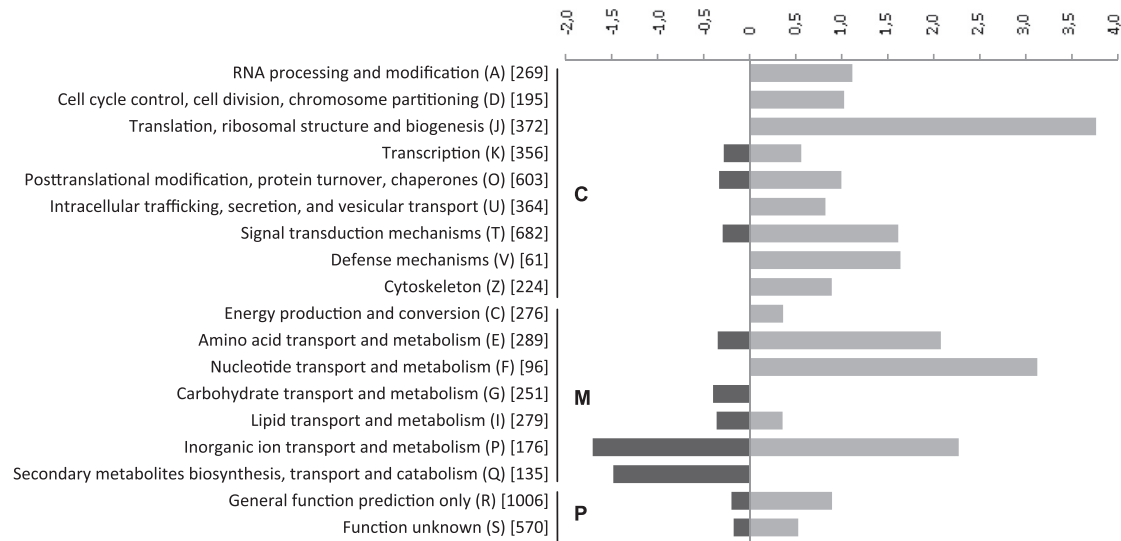


Fig. 3. Square brackets: Total number of EST's within the *S. crassipalpis* EST database. Round brackets: KOG functional category label. C: cellular processes and signaling. M: metabolism. P: poorly characterized. Dark bars: down-regulation. Gray bars: up-regulation.

fat body) is that patterns of gene expression represent the sum of transcriptional profiles across many specialized cell types. Thus, a transcriptomics study showing some components of energy metabolism by glycolysis that are up-regulated and some pathway members that are down-regulated may reflect the fact that glycolysis is up-regulated in some cells and down-regulated in others, rather than indicating that some components of the pathway are up-regulated and others down-regulated within a single cell type. Furthermore, a single gene that may affect multiple downstream pathways and functional categories (i.e., GO or COG) can also reflect a diversity of biochemical cellular events that indicate multiple different physiological effects on organismal phenotypes. The responses of *Sarcophaga* fly hosts to envenomation by *N. vitripennis* have been studied from organismal and physiological perspectives for two decades (Rivers and Denlinger, 1994a,b, 1995; Rivers et al., 2002a,b, 2010; Rivers and Brogan, 2008) revealing that even without the developmental influence of the parasitoid larva, envenomation alters fly hosts in three major ways: by suppressing host immunity, arresting host development, and altering host metabolism to favor parasitoid development. Below, we interpret our transcriptomic results in the context of

these three host manipulations. Many patterns of transcript abundance are products of host manipulation by the parasitoid (e.g., changes in many downstream players in genetic information processing), but here we consciously focus our discussion on regulatory processes that we believe may be important in modulating host immunity, development, and metabolism to benefit parasitoid development. We acknowledge that other, more-subtle interactions between parasitoid venom and host physiology, beyond those observed in our data or discussed here, are undoubtedly occurring. For example, some of the transcriptional responses we observe may be the product of host responses attempting to deter parasitoid success. However, we cannot currently disentangle host-defensive responses from parasitoid manipulation of hosts. Future work comparing responses to envenomation across populations of strains of hosts that vary in their susceptibility to parasitism by *N. vitripennis* is needed. Because successful envenomation by *N. vitripennis* always leads to developmental arrest and eventual host death in the strains used here, we focus our discussion from the perspective of host molecular responses to parasite manipulation. We hope that our data, the first to our knowledge on transcriptomic responses to ectoparasitoid envenomation, will

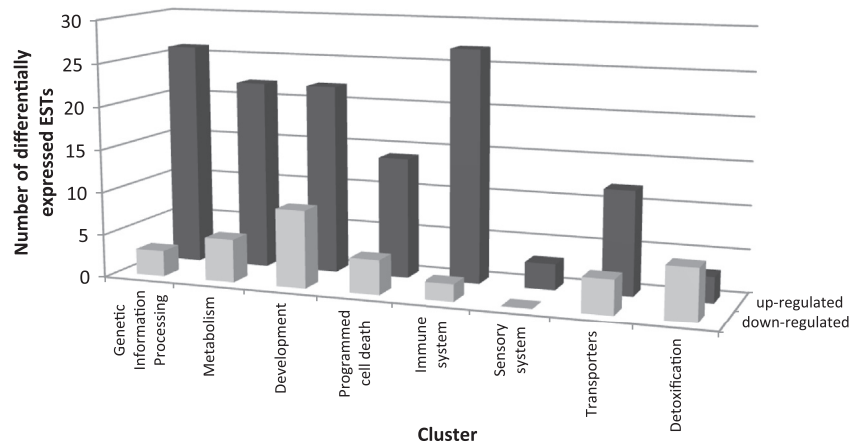


Fig. 4. Differentially expressed EST's for comparisons Contr_25h vs. Paras_25h, manually clustered into 8 different classes. Schematically presented. Y-axis represents the number of differentially expressed ESTs, the x-axis represents the different clusters.

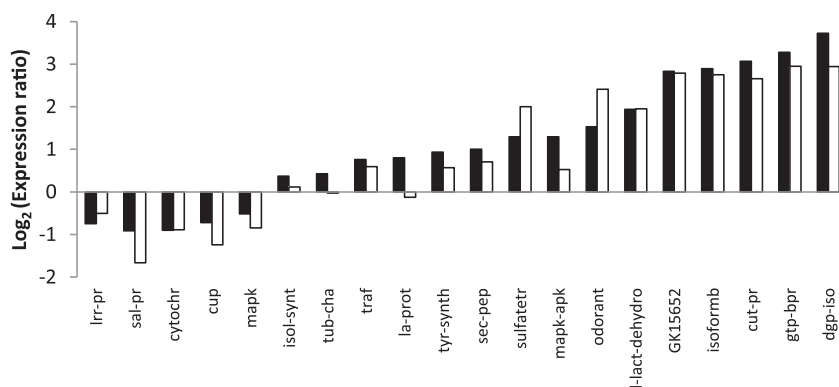


Fig. 5. Validation of microarray data with RT-qPCR. Log₂-transformed expression ratio of Paras_3h compared to Contr_3h and of Paras_25h compared to Contr_25h. White bars: RT-qPCR experiment. Black bars: microarray experiment. +: significant differential expression ($p < 0.05$). -: no significant expression ($p \geq 0.05$).

motivate additional hypothesis building that leads to careful biochemical and cellular studies of venom modes of action in the *N. vitripennis*-*Sarcophaga* interaction and other host-parasitoid systems.

3.5. Immunity

From the perspective of host exploitation, venoms should inhibit components of the host immune response that target parasitoid larvae while either maintaining or even enhancing components of the host immune system that target other invaders, like bacteria or fungi, that may promote degrading host quality, compete for parasitoid larvae for host resources, or infect the parasitoids themselves (Asgari and Rivers, 2011). Hosts will react to the invasion of foreign agents by producing antimicrobial peptides and reactive oxygen species by contact epithelia, fat body and hemocytes and more directly by phagocytosis, encapsulation and nodule formation in which specialized hemocytes interplay (Danneels et al., 2010). Studies of hemocyte dynamics showed that envenomation of *Sarcophaga bullata* pupae by *N. vitripennis* resulted in the rapid death of plasmatocytes, inhibited proliferation and differentiation of pro-hemocytes into plasmatocytes, and diminished granulocyte spreading (Rivers and Denlinger, 1995; Rivers et al., 2002a,b).

