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PARASITOID VENOM INDUCES METABOLIC CASCADES IN FLY HOSTS

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Abstract

Parasitoid wasps inject insect hosts with a cocktail of venoms to manipulate the physiology, development, and immunity of the hosts and to promote development of the parasitoid offspring. The jewel wasp *Nasonia vitripennis* is a model parasitoid with at least 79 venom proteins. We conducted a high-throughput analysis of *Nasonia* venom effects on temporal changes of 249 metabolites in pupae of the flesh fly host (*Sarcophaga bullata*), over a five-day time course. Our results show that venom does not simply arrest the metabolism of the fly host. Rather, it targets specific metabolic processes while keeping hosts alive for at least five days post venom injection by the wasp. We found that venom: (a) Activates the sorbitol biosynthetic pathway while maintaining stable glucose levels, (b) Causes a shift in intermediary metabolism by switching to anaerobic metabolism and blocking the tricarboxylic acid cycle, (c) Arrests chitin biosynthesis that likely reflects developmental arrest of adult fly structures, (d) Elevates the majority of free amino acids, and (e) May be increasing phospholipid degradation. Despite sharing some metabolic effects with cold treatment, diapause, and hypoxia, the venom response is distinct from these conditions. Because *Nasonia* venom dramatically increases sorbitol levels without changing glucose levels, it could be a useful model for studying the regulation of the sorbitol pathway, which is relevant to diabetes research. Our findings generally support the view that parasitoid venoms are a rich source of bioactive molecules with potential biomedical applications.

Keywords

Venom; *Nasonia*; Sorbitol; Anaerobic respiration; Chitin; Amino acids

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Conflict of Interest Statement

Authors Mrinalini, Aisha Siebert, Jeremy Wright, Ellen Martinson, David Wheeler, and John H. Werren, declare that they have no conflict of interest.

Animal Studies

All institutional and national guidelines for the care and use of laboratory animals were followed.

1. Introduction

The venom systems of most animals are used for defense against predators, immobilization of prey, and/or prey digestion. In contrast, the sophisticated venom systems of the parasitoid wasps (Order Hymenoptera) have evolved to manipulate host physiology in diverse ways to provide a food source for their developing young (Pennacchio and Strand, 2006).

Consequently, parasitoid venoms are potentially a rich source of novel bioactive molecules for drug discovery (Werren et al., 2010; Danneels et al., 2010; Moreau and Guillot, 2005; Asgari and Rivers., 2011). The wasp's venom gland and reservoir are part of the reproductive system and venom is injected into the host through the ovipositor (Zhu et al., 2008; Wan et al., 2006; de Graaf et al., 2010). Parasitoid venom can consist of proteins, ovarian fluids, and (in some cases) polydnaviruses (PDV) and/or virus-like particles (VLP), which are injected into arthropod hosts prior to or at the time of egg laying (Asgari and Rivers, 2011; Mabiala-Moundougou et al., 2010; Moreau and Guillot, 2005; Beckage and Gelman, 2004).

There are two basic egg-laying patterns in parasitoids. Endoparasitoids lay their eggs within insect hosts, which typically continue to develop until the eventual emergence of the parasitoid young results in host death. In these situations, venoms play a major role in suppressing immune responses of the host to protect parasitoid eggs and larvae (Asgari et al., 2003; Strand and Pech, 1995; Parkinson et al., 2001). In contrast, ectoparasitoids lay their eggs on the integument of the host, with the venoms in these systems typically inducing paralysis, developmental arrest, and physiological alterations in growth and metabolism, presumably enhancing host nutritional quality for parasitoid larval consumption (Rivers and Denlinger, 1995a; Rivers and Denlinger, 1995b; Doury et al., 1995; Soller and Lanzrein, 1996; Kaeslin et al., 2010; Danneels et al., 2010). An advantage of ectoparasitoids for studying venom effects is that the eggs are external and can be removed prior to hatching, allowing evaluation of venom-induced effects on hosts independent of alterations induced by feeding larvae. Characterizing parasitoid venom functions requires elucidation of venom induced shifts in host physiology and biochemistry (Poulin, 2010; Doury et al., 1995).

Nasonia vitripennis is a widely studied, gregarious ectoparasitoid wasp that parasitizes a broad range of hosts, but prefers the pupae of blow flies and flesh flies (e.g., *Sarcophaga bullata*) (Whiting, 1967). With the availability of a sequenced genome (Werren et al., 2010) and systemic RNA interference (RNAi) methods (Lynch and Desplan, 2006; Werren et al., 2009), *N. vitripennis* has emerged as a model organism in parasitoid research (Werren and Loehlin, 2009a). The *N. vitripennis* venom proteome is also well-characterized and consists of at least 79 proteins, with greater than 25% of these being novel proteins that have no known homology to any other proteins (de Graaf et al., 2010; Danneels et al., 2010; Werren et al., 2010).