3.5.1. Programmed cell death in hemocytes and other tissues

In vivo studies in *Sarcophaga* hosts and *in vitro* work on lepidopteran cells suggests that venom-induced hemocyte cell death occurs by apoptosis signaled by phospholipase A₂ (PLA₂) that alters cellular Ca²⁺ signaling and Na⁺ homeostasis leading to characteristic changes in cell shape, including blebbing caused by membrane separation from the cytoskeleton, cellular swelling, DNA fragmentation, phosphatidylserine externalization and activation of caspase activity (Rivers and Denlinger, 1994a, 1994b; Rivers et al., 2002a,b; 2010; Formesyn et al., 2013). We show differential regulation of apoptotic transcripts, including up-regulation (2.15-fold) of a secretory PLA₂ transcript that may activate caspase activity. Consistent with the previous observations that PLA₂ activity is associated with changes in cellular Na⁺ and Ca²⁺ homeostasis that may induce apoptosis, we found a putative small mitochondrial calcium-binding protein transcriptionally up-regulated 25 h after envenomation (1.83-fold), as well as a putative calyphosine that was also up-regulated (1.44-fold). Up-regulation of a putative cytochrome oxidase III subunit (3.81-fold) may also be related to mitochondrial dysfunction induced by PLA₂ signaling. Over-expression of cytochrome III may help to induce apoptosis by affecting the redox state of cytochrome C, a known effector of

caspase activity and apoptosis (Brown and Borutaite, 2008; Wu et al., 2009).

Three transcripts annotated to the Rho family of GTP-binding proteins and a guanine nucleotide exchange factor were highly up-regulated 25 h after envenomation (13.2, 9.7, 1.38, and 1.3-fold enrichment respectively, Table S2). Rho GTPases belong to the Ras superfamily and play diverse roles in intracellular signaling (Rossman et al., 2005; Cox and Der, 2003). We propose that Rho GTP-binding proteins and the observed guanine nucleotide exchange factor modulate Rho signaling and promote apoptosis in hemocytes and other tissues by activating the RASSF1/Nore1/Mst1 signaling pathway that leads eventually to caspase activation. Rho signaling pathway members were also found to be differentially regulated in a study of the evolution of resistance of *D. melanogaster* hosts to the parasitoid wasp *A. tabida* (Wertheim et al., 2011). Thus, further interrogation of the potential roles of Rho signaling is needed to understand how *N. vitripennis* venom affects *Sarcophaga* hosts from the perspectives of both immune modulation by hemocyte cell death and selective programmed cell death in other tissues (see 3.6.1.).

Rho GTPases are also known for their involvement in cytoskeletal actin reorganization (Coleman and Olson, 2002; Fiorentini et al., 2003). Blebbing, where the cell membrane separates from the cytoskeleton, is one of the phenotypic hallmarks of cell death when cultured lepidopteran cells are exposed to *N. vitripennis* venom *in vitro* (Rivers et al., 2002a, 2002b, 2010), and Rho signaling has been shown to promote blebbing by actin cytoskeleton reorganization in cultured mammalian cells (Azhar and Lacal, 2001). Consistent with this view, several cytoskeleton-related transcripts were up-regulated by envenomation in our study (Table S2). Cytoskeletal disorganization could also reduce the ability of hemocytes to produce pseudopodial projections critical for endocytosis or spreading responses, and venom of several endoparasitoids has been shown to reduce the efficacy of spreading by disrupting hemocyte cytoskeletal structure (Gillespie et al., 1997; Strand, 2008; Asgari and Rivers, 2011; Richards et al., 2013). Differential regulation of cytoskeletal transcripts has been observed in other transcriptional studies of parasitism (Wertheim et al., 2005; Schlenke et al., 2007; Mahadav et al., 2008; Fang et al., 2010, 2010; Bitra et al., 2011; Provost et al., 2011; Wertheim et al., 2011), suggesting conservation of this mode of action between endoparasitoid and ectoparasitoid venoms.

3.5.2. Cellular and humoral immune responses

Although *N. vitripennis* venom can dramatically suppress *Sarcophaga* immunity by rapidly killing hemocytes, hosts still do mount an immune response characterized by both cellular and

humoral mechanisms (Rivers et al., 2002a,b). The p38K and JNK cascades of the multifunctional mitogen-activated protein kinase (MAPK) pathway are promising candidates for regulating both the cellular immune response to envenomation and hemocyte apoptosis (Concannon et al., 2003; Rane et al., 2003). MAPKs are cytosolic proteins that translocate into the nucleus to regulate transcription when activated. The p38K and JNK signaling cascades are associated with cellular stress responses including apoptosis, immunity, and cell cycle arrest. After envenomation, a putative MAPK 3 or MAPK 4 transcript possibly belonging to either the p38 or JNK cascade was down-regulated (–1.46-fold) and transcript abundance of a putative MAPK 3 or MAPK 4-phosphatase that suppresses MAPK activity was up-regulated (1.86-fold). We currently cannot distinguish whether this transcript functions as MAPK 3 by activating the p38K cascade or acting as MAPK 4 by activating the JNK cascade because we did not detect other transcripts clearly assigned to either cascade. However, both signaling cascades participate in immune system function, and down-regulating this arm of MAPK signaling may be an important part of the parasitoid's strategy for host immune suppression. MAPKs are activated post-translationally by phosphorylation, thus testing whether any of the arms of MAPK signaling are important in immune modulation at envenomation will involve further biochemical work at the level of immune cells and other tissues.

Parasitoid detection and immune system activation may also be modulated by the NF- κ B pathway (Strand, 2008). A transcript for a putative tumor necrosis factor (TNF) receptor associated factor (TRAF) known to regulate NF- κ B activity in *Drosophila* (Zapata et al., 2000) was up-regulated 25 h after envenomation (1.7-fold). A protein tyrosine phosphatase involved in intracellular immune signaling pathways that had previously been implicated in the immune response of *D. melanogaster* to the wasp *A. tabida* (Wertheim et al., 2005) was also up-regulated (2.17-fold).

The immune response in all organisms, including insects, has been associated with microRNA (miRNA) activity (O'Connell et al., 2010; Asgari, 2013). Two different transcripts that annotated to the host argonaute-2 protein, a critical mediator of the RNAi response, were up-regulated 25 h after envenomation (1.36 and 1.35-fold respectively). In addition to their known roles in antimicrobial immunity in insects (Fullaondo and Lee, 2012; Asgari, 2013), microRNAs have also recently been implicated in the response of a lepidopteran host, *P. xylostella*, to the parasitoid wasp *Diadegma semiclausum* (Etebari et al., 2013), motivating further study of microRNAs in regulating envenomation responses. We also observed up-regulation of transcripts for two odorant-binding proteins (OBPs). Beyond their activities in chemoreception, OBPs have been implicated in diverse cellular responses including pathogen recognition and neutralization of invading microorganisms (Levy et al., 2004). Of further interest, two distinct OBPs were also discovered in *N. vitripennis* venom (de Graaf et al., 2010), but whether and how endogenous host OBPs and venom-derived parasitoid OBPs may interact is unknown and merits further study.

Envenomation by *N. vitripennis* has long been associated with decreased melanization of *Sarcophaga* host hemolymph (Rivers and Denlinger, 1994a,b; Rivers et al., 2002a,b). The deposition of melanin around the intruding object forms a physical shield and prevents or retards the growth of the intruder. Given such a crucial role for melanization in host immunity, alterations of this process in hosts likely represents a strategy for host manipulation. Analysis of *N. vitripennis* venom has revealed both serine protease inhibitors (serpins) and cysteine-rich protease inhibitors that may impede the activity of pro-phenoloxidase and the melanization response (de Graaf et al., 2010). We found substantial up-regulation of transcripts for two serine proteases that could compete with venom-derived protease inhibitors in an effort to disrupt the parasitoid's

immune-suppressive strategy, similar to the enhanced expression of melanization cascade transcripts observed when *D. melanogaster* was parasitized by the virulent parasitoid wasp *Leptopilina boulardi* (Schenkle et al., 2007).

A targeted oxidative burst that is facilitated by both cellular and humoral elements of immunity is associated with melanization (Strand, 2008). For hosts, this targeted burst of free radicals and pro-oxidants must be delivered precisely to stress the invader while protecting host tissues. Concomitantly, parasitoids must reduce effects of oxidative damage on their own bodies and critical host tissues such as the fat body. Consistent with this dynamic interplay of host and parasitoid control of free radical damage, we found enrichment of detoxification proteins that could control redox events and may play roles in immune oxidative burst reactions, including down-regulation of a putative metallothionein family 5 protein (–2.01-fold), cytochrome p450-28a1 (–1.91-fold), copper uptake protein (–1.65-fold), and up-regulation of transcripts for putative myoinositol oxygenase (1.83-fold) and ribonucleoside-diphosphate reductase (1.46-fold). These redox proteins may be important in detoxification responses that facilitate immunity. Other detoxification transcripts were also differentially regulated, including down-regulation of the glutathione metabolism enzyme alanyl aminopeptidase (–2.93-fold), and an apparent ABC transporter was up-regulated (2.75-fold).