N. vitripennis venom causes several changes in *S. bullata*. Following venom injection by the wasp, *S. bullata* pupae undergo developmental arrest, which includes failure of deposition of eye pigments and formation of body bristles (Figure 1). It has been proposed that developmental arrest due to *N. vitripennis* venom could be similar to naturally occurring over-wintering diapause in *S. bullata* (Rivers and Denlinger, 1994a; Rivers and Denlinger,

1994b), which also occurs in the pupal stage and involves developmental arrest (Fraenkel and Hsiao, 1968). It is also known that *Nasonia* venom induces a reduction in oxygen consumption but does not cause pupal death until several days post venom exposure (Rivers and Denlinger, 1994b). Differential regulation of several genes involved in fly metabolism, development, and immunity indicate that flesh fly pupae continue to be transcriptionally active following venomation (Danneels et al., 2013). Venom induced changes in cell defense pathways and the abundance of several metabolites has previously been reported for *S. bullata* (Rivers and Denlinger, 1994b, Rivers et al., 2002; Asgari et al., 2003; Zhang et al., 2004; Colinet et al., 2013; Rivers et al., 1999; Er et al., 2001; Rivers et al., 2005). However, so far there has been no comprehensive study on the global effects of *N. vitripennis* venom on the metabolism and biochemistry of *S. bullata*.

We studied the effects of *N. vitripennis* venom in *S. bullata* pupae by comparing the metabolic changes after venom injection by *Nasonia* against those in control pupae. Using high-throughput, global metabolomic approach to characterize the relative abundance of 249 diverse metabolites and small molecules from eight metabolic categories. We compared the metabolomes of control and venom-treated pupae over five days. Our results show that venom manipulates sugar metabolism, oxidative metabolism, chitin synthesis, and amino acid metabolism in the host while it remains alive even five days following stinging and venom exposure.

2. Materials and Methods

2.1 Experimental Design and Sampling

Adult female *N. vitripennis* (24 - 48 hours after emergence) were pre-exposed to *S. bullata* in cotton-plugged glass vials for 6 hours at 25° C. (Rearing of *S. bullata* was as per Werren and Loehlin (2009b)). After 6 hours, hosts were removed and wasps were left without hosts overnight. Recently-pupated (4-5 days after the start of pupation) fresh hosts were placed in foam plugs with the anterior end exposed to a pre-conditioned female wasp inside a glass vial. The wasp was allowed to sting the host (at which time venom is injected by the wasps), feed and lay eggs over a 4-hour window. Wasps were then removed and the anterior ends of host puparial wall were opened with a probe. Stinging was confirmed by identifying melanization at the sting site (see Figure 1). To isolate venom effects from the consequences of wasp larval feeding, wasp eggs were gently removed with a brush at this stage. To prevent desiccation, the exposed ends of the host were capped using gel capsules and hosts were stored at 25° C. Hosts were also set up similarly without exposure to wasps to serve as controls. Henceforth we will refer to hosts that were exposed to wasp stinging as **Venomated (V)** hosts and those not exposed to wasp stinging as **Control (C)** hosts.

Control and Venomated hosts were collected at 24, 72, and 120 hours (1, 3, and 5 days) after the removal of wasp from host. For metabolomic processing of pupae, each was extracted from its puparium using a clean pair of forceps and cryopreserved immediately in liquid nitrogen. Five individuals were pooled per sample. Biological replication is critical for robust statistical analyses of metabolomic datasets (Fernie et al., 2011). We therefore collected 10 samples (five pooled hosts per sample) for Control and Venomated treatments at

each time point in two replicated experiments. All samples were stored at -80°C until they were ready for shipping on dry ice to Metabolon Inc. for global metabolomic quantification.

2.2 Metabolomics

2.2.1 Extraction and Sample Preparation Procedures—At Metabolon Inc., the flash-frozen hosts were homogenized into a powder, thoroughly mixed, and 500 mg weighed out for analysis. Recovery standards were added prior to the extraction process for quality control (QC). A series of proprietary organic and aqueous extractions were performed with vigorous shaking to remove protein, dissociate small molecules bound to protein or trapped in precipitated protein matrix, and to recover the chemically diverse metabolites. The extract was centrifuged and the supernatant removed for analysis on Liquid Chromatography (LC) and Gas Chromatography (GC) platforms. Organic solvents were removed and each sample was frozen and dried under vacuum.

2.2.2 Measurements and Compound Identification—Sample extracts were prepped separately for positive and negative ions in LC/MS and LC/MS² analysis. Injection standards were used to ensure injection and chromatographic consistency. The LC/MS platform was based on Waters ACQUITY UPLC and Thermo-Finnigan LTQ MS, consisting of electrospray ionization (ESI) source and linear ion-trap (LIT) mass analyzer front end and a Fourier transform ion cyclotron resonance (FT-ICR) MS back end. Ions with counts > 2 million, could be measured with mass error of < 5 ppm. GC/MS analysis was performed using Thermo-Finnigan Trace DSQ fast-scanning single-quadrupole MS using electron impact ionization.

Stringent QC practices were employed with addition of QC compounds, monitoring of process variation and data output quality. All experimental samples were randomly distributed throughout each day's run. Data was extracted, examined, and appropriate QC limits were imposed. Compounds were identified based on a metabolomic library of more than 1000 purified standards. Curation procedures ensured a high quality dataset for statistical analysis and data interpretation. Confirmation of consistency in peak identification was also performed manually.

2.2.3 Raw MS Data Normalization and Imputation—MS raw ion count for each metabolite was normalized in terms of raw area counts and calibrated in run-day blocks by registering the medians to 1.00. Data was block-corrected across run days to control for instrument inter-day tuning differences and then normalized to protein concentration estimated from Bradford assay.

As missing values likely reflect levels below detection limit rather than missed data points, imputation of such datapoints with minimum value is a widely used method in metabolomics (e.g., Delgado et al., 2012; Nieman et al., 2013). We therefore used a minimum value imputation for datapoints below detection limit in our dataset and readjusted the median to 1.00 for metabolites with imputed data points.

2.3 Data Analysis and Statistics

2.3.1 Dataset optimization—Our full dataset consisted of 307 metabolites measured from 60 samples. Although minimum value imputation is widely used, a high proportion of imputations could induce statistical noise and increase the chances of recovering skewed results. Therefore, out of 55 metabolites that required imputation, we excluded 16 which had > 60% imputation/time point/treatment.

Additionally, metabolomics involves profiling metabolites with diverse properties and abundances, which comes at the cost of technical procedures not being optimal for every class of metabolite (Ferne et al., 2011). In particular, the technical procedures used by Metabolon, Inc., were not optimal for lipid extraction and analysis. We therefore performed an initial data assessment using Principal Component Analysis (PCA) in Multibase 2013 (www.numericaldynamics.com) in Microsoft Excel®. A Euclidean distance-based heatmap with no sample clustering was also generated using *PermutMatrix* V1.9.3 (Eisen et al., 1998; Caraux and Pinloche, 2005). The PCA score plot and heatmap revealed a set of 42 water-insoluble lipids from multiple lipid classes to be especially noisy compared to all other metabolites. The patterns of noise were identical across all 42 lipids in samples from all treatments and time points. Further investigation revealed no correlation between these samples, experimental batches, MS run-days, or personnel handling conditions. Given that all 42 metabolites were lipids and that the extraction protocol was sub-optimal for lipid analyses, there was a high possibility that these abnormal patterns were due to technical limitations of the procedure. We therefore excluded the 42 lipids from subsequent analyses.

Our final dataset for analysis consisted of a concentration matrix for 249 metabolites from 60 samples. Datasets for all further statistical analyses were prepared separately in MetaboAnalyst 2.0 (Xia et al., 2012; Xia et al., 2009). Data was log or cube-root transformed and/or scaled to improve overall consistency of the metabolite concentration matrix and obtain a normal distribution. Some subsets of our data required a combination of transformation and scaling to reduce large values to smaller values and also fully adjust for differences in magnitude (Van Den Bert et al. 2006).

2.3.2 Principal Component Analysis—We used our full dataset of 60 samples (n=10 collected per 1, 3, and 5 day time points from Control and Venomated conditions) to identify treatment and time based shifts in the whole metabolome. We performed a PCA in Multibase 2013 (www.numericaldynamics.com) in Microsoft Excel® using the concentration matrix of 249 metabolites (after dataset optimization) for all 60 samples. A two-dimensional (2D) score plot was plotted using PC1 & PC2 to visualize time and treatment based clustering of samples. A 2D loading plot of PC1 & PC2 gave the relative contribution of each metabolite to the separation of sample clusters.

2.3.3 Hierarchical clustering—We visualized the metabolite concentrations in a heatmap generated in *PermutMatrix* V1.9.3. We used a Euclidean distance matrix of dissimilarity, and samples were clustered based on McQuitty's method of unsupervised hierarchical clustering. Multiple fragment heuristic seriation rule was used, with red signifying elevation and cyan signifying decrease in concentration with respect to the

median. Row-wise (metabolite) enumeration was optimized to improve visualization of data structure. We used the PCA and heatmap results to design our subsequent analyses.

2.3.4 Temporal shifts in metabolites in Control and Venomated hosts—We initially tracked the temporal changes in metabolism of Control and Venomated *S. bullata* pupae. Based on the PCA and heatmap results, we then used 1 and 5 day Control and Venomated samples in the following analyses. A one-way ANOVA was performed in MetaboAnalyst 2.0 to identify metabolites that changed significantly ($p < 0.05$) between any time points and treatments. False discovery rate (FDR) of significantly changing metabolites (q -value) was estimated to take into account multiple comparisons. Fisher's Least Significant Difference (Fisher's LSD) *post-hoc* test was used to identify the relationship between time points. A cut off of $p < 0.05$ and $q < 0.10$ was used for significant, high confidence results.