We detected no change in abundance of antimicrobial peptide transcripts, an important component of humoral immunity. Other studies on host-parasitoid responses also showed minimal up-regulation of antimicrobial peptides in responses to parasitoids relative to exposure to microorganisms (Ross and Dunn, 1989; Nicolas et al., 1996; Masova et al., 2010; Wertheim et al., 2005).

3.6. Development

Delaying or arresting host development is a common life history tactic for parasitoids. In endoparasitoids, suppressive actions are begun by venom components, and sometimes symbiotic viruses, then reinforced by developing parasitoids (Strand, 2008; Asgari and Rivers, 2011). An ectoparasitoid without obvious viral symbionts, *N. vitripennis* uses venom to arrest host development until external parasitoid eggs can hatch and larvae can affect host physiology directly. Even without the influence of a developing wasp larva, *Sarcophaga* pupae envenomated by *N. vitripennis* enter an irreversible state of developmental arrest that can last more than a month before the host dies (Rivers and Denlinger, 1994b; Rivers and Brogan, 2008). Envenomation-induced developmental arrest in *Sarcophaga* pupae superficially resembles the developmental arrest induced at pupal diapause in this species. However, within 24 h of envenomation the brain of a *Sarcophaga* host pupa undergoes substantial programmed cell death and thus will never be able to develop into a functional adult (Rivers and Brogan, 2008).

Degeneration of the brain contributes to envenomation-induced developmental arrest because the release of ecdysteroids from the brain is needed to coordinate pupal-adult metamorphosis. However, unlike diapausing pupae that will resume development with exogenous ecdysteroid exposure, exogenous ecdysteroids cannot restart development in envenomated pupae (Rivers and Denlinger, 1994a,b). This suggests that the developmental arrest in envenomated *Sarcophaga* pupae is regulated differently than pupal diapause. Envenomation-induced developmental arrest must somehow disrupt ecdysteroid signaling and may also include coordinated cell death in other critical tissues, like partially differentiated imaginal wing or antennal discs. In contrast to the programmed cell death that occurs after envenomation in hemocytes and brain cells, cells of the host fat body remain healthy (Rivers and Brogan, 2008) and continue participating in

intermediary metabolism of the pupae, including the accumulation of greater fat reserves (Rivers and Denlinger, 1995). This clear contrast in the cellular viability responses amongst the three *Sarcophaga* tissues that have been studied is consistent with venom manipulating the host environment to favor parasitoid larvae. Further examination may reveal that other host tissues benefiting larval development, like the heart and respiratory system, are also selectively maintained. An important question is, what cellular and biochemical factors promote survival and viability in some *Sarcophaga* pupal tissues, like the fat body which survives for weeks despite exposure to *N. vitripennis* venom, when other tissues undergo programmed cell death within hours of envenomation?

3.6.1. Developmental signaling pathways

One third of the differentially expressed genes at 25 h post-envenomation have putative roles in developmental processes, with 70% of these transcripts up-regulated and 30% down-regulated (Tables S2 and S3). Many genes classified as promoting development by cellular proliferation and growth also have functions in apoptosis (discussed above). A possible regulator of developmental arrest is down-regulation (–2.87-fold) of a putative alkylidihydroxy-acetonephosphate synthase (ADHAPS) required for normal development in humans and *Caenorhabditis elegans* (Motley et al., 2000). Understanding the molecular basis of selective maintenance of some tissues during developmental arrest while others are destroyed will require picking apart the activity of candidate genes and signaling pathways in individual tissues.

As mentioned above (see 3.5.2.), 25 h after envenomation we observed up-regulation in transcripts of a putative MAPK 3 or MAPK 4-phosphatase (1.86-fold) that could inhibit MAPK signaling through the p38K or JNK cascades, and down-regulation of transcripts for a putative MAPK 3 or MAPK 4 protein that would activate p38K or JNK signaling (–1.46-fold) and thus modulate development and immunity. Because envenomation initiates a developmental arrest in pupae preventing pharate adult metamorphosis, we expected that ERK-signaling, which promotes growth and morphogenesis, would be down-regulated after envenomation. Interestingly, Torso, a peptide-hormone receptor that activates ERK signaling was up-regulated 25 h after envenomation (2.17-fold). Torso is the prothoracicotrophic hormone (PTTH) receptor that signals the prothoracic glands to produce ecdysone to precipitate molting and morphogenesis via ERK signaling (Rewitz et al., 2009). It may seem counterintuitive for transcripts of the PTTH receptor to be in greater abundance in developmentally arrested pupae compared to control animals already undergoing pupal-adult metamorphosis by 25 h after envenomation. However, by the time of metamorphosis developing flies have already completed PTTH signaling and released ecdysteroids so PTTH reception may not be necessary. In contrast, envenomated, developmentally arrested pupae may still express the PTTH receptor even though exogenous ecdysteroids cannot trigger the resumption of development (Rivers and Denlinger, 1994a,b), indicating that these pupae are stuck perpetually in molecular stasis. A putative Torso/PTTH-receptor transcript was also up-regulated in larvae of *P. xylostella* that failed to successfully pupate when parasitized by *D. semiclausum* (Etebari et al., 2011), again suggesting developmental arrest may occur up-stream of ecdysone reception. A putative cytochrome p450-28a1 that is down-regulated (–1.91-fold) could also be involved in developmental arrest. This p450 is similar to *C. elegans* daf-9, an enzyme that regulates the dauer developmental arrest by producing the steroid hormone dafachronic acid that acts through a nuclear steroid hormone receptor, daf-12 (Gerisch and Antebi, 2004). Further detailed investigations of both steroid hormone production and signaling are needed to tease apart potential regulatory roles in developmental arrest in host-

parasitoid interactions. We expect that investigation of sensitivity to the action of PTTH and ecdysteroids through the ERK signaling cascade relative to p38K and JNK signaling holds promise for understanding the regulation of envenomation-induced arrest of host development.

Rho signaling, mentioned above in the context of immunity (see 3.5.1.), may also play a critical role in envenomation-induced developmental arrest as suggested by high levels of up-regulation in three Rho-family GTP-binding proteins and a guanine nucleotide exchange factor (13.2, 9.7, 1.38, and 1.3-fold enrichment respectively, Table S2). Rho signaling affects cytoskeletal structure in embryonic and pupal morphogenesis in *Drosophila* (Chen et al., 2004). Rho signaling is GTP-mediated and mutations or transgenes that enhance Rho signaling increase cellular proliferation and yield tissue overgrowth (Clark et al., 2000). Thus, up-regulation of Rho family GTP-binding proteins and a guanine nucleotide exchange factor that regulates cyclic GMP levels may help induce developmental arrest by sequestering and decreasing GTP to inhibit the Rho signaling that would normally lead to pupal-adult metamorphosis (Chang et al., 1998). Rho signaling can also interact with signaling in another pathway affecting cellular proliferation and morphogenesis, the SH2 domain ankyrin repeat kinase (Src) pathway (Chan et al., 1994; Pedraza et al., 2004). Transcripts for a putative inhibitor protein of Src signaling, a Prl protein-tyrosine phosphatase (Pagarigan et al., 2013), are up-regulated 25 h after envenomation (2.17-fold), suggesting inhibition of Src signaling may contribute to the envenomation-induced developmental arrest.

3.6.2. Other regulators of development

The only transcript to be detectably differentially expressed 3 h after envenomation was a down-regulated leucine rich-repeat protein (–0.74-fold). Putative homologues of this transcript are highly expressed during the pupal-adult transition in *D. melanogaster* where they regulate programmed cell death of larval-pupal structures as the animal undergoes adult morphogenesis (Berry and Baehrecke, 2007). Down-regulation of this protein may contribute to an early developmental halt upon envenomation, preventing the pupal host from further morphogenesis that may make it less suitable for parasitoid larvae (Rivers and Denlinger, 1995). Besides their possible involvement in immunity (see 3.5.2.), miRNAs can be important for regulating development in insects because they can modulate major transcriptional programs and RNA processing (Asgari, 2013). Up-regulation of transcripts for two putative argonaute-2 proteins (1.35 and 1.16-fold respectively) in our data combined with similar observations in the interaction between the lepidopteran host *P. xylostella* and the wasp *D. semiclausum* (Etebari et al., 2013) suggests that miRNAs may play important roles in regulating host developmental arrest.