Although some metabolites changed in the same direction, fold changes between Control and Venomated hosts could be vastly different. We performed a fold change analysis on untransformed concentrations of 249 metabolites using Metaboanalyst 2.0. We used the non-parametric Mann-Whitney *U*-test (MW *U*), with unequal group variance and used $p < 0.05$ for significant results. Fold change for each metabolite was estimated within treatment from day 1 to 5 and between treatments at day 1 and 5.

In addition to metabolites changing significantly, we recognized that a suite of metabolites could potentially show low variance from day 1 to 5 in Control and Venomated hosts. We therefore measured the Co-efficient of Variation (CV) to identify such metabolites. CV was appropriate to compare the relative dispersion of metabolites since widely different means could be expected between time points. Mean metabolite concentration and percent CV for each metabolite was calculated and plotted in Microsoft Excel®, and the top metabolites showing low variance in both between treatments were identified. These low variance metabolites are not a result of metabolite peaks being close to detection limit. Lipids had to be excluded from this analysis since our study was not optimized for lipid extraction and could likely confound our results.

2.3.5 Biomarkers for differentiating Venomated hosts from Control hosts—We used Partial Least Squared-Discriminant Analysis (PLS-DA) in MetaboAnalyst 2.0 to identify metabolites that can successfully distinguish between 1 day old Control and Venomated hosts. Cross-validation using Q2 performance measure was used to estimate the optimal number of components. Metabolites were ranked by variable importance projection (VIP) score based on the weighted sum of squares of the PLS loadings. These biomarkers could be used in preliminary assays for differentiating between early Control and Venomated hosts.

2.3.6 Functional interpretation of metabolite changes—Metabolite Set Enrichment Analysis (MSEA) was performed in MetaboAnalyst 2.0 to identify significantly enriched pathways and functional categories in Control and Venomated hosts. All significantly changing metabolites ($p < 0.05$, $q < 0.10$) from the one-way ANOVA were standardized to their metabolite names in MetaboAnalyst 2.0. *Holm-p value*, the *p-value* corrected using

Holm-Bonferroni method, and *Q-statistic*, the correlation between compound concentrations and functional outcome, were estimated.

2.4 Hemolymph pH Measurement

2.4.1 pH measurement procedures—Hemolymph of Venomated and Control *S. bullata* was tested for pH differences. Venomated and Control *S. bullata* were hosted under the conditions as previously discussed. After the anterior end of the puparial wall was removed off, whole Control and Venomated *S. bullata* were placed in 1.5 ml microcentrifuge tubes with anterior end facing upward and centrifuged for 5 minutes at 2000g. Hemolymph was collected by puncturing the exposed head with a pair of sharp forceps, and pipetting out 3ul of hemolymph per sample. This was quickly applied to micro pH paper of range 6.0 to 8.0 (based on an initial test of hemolymph pH ranges) to minimize oxidation. Readings were taken immediately and 10 replicates were collected per treatment at 4 hours as well as 1, 2, 3, 4, and 5 days following venom treatment.

2.4.2 Data analysis—pH data was analyzed in the statistical package *SPSS 22.0.0*. In each treatment, we used 4hr pupa as our baseline to test for significant changes in hemolymph pH at day 1,2,3,4, & 5. We used an independent t-test for unequal group variances and $p<0.05$ to estimate significant changes over time.

3. Results and Discussion

3.1 Temporal changes in the metabolism of Control and Venomated *Sarcophaga bullata*

Control and Venomated samples separate into two super-clusters along Principal Component 1 (PC1) (Figure 2), indicating that their metabolic profiles are distinct from each other. A total variation of 51.2% between Control and Venomated samples is described by PC1 and PC2 (PC1=36.7% and PC2=14.5%). On PC1, sorbitol, ethanolamine, lactate, alanine, and 2-hydroxyglutarate, dominate the positive loadings whereas 4-hydroxy-2-oxoglutaric acid dominates the negative loading (Figure S1). These metabolites are the top contributors to separation of Control and Venomated hosts. Both Control and Venomated hosts show the same general trend along PC2, but with some differences. Control host metabolism changed over time, with 1, 3, and 5 day sample clusters being highly differentiated by PC2. In Venomated hosts, however, temporal separation of samples is less distinct. The 3 and 5 day samples overlap to a considerable extent but are separated from the 1 day samples. This suggests that Venomated host metabolism at day 3 and 5 are more similar to one another than to 1 day.

Control and Venomated hosts are also highly differentiated using hierarchical clustering analysis (Figure S2). Among Control hosts, the 1, 3, and 5 day samples form three separate clusters indicating a high temporal discrimination. In contrast, the metabolic profiles of 3 and 5 day Venomated hosts are virtually indistinguishable. These results are concordant with the PCA analysis.

3.2 Temporal metabolite changes in normally developing Control *Sarcophaga bullata*

For our baseline, we first evaluate metabolite changes in Control host pupae over the 5-day time course. Our analysis of Control hosts shows that metabolism in metamorphosing *S. bullata* undergoes distinct changes between 1, 3, & 5 days (Figure 1 & 2, and Figure S2). A suite of 133 metabolites change significantly from day 1 to day 5 (One-way ANOVA, $p < 0.05$, $q < 0.10$), with 88 increasing and 45 decreasing (Table S1a). When categorized into functional roles, specific trends are apparent (Figure 3a & b). Glycolysis is the main pathway by which insects metabolize glucose to generate pyruvate for energy metabolism (Nation, 2008). During insect metamorphosis, regulation of glycolysis is thought to play a role in energy supply for development (Agrell, 1953). In our Control hosts, glycolytic intermediates increase from day 1 to 5, with 3-phosphoglycerate and glucose-6-phosphate increasing 3.13 fold ($p = 0.00018$, MWU) and 1.5 fold ($p = 0.00049$, MWU) respectively. These changes could represent an increase in glycolytic pathway activity that is related to growth (Agrell, 1953). In contrast, glucose shows no significant change over time (One-way ANOVA, $p < 0.05$, $q < 0.10$) and has the lowest temporal variance (%CV=7.57) of any measured metabolite (Figure S3a), possibly reflecting homeostatic regulation.

The tricarboxylic acid cycle (TCA cycle) is a mitochondrial pathway that uses *Acetyl CoA* as a carbon source and produces energy in the form of adenosine triphosphate (ATP) for cellular processes. Pyruvate, which is the end product of glycolysis and also involved in fatty acid oxidation in the mitochondria, is a potential source of *Acetyl-CoA* for the TCA cycle. In our Control hosts, pyruvate increases 1.5 fold ($p = 7.58E-05$, MWU) from day 1 to 5. Moreover, α -ketoglutarate, a key metabolite in the TCA cycle, increases 1.65 fold ($p = 0.0081$, MWU) from day 1 to 5. These changes could indicate increased aerobic respiration to meet increased energy demands in normally metamorphosing Control hosts.

We observed small increases from day 1 to 5 in levels of several polyol sugars, such as sorbitol and xylitol, which increase 1.44 fold ($p = 0.01$, MWU) and 2 fold ($p = 0.00018$, MWU) respectively. However, the functions of these metabolites in insect development are not yet established. Metabolites involved in chitin synthesis and chitinolysis also increase over time, probably reflecting the differentiation and remodeling of body parts in metamorphosing hosts (Merzendorfer and Zimoch, 2003).

Whereas peptides and related metabolites involved in amino acid metabolism show changes in both directions from day 1 to 5, the levels of ten standard free amino acids (FAAs) decrease and six remain unchanged (Figure 3b). It is known that insects sequester FAAs into storage proteins in fat bodies and re-release them during metamorphosis to synthesize other proteins (Chandrasekar et al., 2008; Hunt et al., 2003; Wang and Haunerland, 1993; Wheeler et al., 2000), which likely explain this pattern. Tyrosine and tryptophan metabolism increase in Control hosts and are known to play key roles in cuticular sclerotization (Andersen, 2010) and development of the eye (Linzen and Schartau, 1974; Han et al., 2007).

3.3 Metabolic effects of parasitoid venom

3.3.1 Overview—*N. vitripennis* venom induces many changes in *S. bullata* metabolism relative to Controls (Table S1a & b). Out of 249 metabolites, only 75 remain stable across 1

to 5 days in both Control and Venomated samples. Notably glucose, urate (uric acid), and glycerol, show low variance over time in both treatments (Figure S3). Of the 118 metabolites that change significantly from day 1 to 5 in Venomated hosts, 53 share the same pattern of change as in Control (37 increasing + 16 decreasing). Among those that increase, several are involved in carbohydrate metabolism, including sugar acids and polyols (sugar alcohols). However, some increasing metabolites show large differences in the magnitude of change between Venomated and Control hosts. For example, sorbitol levels increase from day 1 to 5 in both, however the increase in Venomated hosts is 6.81 fold ($p=0.0001$, MW *U*), whereas in Controls, it is only 1.44 fold ($p=0.01$, MW *U*). Moreover, even at day 1, we found that sorbitol levels in Venomated hosts is 48 fold higher than in time-matched Controls ($p=0.00017$, MW *U*). This contrasts with the very low variance in metabolites such as glucose, urate, and glycerol (Figure S3, Supplementary Material 1a).