3.7. Metabolism

Many parasitoids manipulate host intermediary metabolism to improve the nutritional milieu for larval development through venom and symbiotic viruses, followed by the developing parasitoid and teratocytes (Dahlman et al., 2003; Nakamatsu and Tanaka, 2003, 2004b; Nurullahoglu et al., 2004; Formesyn et al., 2010). Over the 25 h timeframe of our study the egg is external to the host body and would not have yet hatched (Whiting, 1967). Thus the effects we observe are due to envenomation. Envenomation causes a precipitous drop in the metabolic rates of hosts (decreased O₂ consumption – Rivers and Denlinger, 1994a, 1995). Although decreased host metabolism upon envenomation may be partly due to arresting host development, *N. vitripennis* venom clearly manipulates host intermediary metabolism because just after envenomation hosts increase levels of alanine and pyruvate,

but decrease oxaloacetate (Rivers and Denlinger, 1994a,b). Perhaps most striking from a metabolic perspective is that envenomation by *N. vitripennis* causes flesh fly pupae to increase the lipid content of their fat bodies while decreasing circulating blood lipids (Rivers and Denlinger, 1994a, 1995). We construct hypotheses for regulation of increased lipid storage and metabolic depression based on transcript abundance. Because our data are from whole bodies, we interpret these data in the context of intermediary metabolism being increased in some tissues and decreased in others.

3.7.1. Reorganizing metabolism to increase fat body lipids

Because many insect parasitoids, including *N. vitripennis*, show limited or no capacity to synthesize lipids themselves, they must rely on host lipids for fatty acids necessary for juvenile growth and adult reproduction (Visser et al., 2010, 2012). Previous work on several systems has shown parasitoid manipulation of host lipid metabolism from physiological (Rivers and Denlinger, 1994a, 1995; Dahlman et al., 2003; Nakamatsu and Tanaka, 2003, 2004b; Nurullahoglu et al., 2004), proteomic (Song et al., 2008), and transcriptomic perspectives (Fang et al., 2010; Etebari et al., 2011; Provost et al., 2011).

Considering that *Sarcophaga* host pupae are a nutritionally-sealed system that cannot feed, *N. vitripennis* venom must trigger a tissue-specific starvation response so nutrients are mobilized from peripheral tissues destined to degenerate, like the brain and thoracic muscles, while maintaining metabolic and synthetic function in the fat body. Autophagy is a controlled process wherein cells selectively degrade sub-cellular components, recruiting components (e.g., mitochondria) to intracellular lysosomes to break them down into trafficable units like amino acids that can be reused elsewhere (Cooper and Mitchell-Foster, 2011; Kroemer and Levine, 2008). Autophagy is a critical component of both starvation responses and responses to mild cellular damage. Mild starvation or cellular damage that leads to autophagy will induce cell cycle arrest by p53-dependent pathways to stop growth and will trigger lysosomal clearance of some sub-cellular structures, but cells can typically rebound function and resume growth when nutrients become available again or the stress abates (Lee et al., 2012). Prolonged starvation or chronic stress will induce a shift from the milder autophagy response to trigger apoptotic pathways. Both autophagy and apoptosis allow organisms to control the breakdown of cellular components into trafficable units that could be recycled and used to produce fat body lipid stores. Because autophagy has been associated with both starvation/nutrient recycling and cell cycle arrest, regulation of autophagy pathways provides an opportunity for parasitoids to manipulate both host development and intermediary metabolism to favor offspring production. We observe enrichment in several GO categories that are consistent with our expectation that envenomation promotes autophagy and nutrient mobilization in peripheral host tissues to support lipid synthesis in the fat body, including: cell cycle control, intracellular trafficking/vesicular transport, energy production and conversion, amino acid transport and metabolism, nucleotide transport and metabolism, and lipid transport and metabolism (Fig. 3).

When assessing our gene-by-gene analysis (Table S3) several autophagy-related transcripts were differentially expressed. Damage-related autophagy modulator (DRAM), shown to control autophagy in *D. melanogaster* (O'Prey et al., 2009), was up-regulated 25 h after envenomation (1.47-fold). Mitochondria play an important role in promoting or inhibiting autophagy (Cooper and Mitchell-Foster, 2011; Kroemer and Levine, 2008). Specifically, damaged or energy-stressed alterations in the mitochondrial trans-membrane potential regulated by mitochondrial Ca^{2+} signaling can trigger recruitment of mitochondria to autolysosomes (e.g., mitophagy). Two possible regulators of mitochondrial Ca^{2+}

homeostasis associated with autophagy were up-regulated 25 h after envenomation (Cardenas and Foskett, 2012; Lin et al., 2012), a putative calcyphosine (1.44-fold) and a putative small mitochondrial calcium-binding protein (1.83-fold). A putative DGP-1, an elongation factor that regulates taking damaged cells out of the cell cycle and inducing autophagic repair (Gruenewald et al., 2009; Blanco et al., 2010) was highly up-regulated after envenomation (13.2-fold). A putative lysosomal aspartic protease was also up-regulated (1.74-fold) 25 h after envenomation. This transcript has similarities to cathepsin D, a lysosomal protease activated in cells undergoing autophagy, apoptosis, and even necrotic cell death (Benes et al., 2008; Guicciardi et al., 2004). Cathepsins specifically, and other pro-autophagic genes more generally have been implicated in host responses to parasitism in other transcriptomic (Etebari et al., 2011; Fang et al., 2010) and proteomic studies (Song et al., 2008). Although we have couched most of the autophagy-related responses as important to nutritional manipulation of host tissues, there is substantial overlap between autophagic and apoptotic genes such that it is difficult to determine from simple snapshots of transcripts or proteins what specific pathways are being triggered across studies. Clearly pro-autophagy pathways could be playing roles in the apoptotic response of host tissues after envenomation in the *Sarcophaga-Nasonia* interaction (see 3.6; Rivers et al., 2002a,b; Rivers and Brogan, 2008) and other parasitoid-host manipulation systems (Song et al., 2008; Fang et al., 2010; Etebari et al., 2011). We expect apoptotic pathways will be rapidly initiated for parasitoid suppression of host immunity, then autophagic responses will be important for longer-term manipulation of host development and nutritional state. Careful investigation of time-course patterns of autophagy and apoptotic responses are needed across multiple tissue types to test our hypotheses about the relative contributions of autophagy and apoptosis to the three major axes of host manipulation: immunity, development, and nutrition.

Amino acids are a major product of cellular autophagy responses, and we expect that these amino acids liberated from the peripheral host tissues by envenomation are being deaminated to provide carbon skeletons for metabolic fuel as substrates for anabolic lipid synthesis. Gene Ontology categories including energy production and conversion, amino acid transport and metabolism, nucleotide transport and metabolism, and lipid transport and metabolism were all differentially expressed in hosts 25 h after envenomation (Fig. 3). When considering lipid synthesis from amino acids produced by autophagy, some transporters were up-regulated including a cationic amino acid transporter (2.13-fold) and an oligopeptide transporter (YIN) (2.87-fold) associated with transport of small peptides (especially alanylalanine) in *D. melanogaster* (Charriere et al., 2010). 2-amino-3-ketobutyrate coenzyme A ligase (Edgar and Polak, 2000) plays a critical role in the metabolism of serine, threonine, and glycine into acetyl-CoA for use in fatty acid synthesis and was up-regulated (1.63-fold). Up-regulation (2.05-fold) of a putative sodium-associated monocarboxylate transporter 25 h after envenomation also suggests amino acid catabolism because monocarboxylate transporters may be moving metabolic intermediates of catabolism of peripheral tissue protein, like pyruvate, acetate, or propionate, into the fat body for lipid synthesis. A putative hydroxy-acid oxidase was also up-regulated (7.43-fold). Hydroxy-acid oxidase plays a critical role in glyoxylate and dicarboxylate metabolism following serine, threonine, and glycine metabolism. Envenomation could also trigger glyoxylate and dicarboxylate metabolism in host peripheral tissues, where two-carbon precursors could be used for gluconeogenesis; simple carbohydrates or Krebs-cycle intermediates could be trafficked to the host fat body for fatty-acid synthesis (Voet and Voet, 2011). Our data suggest that envenomation of *Sarcophaga*

host pupae by *N. vitripennis* causes a substantial shift in protein and amino-acid metabolism to mobilize nutrients from peripheral tissues to the host fat body to support larval parasitoid growth, ensuring that parasitoids can acquire enough lipids during larval feeding to compensate for their inability to synthesize lipids *de novo* in adulthood (Visser et al., 2010; Visser et al., 2012). Although several candidate metabolic pathways emerge above, particularly serine, threonine, and glycine metabolism coupled to the glyoxylate/dicarboxylate cycle, future work should include testing which pathways of intermediary metabolism are most affected by envenomation, using techniques to carefully track labeled amino acid substrates through their metabolic intermediates (Zera, 2011; Visser et al., 2012).