Venom induced changes that differ uniquely from Controls include 41 metabolites that only change in Venomated hosts. Notable among these are the polyol ribitol and the monosaccharide fructose (Table S2a), which increase 3.78 fold ($p=0.0001$, MW *U*) and 2.89 fold ($p=1.08E-05$, MW *U*) respectively. Additionally, 24 metabolites change in the opposite direction in Venomated hosts relative to Controls over time. These include the TCA cycle metabolite α -ketoglutarate which decreases from day 1 to 5 and free amino acids (FAAs) alanine, methionine, lysine, and valine, which increase in Venomated hosts (Table S2b). The metabolites increasing in Venomated hosts also include lipid metabolites such as ethanolamine, palmitoylcarnitine, and oleoylcarnitine (Supplementary Material 1b). Finally, a set of 56 metabolites that change from day 1 to 5 in Control hosts did not change in Venomated hosts, possibly reflecting an association with developmental arrest. These include several dipeptides and γ -glutamyl amino acids (Table S2b), which could be an indication of protein biosynthesis being arrested in Venomated hosts.

In general, venom up regulates polyol and amino acid biosynthesis, down regulates glycolysis and oxidative metabolism, and arrests chitin biosynthesis (Figure 3).

Partial Least Squared-Discriminant Analysis (PLS-DA) was used to identify the top ten biomarkers that distinguish Control and Venomated hosts as early as 24 hours post venom treatment (Figure S4). Sorbitol is the top differentially regulated metabolite with VIP=3.51 (48 fold higher in Venomated hosts, $p=0.00017$, MW *U*). Other key metabolites include the amino acid glutamine (VIP=3.37), carbohydrate metabolites lactate (VIP=2.0) and fructose (VIP=1.98), lipid metabolite ethanolamine (VIP=2.41), and the TCA cycle metabolite α -ketoglutarate (VIP=1.92).

Nasonia stung hosts have been frequently referred to as being developmentally arrested (e.g., Rivers and Denlinger, 1994a & b; Rivers and Denlinger, 1995b), and it has been proposed that *Nasonia* venom effects may mimic or activate the same pathways as pupal diapause and/or low-temperature treatment (Rivers and Denlinger, 1994a; Rivers and Denlinger, 1994b). We therefore compared our results to results from studies on *Sarcophaga* undergoing developmental arrest due to low-temperature treatment (Teets et al., 2012) and diapause (Michaud and Denlinger, 2007). We found that, while some metabolites in Venomated hosts are regulated similarly to both diapause and low-temperature treatment, the effect of venom

on *Sarcophaga* metabolism can be clearly differentiated from both conditions (Table 1a). Furthermore, while some aspects of Venomated host metabolite regulation are similar to flies under conditions of hypoxia (Feala et al., 2007), the venom response is also distinct from the hypoxia response (Table 1b). These comparisons are discussed in more detail in the sections below, which evaluate venom effects on specific metabolic pathways.

3.3.2 Regulation of sugar metabolism

(i) Activation of polyol biosynthetic pathways: An interesting outcome of *Nasonia* venom treatment is the dramatic activation of polyol biosynthetic pathways (Figure 4a). Until now, insects were known to produce polyols or sugar alcohols as a cryoprotective physiological response during photoperiod-induced diapause or temperature-induced rapid cold hardening (see reviews Danks, 2000; Clark and Worland, 2008; Denlinger, 2002). Sorbitol is also thought to play a central role in maintaining insect larvae in their developmental state (Horie et al., 2000; Iwata et al., 2005; Nation, 2008). We found that one of the main effects of venom is to increase polyol biosynthesis from day 1 to 5, specifically sorbitol, ribitol, mannitol, erythritol, xylitol, and arabitol (Figure 4a). Only Glycerol did not change and exhibited low variance in both treatments (Figure S3).

The most dramatic among polyol increases is that of sorbitol (Figure 5a). Whereas sorbitol also generally increases over time in Control hosts, the fold increase from day 1 to 5 is much larger in Venomated hosts (6.81 fold, $p=0.0001$, MWU). Furthermore, at both day 1 and 5, sorbitol levels are dramatically higher in Venomated hosts compared to Controls by 48 fold ($p=0.0001$, MWU) and 226 fold ($p=0.0001$, MWU) respectively. To identify which pathways are affected in Venomated hosts between day 1 and 5, we conducted a metabolite set enrichment analysis (Xia et al., 2012). Metabolite sets for the fructose and mannose degradation pathway, in which sorbitol is produced, is enriched >10-fold (*Holm*- $p=2.55E-08$) (Figure S5a).

Sorbitol accumulation has been implicated in diabetic microvascular damage in insulin-independent tissues of kidneys, retinal, and nervous system in humans (see review Forbes and Cooper, 2013). Sorbitol synthesis is generally a highly regulated metabolic process, where glucose is converted to sorbitol by the rate-limiting enzyme *aldose reductase* (Hamada et al., 1991). *Aldose reductase* has low affinity to glucose, and hence hyperglycaemia (elevated glucose levels) is typically necessary for excess sorbitol synthesis and accumulation (Hamada et al., 1991). However, glucose levels appear to remain tightly regulated (i.e., show low variance) within and between treatments (Figure S3). Therefore in Venomated hosts, the dramatic increase in sorbitol biosynthesis (Figure 5a) could be mediated by altered *aldose reductase* function rather than elevated glucose levels (Figure 5b). This could present a potential application of *Nasonia* venom to diabetes research for understanding the mechanism of sorbitol pathway activation and accumulation. .

Under unfavorable conditions in nature, sorbitol increases could aid in cryoprotection (Teets et al., 2012) and/or maintenance of larvae in their developmental state (Horie et al., 2000; Iwata et al., 2005; Nation, 2008). The dramatically high levels of sorbitol in Venomated hosts (compared to Control) resembles *Sarcophaga* subjected to low-temperature treatment (Table 1a), where sorbitol was nearly 70-fold higher (Teets et al., 2012). In contrast,

diapausing hosts regulate sorbitol differently, with the levels being lower compared to controls (Michaud and Denlinger, 2007). Venomated hosts, however, differ from both diapause and low-temperature treatment hosts in glycerol regulation (Table 1a). While there is no significant difference in glycerol levels between Venomated and Control hosts, it is known to increase 4-8 fold in diapausing and low-temperature treated flies (Michaud and Denlinger, 2007; Teets et al., 2012). Finally, several other polyols have also been found to be higher in low-temperature treated hosts, whereas only ribitol was higher in Venomated hosts (Table 1a). Although there are some similarities in polyol regulation, the pattern of polyol elevation in venom treatment is different from low-temperature treated hosts. Whether sorbitol and ribitol elevation in Venomated hosts (compared to Controls) could provide a source of nutrition to developing parasitoid young is not yet known.

(ii) Gluconeogenesis and Glycolysis: Gluconeogenesis and glycolysis involve a set of reactions occurring in opposite directions in the same basic pathway (Figure 4a). Gluconeogenesis regenerates glucose, either from various non-carbohydrate substrates or from degradation of storage carbohydrates, to regulate blood glucose levels. In insects, trehalose is the main storage sugar used to generate glucose for glycolysis (Nation 2008). Under normoxic conditions, glycolysis involves conversion of glucose to pyruvate, which is then used for ATP production in the mitochondria (via the TCA cycle and oxidative phosphorylation). A key effect of *N. vitripennis* venom in *S. bullata* is modulation of gluconeogenesis/glycolysis (Figure 4a). Several intermediate compounds in this pathway decrease from day 1 to 5. Strikingly, however, glucose levels show no significant change (One-way ANOVA, $p < 0.05$, $q < 0.10$) from day 1 to 5 day in either Venomated or Control hosts. Glucose levels in Venomated hosts are also not significantly different from time matched Control hosts at both day 1 and 5 (One-way ANOVA, $p < 0.05$, $q < 0.10$). Moreover, we found low temporal variation in glucose in both treatments (Figure S3). Finally, in insects excess hemolymph glucose is managed by conversion to trehalose via trehalose-6-phosphate. In Venomated hosts, trehalose-6-phosphate did not show significant change from day 1 to 5 ($p > 0.05$, one-way ANOVA). While it was previously hypothesised that *Nasonia* venom could be increasing gluconeogenesis by increasing the mobilization of storage carbohydrates (Rivers and Denlinger, 1994b), our results do not lend support to this hypothesis. Previous studies have also reported stable trehalose levels in venom-exposed hosts (Rivers and Denlinger, 1994b). Parasitized larvae of *Manduca sexta* maintain hemolymph sugar levels via gluconeogenesis when on a reduced carbohydrate diet (Thompson and Dahlgren, 1998; Thompson, 2001). Our hosts are pupae and do not consume food and could likely be maintaining stable glucose levels by a similar mechanism.

Gluconeogenic/glycolytic compounds and pyruvate decrease from day 1 to 5 in Venomated hosts, indicating that glycolysis is not occurring normally as compared to Controls. Lactic acidosis (increase in lactate) is an indication of anaerobic respiration. Lactate levels increase 3.12 fold ($p = 0.0001$, MW U) from day 1 to 5 in Venomated hosts and, even at day 1, lactate is among the top biomarkers that differentiates Control and Venomated hosts (Figure S4). Furthermore, the hemolymph of Venomated hosts gradually acidifies from day 2 after venom exposure (Table S3), which is an indication of lactic acidosis.

We compared the venom-response in *Sarcophaga* to the hypoxia response in *Drosophila* (Table 1b) by Feala et al. (2007). Hypoxia induces an elevation in both lactate and alanine as a result of glycolysis occurring under anaerobic conditions (Feala et al., 2007). Stress response to hypoxia is thought to increase conversion of pyruvate to lactate, to regenerate NAD⁺ for glycolysis (Feala et al., 2007). Even at day 1, both lactate and alanine are several fold higher in Venomated hosts compared to Controls by 9.56 fold ($p=0.0001$, MW *U*) and 2.51 fold ($p=0.0001$, MW *U*) respectively. This provides further support for anaerobic respiration in Venomated hosts.