3.7.2. Metabolic depression

Upon envenomation by *N. vitripennis* metabolic rates of *Sarcophaga* pupae decline precipitously (Rivers and Denlinger, 1994a) and within 24 h pupae increase alanine and pyruvate levels, but not the Krebs-cycle intermediate oxaloacetate (Rivers and Denlinger, 1994a). Although pyruvate levels increase initially they later drop as lipid is accumulated in the host fat body, suggesting that early high levels of pyruvate may support envenomation-induced alterations in host lipid metabolism (Rivers and Denlinger, 1994a). As in other parasitoid-host interactions that have been studied from a transcriptomic or proteomic perspective (Etebari et al., 2011; Song et al., 2008; Nguyen et al., 2008; Wertheim et al., 2005, 2011; Zhu et al., 2009), genes associated with energy metabolism were differentially expressed in our study (Fig. 3, Table S2). Across a wide range of taxa from turtles to insects, metabolic depression is associated with inducing hypoxic-like states that induce greater anaerobic metabolism through glycolytic/gluconeogenic pathways and decreased reliance on the Krebs cycle (Guppy and Withers, 1999). A shift away from aerobic metabolism towards increased glycolysis and gluconeogenesis appears to occur with metabolic depression during pupal diapause in *Sarcophaga* flies despite the fact that diapausing pupae remain normoxic (Michaud and Denlinger, 2007; Ragland et al., 2010), and several observations suggest envenomation may encourage a shift towards increased anaerobic metabolism. Lactate dehydrogenase regenerates NAD^+ by converting pyruvate to lactate under anaerobic conditions (Voet and Voet, 2011), and a putative lactate dehydrogenase was up-regulated 25 h after envenomation (3.83-fold). Elevated pyruvate levels in *Sarcophaga* pupae just after envenomation (Rivers and Denlinger, 1994a) are consistent with envenomation causing increased glycolysis relative to aerobic Krebs-cycle activity, and lactate dehydrogenase may help maintain NAD^+ levels to facilitate glycolysis. Up-regulation of a putative myoinositol oxygenase (1.83-fold) also suggests greater glycolysis in envenomated hosts. Myoinositol can be used for glycolysis or to produce the lipid-precursor inositol, and myoinositol oxygenases have been implicated in sugar balance and diabetes (Ganapathy et al., 2008; Nayak et al., 2011).

Envenomated pupae were kept in normal-oxygen atmospheres, so a shift towards anaerobic metabolism favoring glycolysis and reducing the activity of the Krebs cycle could be caused by components of *N. vitripennis* venom altering pathways that regulate metabolic responses to hypoxia. A putative hypoxia-inducible domain family 1 protein was up-regulated 25 h after envenomation (2.22-fold). This protein is a downstream effector of the HIF signaling pathway and may be playing a role in directly reducing mitochondrial activity (Gorr et al., 2004; Hayashi et al., 2012). Future physiological and biochemical studies will be needed to test our hypothesis that envenomated host pupae are in a hypoxia-like state despite being normoxic. Although most studies of parasitoid manipulation of hosts focus on modulation of the immune

response, the mechanisms that parasitoids use to manipulate host metabolism may provide new perspectives into states of metabolic depression.

3.8. Conclusions

This paper supports earlier studies demonstrating that *N. vitripennis* venom influences diverse physiological processes in one of its preferred host organism *S. crassipalpis*. Our molecular assay to confirm parasitization *post hoc* is unique because it keeps the samples intact, avoiding adverse effects caused by manipulation. Furthermore, to our knowledge this is the first transcriptomic study of the response of a host insect to attack by an ectoparasitoid, complementing a substantial body of physiological literature on the *N. vitripennis*-*Sarcophaga* interaction. Overall, fewer genes were found to be differentially expressed after parasitization with *N. vitripennis* than have been observed in studies with endoparasitoids. This observation is at least partly the result of venom injection alone rather than feeding by the wasp larvae because our samples were taken prior to hatching of the wasp eggs. The patterns of differential expression we observed suggest several clear candidate pathways for the molecular regulation of immune suppression, host developmental arrest, and alteration of host metabolism in response to ectoparasitoid envenomation. Many of these same pathways have also been implicated in endoparasitoid attack of hosts, suggesting some fundamental, conserved aspects of host-parasitoid interactions to be further investigated.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at <http://dx.doi.org/10.1016/j.ibmb.2013.10.003>.

References

- Asgari, S., 2013. MicroRNA functions in insects. *Insect Biochem. Mol. Biol.* 43, 388–397.
- Asgari, S., Rivers, D.B., 2011. Venom proteins from endoparasitoid wasp and their role in host-parasite interactions. *Annu. Rev. Entomol.* 56, 313–335.
- Aznar, S., Lacal, J.C., 2001. Rho signals to cell growth and apoptosis. *Cancer Lett.* 165, 1–10.
- Benes, P., Vetvicka, V., Fusek, M., 2008. Cathepsin D—Many functions of one aspartic protease. *Crit. Rev. Oncol. Hematol.* 68, 12–28.
- Benjamini, Y., Hochberg, Y., 1995. Controlling the false discovery rate — a practical and powerful approach to multiple testing. *J. R. Stat. Soc. Ser. B-Methodol.* 57, 289–300.
- Berry, D.L., Baehrecke, E.H., 2007. Growth arrest and autophagy are required for salivary gland cell degradation in *Drosophila*. *Cell* 131, 1137–1148.
- Bitra, K., Zhang, S., Strand, M.R., 2011. Transcriptomic profiling of *Microplitis demolitor* bracovirus reveals host, tissue and stage-specific patterns of activity. *J. Gen. Virol.* 92, 2060–2071.
- Blanco, E., Ruiz-Romero, M., Beltran, S., Bosch, M., Punset, A., Serras, F., Corominas, M., 2010. Gene expression following induction of regeneration in *Drosophila* wing imaginal discs. Expression profile of regenerating wing discs. *BMC Develop. Biol.* 10.
- Brown, G.C., Borutaite, V., 2008. Regulation of apoptosis by the redox state of cytochrome c. *Biochim. Biophys. Acta-Bioenerg.* 1777, 877–881.
- Cardenas, C., Fokkett, J.K., 2012. Mitochondrial Ca^{2+} signals in autophagy. *Cell Calcium* 52, 44–51.
- Charriere, G.M., Ip, W.K.E., Dejardin, S., Boyer, L., Sokolovska, A., Cappillino, M.P., Cherayil, B.J., Podolsky, D.K., Kobayashi, K.S., Silverman, N., Lacy-Hulbert, A.,

- Stuart, L.M., 2010. Identification of *Drosophila* Yin and PEPT2 as evolutionarily conserved phagosome-associated muramyl dipeptide transporters. *J. Biol. Chem.* 285, 20147–20154.
- Chan, T.A., Chu, C.A., Rauen, K.A., Krohier, M., Tatarewicz, S.M., Steele, R.E., 1994. Identification of a gene encoding a novel protein-tyrosine kinase containing Sh2 domains and ankyrin-like repeats. *Oncogene* 9, 1253–1259.
- Chang, J.H., Pratt, J.C., Sawasdikosol, S., Kapeller, R., Burakoff, S.J., 1998. The small GTP-binding protein rho potentiates AP-1 transcription in T cells. *Mol. Cell Biol.* 18, 4986–4993.
- Chen, G.C., Gajowniczek, P., Settleman, J., 2004. Rho-LIM kinase signaling regulates ecdysone-induced gene expression and morphogenesis during *Drosophila* metamorphosis. *Curr. Biol.* 14, 309–313.
- Clark, E.A., Golub, T.R., Lander, E.S., Hynes, R.O., 2000. Genomic analysis of metastasis reveals an essential role for RhoC. *Nature* 406, 532–535.
- Coleman, M.L., Olson, M.F., 2002. Rho GTPase signalling pathways in the morphological changes associated with apoptosis. *Cell Death Differ.* 9, 493–504.
- Concannon, C.G., Gorman, A.M., Samalli, A., 2003. On the role of Hsp27 in regulating apoptosis. *Apoptosis* 8, 61–70.
- Conesa, A., Gotz, S., Garcia-Gomez, J.M., Terol, J., Talon, M., Robles, M., 2005. Blast2GO: a universal tool for annotation, visualization and analysis in functional genomics research. *Bioinformatics* 21, 3674–3676.
- Cooper, D.M., Mitchell-Foster, K., 2011. Death for survival: what do we know about innate immunity and cell death in insects? *Invertebr. Surv. J.* 8, 162–172.
- Cox, A.D., Der, C.J., 2003. The dark side of Ras: regulation of apoptosis. *Oncogene* 22, 8999–9006.
- Dahlman, D.L., Rana, R.L., Schepers, E.J., Schepers, T., Diluna, F.A., Webb, B.A., 2003. A teratocyte gene from a parasitic wasp that is associated with inhibition of insect growth and development inhibits host protein synthesis. *Insect Mol. Biol.* 12, 527–534.
- Danneels, E.L., Rivers, D.B., de Graaf, D.C., 2010. Venom proteins of the parasitoid wasp *Nasonia vitripennis*: recent discovery of an untapped pharmacopee. *Toxins (Basel)* 2, 494–516.
- de Graaf, D.C., Aerts, M., Brunain, M., Desjardins, C.A., Jacobs, F.J., Werren, J.H., Devreese, B., 2010. Insights into the venom composition of the ectoparasitoid wasp *Nasonia vitripennis* from bioinformatic and proteomic studies. *Insect Mol. Biol.* 19, 11–26.
- Denlinger, D.L., 1972. Induction and termination of pupal diapause in *Sarcophaga* (Diptera-Sarcophagidae). *Biol. Bull.* 142, 11–24.
- Denlinger, D.L., Zdzarek, J., 1994. Metamorphosis behavior of flies. *Annu. Rev. Entomol.* 39, 243–266.
- Edgar, A.J., Polak, J.M., 2000. Molecular cloning of the human and murine 2-amino-3-ketobutyrate coenzyme A ligase cDNAs. *Eur. J. Biochem.* 267, 1805–1812.
- Etebari, K., Palfreyman, R.W., Schlipalius, D., Nielsen, L.K., Glatz, R.V., Asgari, S., 2011. Deep sequencing-based transcriptome analysis of *Plutella xylostella* larvae parasitized by *Diadegma semiclausum*. *BMC Genomics* 12, 446–463.
- Etebari, K., Hussain, M., Asgari, S., 2013. Identification of microRNAs from *Plutella xylostella* larvae associated with parasitization by *Diadegma semiclausum*. *Insect Biochem. Mol. Biol.* 43, 309–318.
- Fang, Q., Wang, L., Zhu, J.Y., Li, Y.M., Song, Q.S., Stanley, D.W., Akhtar, Z.R., Ye, G.Y., 2010. Expression of immune-response genes in lepidopteran host is suppressed by venom from an endoparasitoid, *Pteromalus puparum*. *BMC Genomics* 11, 484–500.
- Fiorentini, C., Falzano, L., Travaglione, S., Fabbri, A., 2003. Hijacking Rho GTPases by protein toxins and apoptosis: molecular strategies of pathogenic bacteria. *Cell Death Differ.* 10, 147–152.
- Formesyn, E.M., Danneels, E.L., de Graaf, D.C., 2010. Chapter 19-Proteomics of the venom of the parasitoid *Nasonia vitripennis*. In: Beckage, N.E., Drezner, J.M. (Eds.), *Parasitoid Viruses: Symbionts and Pathogens*. Elsevier Inc., pp. 233–246.
- Formesyn, E.M., Heyninck, K., de Graaf, D.C., 2013. The role of serine- and metalloproteases in *Nasonia vitripennis* venom in cell death related processes towards a *Spodoptera frugiperda* Sf21 cell line. *J. Insect Physiol.* 59, 795–803.
- Fullaondo, A., Lee, S.Y., 2012. Identification of putative miRNA involved in *Drosophila melanogaster* immune response. *Develop. Comp. Immunol.* 36, 267–273.
- Ganapathy, V., Thangaraju, M., Gopal, E., Martin, P.M., Itagaki, S., Miyauchi, S., Prasad, P.D., 2008. Sodium-coupled monocarboxylate transporters in normal tissues and in cancer. *AAPS J.* 10, 193–199.
- Gentleman, R.C., Carey, V.J., Bates, D.M., Bolstad, B., Dettling, M., Dudoit, S., Ellis, B., Gautier, L., Ge, Y.C., Gentry, J., Hornik, K., Hothorn, T., Huber, W., Iacus, S., Irizarry, R., Leisch, F., Li, C., Maechler, M., Rossini, A.J., Sawitzki, G., Smith, C., Smyth, G., Tierney, L., Yang, J.Y.H., Zhang, J.H., 2004. Bioconductor: open software development for computational biology and bioinformatics. *Genome Biol.* 5, R80.
- Gerisch, B., Antebi, A., 2004. Hormonal signals produced by DAF-9/cytochrome P450 regulate *C. elegans* dauer diapause in response to environmental cues. *Development* 131, 1765–1776.
- Gillespie, J.P., Kanost, M.R., Trenczek, T., 1997. Biological mediators of insect immunity. *Annu. Rev. Entomol.* 42, 611–643.
- Glatz, R.V., Asgari, S., Schmidt, O., 2004. Evolution of polydnviruses as insect immune suppressors. *Trends Microbiol.* 12, 545–554.
- Gorr, T.A., Tomita, T., Wappner, P., Bunn, H.F., 2004. Regulation of *Drosophila* hypoxia-inducible factor (HIF) activity in SL2 cells – identification of a hypoxia-induced variant isoform of the HIF alpha homolog gene similar. *J. Biol. Chem.* 279, 36048–36058.
- Gotz, S., Garcia-Gomez, J.M., Terol, J., Williams, T.D., Nagaraj, S.H., Nueda, M.J., Robles, M., Talon, M., Dopazo, J., Conesa, A., 2008. High-throughput functional annotation and data mining with the Blast2GO suite. *Nucleic Acids Res.* 36, 3420–3435.
- Gruenewald, C., Botella, J.A., Bayersdorfer, F., Navarro, J.A., Schneuwly, S., 2009. Hyperoxia-induced neurodegeneration as a tool to identify neuroprotective genes in *Drosophila melanogaster*. *Free Radic. Biol. Med.* 46, 1668–1676.
- Guicciardi, M.E., Leist, M., Gores, G.J., 2004. Lysosomes in cell death. *Oncogene* 23, 2881–2890.
- Guppy, M., Withers, P., 1999. Metabolic depression in animals: physiological perspectives and biochemical generalizations. *Biol. Rev. Cambridge Phil. Soc.* 74, 1–40.
- Hahn, D.A., Ragland, G.J., Shoemaker, D.D., Denlinger, D.L., 2009. Gene discovery using massively parallel pyrosequencing to develop ESTs for the flesh fly *Sarcophaga crassipalpis*. *BMC Genomics* 10, 234–242.
- Hayashi, H., Nakagami, H., Takeichi, M., Shimamura, M., Koibuchi, N., Oiki, E., Sato, N., Koriyama, H., Mori, M., Araujo, R.G., Maeda, A., Morishita, R., Tamai, K., Kaneda, Y., 2012. HIG1, a novel regulator of mitochondrial gamma-secretase, maintains normal mitochondrial function. *FASEB J.* 26, 2306–2317.
- Kroemer, G., Levine, B., 2008. Autophagic cell death: the story of a misnomer. *Nat. Rev. Mol. Cell Biol.* 9, 1004–1010.
- Labrosse, C., Carton, Y., Dubuffet, A., Drezner, J.M., Poirie, M., 2003. Active suppression of *D. melanogaster* immune response by long gland products of the parasitic wasp *Leptopilina boulardi*. *J. Insect Physiol.* 49, 513–522.
- Lee, I.H., Kawai, Y., Fergusson, M.M., Rovira, I.L., Bishop, A.J.R., Motoyama, N., Cao, L., Finkel, T., 2012. Atg7 modulates p53 activity to regulate cell cycle and survival during metabolic stress. *Science* 336, 225–228.
- Levy, F., Bulet, P., Ehret-Sabatier, L., 2004. Proteomic analysis of the systemic immune response of *Drosophila*. *Mol. Cell Proteomics* 3, 156–166.
- Lin, C.J., Lee, C.C., Shih, Y.L., Lin, C.H., Wang, S.H., Chen, T.H., Shih, C.M., 2012. Inhibition of mitochondria- and endoplasmic reticulum stress-mediated autophagy augments temozolomide-induced apoptosis in glioma cells. *PLoS One* 7.
- Ling, D.J., Salvaterra, P.M., 2011. Robust RT-qPCR data normalization: validation and selection of internal reference genes during post-experimental data analysis. *PLoS One* 6.
- Mahadav, A., Gerling, D., Gottlieb, Y., Czosnek, H., Ghanim, M., 2008. Parasitization by the wasp *Eretmocerus mundus* induces transcription of genes related to immune response and symbiotic bacteria proliferation in the whitefly *Bemisia tabaci*. *BMC Genomics* 9, 342–352.
- Masova, A., Sanda, M., Jiracek, J., Selicharova, I., 2010. Changes in the proteomes of the hemocytes and fat bodies of the flesh fly *Sarcophaga bullata* larvae after infection by *Escherichia coli*. *Proteome Sci.* 8, 1–11.
- Michaud, M.R., Denlinger, D.L., 2007. Shifts in the carbohydrate, polyol, and amino acid pools during rapid cold-hardening and diapause-associated cold-hardening in flesh flies (*Sarcophaga crassipalpis*): a metabolomic comparison. *J. Comp. Physiol. B-Biochem. Syst. Environ. Physiol.* 177, 753–763.
- Motley, A.M., Hettima, E.H., Ketting, R., Plasterk, R., Tabak, H.F., 2000. *Caenorhabditis elegans* has a single pathway to target matrix proteins to peroxisomes. *Embo Rep.* 1, 40–46.
- Nakamatsu, Y., Tanaka, T., 2003. Venom of ectoparasitoid, *Euplectrus* sp near *plathypenae* (Hymenoptera: Eulophidae) regulates the physiological state of *Pseudaletia separata* (Lepidoptera: Noctuidae) host as a food resource. *J. Insect Physiol.* 49, 149–159.
- Nakamatsu, Y., Tanaka, T., 2004b. Venom of *Euplectrus separatae* causes hyperlipidemia by lysis of host fat body cells. *J. Insect Physiol.* 50, 267–275.
- Nayak, B., Kondeti, V.K., Xie, P., Lin, S., Viswakarma, N., Raparia, K., Kanwar, Y.S., 2011. Transcriptional and post-translational modulation of myo-Inositol oxygenase by high glucose and related pathobiological stresses. *J. Biol. Chem.* 286, 27594–27611.
- Nguyen, T.T.A., Boudreault, S., Michaud, D., Cloutier, C., 2008. Proteomes of the aphid *Macrosiphum euphorbiae* in its resistance and susceptibility responses to differently compatible parasitoids. *Insect Biochem. Mol. Biol.* 38, 730–739.
- Nicolas, E., Nappi, A.J., Lemaître, B., 1996. Expression of antimicrobial peptide genes after infection by parasitoid wasps in *Drosophila*. *Develop. Comp. Immunol.* 20, 175–181.
- Nurullahoglu, Z.U., Uckan, F., Sak, O., Ergin, E., 2004. Total lipid and fatty acid composition of *Apanteles galleriae* and its parasitized host. *Ann. Entomol. Soc. Am.* 97, 1000–1006.
- O'Connell, R.M., Rao, D.S., Chaudhuri, A.A., Baltimore, D., 2010. Physiological and pathological roles for microRNAs in the immune system. *Nat. Rev. Immunol.* 10, 111–122.
- O'Prey, J., Skommer, J., Wilkinson, S., Ryan, K.M., 2009. Analysis of DRAM-related proteins reveals evolutionarily conserved and divergent roles in the control of autophagy. *Cell Cycle* 8, 2260–2265.
- Olesnick, E.C., Desplan, C., 2007. Distinct mechanisms for mRNA localization during embryonic axis specification in the wasp *Nasonia*. *Develop. Biol.* 306, 134–142.
- Pagarigan, K.T., Bunn, B.W., Goodchild, J., Rahe, T.K., Weis, J.F., Saucedo, L.J., 2013. *Drosophila* PRL-1 is a growth inhibitor that counteracts the function of the Src oncogene. *PLoS One* 8.
- Pedraza, L.G., Stewart, R.A., Li, D.M., Xu, T., 2004. *Drosophila* Src-family kinases function with Csk to regulate cell proliferation and apoptosis. *Oncogene* 23, 4754–4762.

- Pennacchio, F., Strand, M.R., 2006. Evolution of developmental strategies in parasitic Hymenoptera. *Annu. Rev. Entomol.* 51, 233–258.
- Provost, B., Jouan, V., Hilliou, F., Delobel, P., Bernardo, P., Ravallec, M., Cousserans, F., Wajnberg, E., Darboux, I., Fournier, P., Strand, M.R., Volkoff, A.N., 2011. Lepidopteran transcriptome analysis following infection by phylogenetically unrelated polydnaviruses highlights differential and common responses. *Insect Biochem. Mol. Biol.* 41, 582–591.
- Ragland, G.J., Denlinger, D.L., Hahn, D.A., 2010. Mechanisms of suspended animation are revealed by transcript profiling of diapause in the flesh fly. *Proc. Natl. Acad. Sci. U S A* 107, 14909–14914.
- Rane, M.J., Pan, Y., Singh, S., Powell, D.W., Wu, R., Cummins, T., Chen, Q., McLeish, K.R., Klein, J.B., 2003. Heat shock protein 27 controls apoptosis by regulating Akt activation. *J. Biol. Chem.* 278, 27828–27835.
- Rewitz, K.F., Yamanaka, N., Gilbert, L.I., O'Connor, M.B., 2009. The insect neuropeptide PTTH activates receptor tyrosine kinase Torso to initiate metamorphosis. *Science* 326, 1403–1405.
- Richards, E.H., Dani, M.P., Bradish, H., 2013. Immunosuppressive properties of a protein (rVPr1) from the venom of the endoparasitic wasp, *Pimpla hypochondriaca*: mechanism of action and potential use for improving biological control strategies. *J. Insect Physiol.* 59, 213–222.
- Rivers, D.B., Brogan, A., 2008. Venom glands from the ectoparasitoid *Nasonia vitripennis* (Hymenoptera: Pteromalidae) produce a calreticulin-like protein that functions in developmental arrest and cell death in the flesh fly host, *Sarcophaga bullata* Parker (Diptera: Sarcophagidae). In: Maes, R.P. (Ed.), *Insect Physiology: New Research*. Nova Science Publishers, New York, pp. 259–278.
- Rivers, D.B., Denlinger, D.L., 1994a. Redirection of metabolism in the flesh fly, *Sarcophaga Bullata*, following envenomation by the ectoparasitoid *Nasonia vitripennis* and correlation of metabolic effects with the diapause status of the host. *J. Insect Physiol.* 40, 207–215.
- Rivers, D.B., Denlinger, D.L., 1994b. Developmental fate of the flesh fly, *Sarcophaga bullata*, envenomated by the pupal ectoparasitoid, *Nasonia vitripennis*. *J. Insect Physiol.* 40, 121–127.
- Rivers, D.B., Denlinger, D.L., 1995. Venom-induced alterations in fly lipid-metabolism and its impact on larval development of the ectoparasitoid *Nasonia vitripennis* (Walker) (Hymenoptera, Pteromalidae). *J. Invertebr. Pathol.* 66, 104–110.
- Rivers, D.B., Rocco, M.M., Frayha, A.R., 2002a. Venom from the ectoparasitic wasp *Nasonia vitripennis* increases Na⁺ influx and activates phospholipase C and phospholipase A(2) dependent signal transduction pathways in cultured insect cells. *Toxicol.* 40, 9–21.
- Rivers, D.B., Ruggiero, L., Hayes, M., 2002b. The ectoparasitic wasp *Nasonia vitripennis* (Walker) (Hymenoptera: Pteromalidae) differentially affects cells mediating the immune response of its flesh fly host, *Sarcophaga bullata* Parker (Diptera: Sarcophagidae). *J. Insect Physiol.* 48, 1053–1064.
- Rivers, D.B., Uckan, F., Ergin, E., Keefer, D.A., 2010. Pathological and ultrastructural changes in cultured cells induced by venom from the ectoparasitic wasp *Nasonia vitripennis* (Walker) (Hymenoptera Pteromalidae). *J. Insect Physiol.* 56, 1935–1948.
- Rivers, D.B., Yoder, J.A., Ruggiero, L., 1999. Venom from *Nasonia vitripennis*: a model for understanding the roles of venom during parasitism by ectoparasitoids. *Trends Entomol.* 2, 1–17.
- Ross, D.R., Dunn, P.E., 1989. Effect of parasitism by *Cotesia congregata* on the susceptibility of *Manduca sexta* larvae to bacterial infection. *Develop. Comp. Immunol.* 13, 205–216.
- Rossmann, K.L., Der, C.J., Sondek, J., 2005. GEF means go: turning on Rho GTPases with guanine nucleotide-exchange factors. *Nat. Rev. Mol. Cell Biol.* 6, 167–180.
- Scharlaken, B., de Graaf, D.C., Goossens, K., Brunain, M., Peelman, L.J., Jacobs, F.J., 2008. Reference gene selection for insect expression studies using quantitative real-time PCR: the head of the honeybee, *Apis mellifera*, after a bacterial challenge. *J. Insect Sci.* 8, 33–43.
- Schlenke, T.A., Morales, J., Govind, S., Clark, A.G., 2007. Contrasting infection strategies in generalist and specialist wasp parasitoids of *Drosophila melanogaster*. *PLoS Pathog.* 3, 1486–1501.
- Smyth, G., 2005. *Limma: Linear Models for Microarray Data*. Bioinformatics and Computational Biology Solutions using R and Bioconductor. Springer, New York, pp. 397–420.
- Song, K.H., Jung, M.K., Eum, J.H., Hwang, I.C., Han, S.S., 2008. Proteomic analysis of parasitized *Plutella xylostella* larvae plasma. *J. Insect Physiol.* 54, 1271–1280.
- Strand, M.R., 2008. The insect cellular immune response. *Insect Sci.* 15, 1–14.
- Tatusov, R.L., Galperin, M.Y., Natale, D.A., Koonin, E.V., 2000. The COG database: a tool for genome-scale analysis of protein functions and evolution. *Nucleic Acids Res.* 28, 33–36.
- Untergasser, A., Nijveen, H., Rao, X., Bisseling, T., Geurts, R., Leunissen, J.A.M., 2007. Primer3Plus, an enhanced web interface to Primer3. *Nucleic Acids Res.* 35, W71–W74.
- Van den Assem, J., Jachmann, F., 1999. Changes in male perseverance in courtship and female readiness to mate in a strain of the parasitic wasp *Nasonia vitripennis* over a period of 20+ years. *Neth. J. Zool.* 49, 125–137.
- Visser, B., Le Lann, C., den Blanken, F.J., Harvey, J.A., van Alphen, J.J.M., Ellers, J., 2010. Loss of lipid synthesis as an evolutionary consequence of a parasitic lifestyle. *Proc. Natl. Acad. Sci. U S A* 107, 8677–8682.
- Visser, B., Roelofs, D., Hahn, D.A., Teal, P.E.A., Marien, J., Ellers, J., 2012. Transcriptional changes associated with lack of lipid synthesis in parasitoids. *Genome Biol. Evol.* 4, 864–874.
- Voet, D., Voet, J., 2011. *Biochemistry*, fourth ed. John Wiley & Sons, Inc.
- Werren, J.H., Richards, S., Desjardins, C.A., Niehuis, O., Gadau, J., Colbourne, J.K., Beukeboom, L.W., Desplan, C., Elsik, C.G., Grimmelikhuijzen, C.J.P., Kitts, P., Lynch, J.A., Murphy, T., Oliveira, D.C.S.G., Smith, C.D., van de Zande, L., Worley, K.C., Zdobnov, E.M., Aerts, M., Albert, S., Anaya, V.H., Anzola, J.M., Barchuk, A.R., Behura, S.K., Bera, A.N., Berenbaum, M.R., Bertossa, R.C., Bitondi, M.M.G., Bordenstein, S.R., Bork, P., Bornberg-Bauer, E., Brunain, M., Cazzamali, G., Chaboub, L., Chacko, J., Chavez, D., Childers, C.P., Choi, J.H., Clark, M.E., Claudianos, C., Clinton, R.A., Cree, A.G., Cristino, A.S., Dang, P.M., Darby, A.C., de Graaf, D.C., Devreese, B., Dinh, H.H., Edwards, R., Elango, N., Elhaik, E., Ermolaeva, O., Evans, J.D., Foret, S., Fowler, G.R., Gerlach, D., Gibson, J.D., Gilbert, D.G., Graur, D., Grunder, S., Hagen, D.E., Han, Y., Hauser, F., Hultmark, D., Hunter, H.C., Jhangian, S.N., Jiang, H.Y., Johnson, R.M., Jones, A.K., Junier, T., Kadowaki, T., Kamping, A., Kapustin, Y., Kechavarzi, B., Kim, J., Kim, J., Kiryutin, B., Koevoets, T., Kovar, C.L., Kriventseva, E.V., Kucharski, R., Lee, H., Lee, S.L., Lees, K., Lewis, L.R., Loehlin, D.W., Logsdon, J.M., Lopez, J.A., Lozado, R.J., Maglott, D., Maleszka, R., Mayampurath, A., Mazur, D.J., McClure, M.A., Moore, A.D., Morgan, M.B., Muller, J., Munoz-Torres, M.C., Muzny, D.M., Nazareth, L.V., Neupert, S., Nguyen, N.B., Nunes, F.M.F., Oakeshott, J.G., Okwuonu, G.O., Pannebakker, B.A., Pejaver, V.R., Peng, Z.G., Pratt, S.C., Predel, R., Pu, L.L., Ranson, H., Raychoudhury, R., Rechtsteiner, A., Reese, J.T., Reid, J.G., Riddle, M., Robertson, I.M., Romero-Severson, J., Rosenberg, M., Sackton, T.B., Sattelle, D.B., Schluns, H., Schmitt, T., Schneider, M., Schuler, A., Schurko, A.M., Shuker, D.M., Simoes, Z.L.P., Sinha, S., Smith, Z., Solovyev, V., Souvorov, A., Springauf, A., Stafflinger, E., Stage, D.E., Stanke, M., Tanaka, Y., Telschow, A., Trent, C., Vattathil, S., Verhulst, E.C., Viljakainen, L., Wanner, K.W., Waterhouse, R.M., Whitfield, J.B., Wilkes, T.E., Williamson, M., Willis, J.H., Wolschin, F., Wyder, S., Yamada, T., Yi, S.V., Zeicher, C.N., Zhang, L., Gibbs, R.A., 2010. Functional and evolutionary insights from the genomes of three parasitoid *Nasonia* species. *Science* 327, 343–348.
- Wertheim, B., Kraaijeveld, A.R., Hopkins, M.G., Boer, M.W., Godfray, H.C.J., 2011. Functional genomics of the evolution of increased resistance to parasitism in *Drosophila*. *Mol. Ecol.* 20, 932–949.
- Wertheim, B., Kraaijeveld, A.R., Schuster, E., Blanc, E., Hopkins, M., Pletcher, S.D., Strand, M.R., Partridge, L., Godfray, H.C.J., 2005. Genome-wide gene expression in response to parasitoid attack in *Drosophila*. *Genome Biol.* 6, R94.
- Whiting, A.R., 1967. Biology of parasitic wasp *Mormoniella vitripennis* [= *Nasonia brevicornis*] (Walker). *Q. Rev. Biol.* 42, 333–406.
- Wu, C.G., Yan, L., Depre, C., Dhar, S.K., Shen, Y.T., Sadoshima, J., Vatner, S.F., Vatner, D.E., 2009. Cytochrome c oxidase III as a mechanism for apoptosis in heart failure following myocardial infarction. *Am. J. Physiol.-Cell Physiol.* 297, C928–C934.
- Zapata, J.M., Matsuzawa, S., Godzik, A., Leo, E., Wasserman, S.A., Reed, J.C., 2000. The *Drosophila* tumor necrosis factor receptor-associated factor-1 (DTRAF1) interacts with Pelle and regulates *NF kappa B* activity. *J. Biol. Chem.* 275, 12102–12107.
- Zera, A.J., 2011. Microevolution of intermediary metabolism: evolutionary genetics meets metabolic biochemistry. *J. Exp. Biol.* 214, 179–190.
- Zhu, J.Y., Ye, G.Y., Fang, Q., Hu, C., 2009. Proteome changes in the plasma of *Papilio xuthus* (Lepidoptera: Papilionidae): effect of parasitization by the endoparasitic wasp *Pteromalus puparum* (Hymenoptera: Pteromalidae). *J. Zhejiang Univ.-Sci. B* 10, 445–453.