Oxygen consumption in *S. bullata* is known to decrease after *Nasonia* injects venom (Rivers and Denlinger, 1994b). Additionally, expression of *lactate dehydrogenase*, the enzyme which interconverts pyruvate and lactate, was found to be upregulated in venom exposed hosts (Danneels et al., 2013). These observations, along with our results, indicate that while venom-induced alteration of glucose metabolism is similar to hypoxia, the regulation of glucose itself is different (Table 1b). In hypoxic flies, glucose shows large increases, possibly as a result of a breakdown of homeostasis, failure of utilization and/or continued mobilization of glycogen and trehalose (Feala et al., 2007). In contrast, the low variation in glucose levels in Venomated hosts (Figure S3b) suggests that glucose could be homeostatically regulated for several days..

In diapausing and low-temperature treated *Sarcophaga*, glucose, pyruvate, and glucose-6-phosphate increase in relation to control pupae (Michaud and Denlinger, 2007; Teets et al., 2012) (Table 1a). These increases are speculated to fuel the synthesis of polyols, which serve as cryoprotectants to survive unfavourable conditions (Michaud and Denlinger, 2007; Teets et al., 2012). In contrast, our Venomated hosts show no significant change in glucose (or pyruvate) from Control hosts (One-way ANOVA, $p<0.05$, $q<0.10$), whereas show sorbitol increases of up to 226 fold ($p=0.0001$, MW *U*) compared to Control hosts at day 5 (Figure 5). This suggests that *Nasonia* venom regulates glycolysis and sorbitol synthesis differently from both diapause and low-temperature treatment.

Based on all the above observations, we hypothesise that *Nasonia* venom could be regulating glucose homeostatically. This is distinct from hypoxia, diapause, or low-temperature treatment. A more definitive test will require flux analyses of glycolytic metabolites.

3.3.3 Regulation of energy metabolism

(i) Tricarboxylic acid (TCA) cycle: Although traditionally considered a mitochondrial pathway that produces electrons for oxidative phosphorylation, cytoplasmic isoforms for almost all TCA cycle enzymes have recently been discovered (Raimundo et al., 2011). The only exception to this are enzymes involved in succinate turnover – α -ketoglutarate dehydrogenase, succinate:CoA ligase, and succinate dehydrogenase (Raimundo et al., 2011). α -ketoglutarate, a critical metabolite in the TCA cycle, is synthesized from Acetyl-CoA derived either from glycolysis or from fatty acid oxidation. In insects, α -ketoglutarate is also synthesized in the mitochondria via proline→glutamate→ α -ketoglutarate by a transamination reaction (Nation, 2008).

In Venomated hosts, there is selective regulation of TCA cycle metabolites, with α -ketoglutarate and succinate decreasing and fumarate and malate increasing from day 1 to 5 (Figure 4b). In contrast, in Control hosts, α -ketoglutarate increases 1.65 fold ($p=0.0081$, MWU) from day 1 to 5. Even at day 1, α -ketoglutarate is one of the top differentially regulated metabolites between treatments hosts, being 0.26 fold ($p=0.0001$, MWU) lower in Venomated hosts (Figure S4). Although succinate decreases from day 1 to 5 in both treatments (Figure 4b), the magnitude of decrease in Control hosts is 0.75 fold ($p=0.001$, MWU) whereas in Venomated hosts, it is 0.29 fold ($p=0.0001$, MWU). Additionally, at day 5, succinate is 0.41 fold ($p=0.0001$, MWU) higher in Control hosts as compared to Venomated hosts. Finally, the metabolite set for the TCA cycle shows > 10 fold enrichment ($Holm-p=4.01E-07$), indicating that this pathway is significantly affected by *Nasonia* venom (Figure S5a). However, increase in fumarate and malate from day 1 to 5 in Venomated hosts could be explained by cytoplasmic enzyme activity (Raimundo et al., 2011). It appears that succinate turnover, cycling of metabolites in the TCA cycle, and energy production via TCA cycle is reduced in Venomated hosts, suggesting that *Nasonia* venom may block oxidative metabolism.

The TCA cycle also plays an important role in hypoxic stress response in insects (Feala et al., 2007). Generally, hydroxylation of proline residues is essential for normoxic degradation of α -subunits of Hypoxia Inducible Factors, and this reaction is coupled to oxidation of α -ketoglutarate (Raimundo et al., 2011). Under hypoxic conditions however, α -subunits of Hypoxia Inducible Factors accumulate and are translocated to the nucleus, where they start the transcriptional cascade for hypoxia response, such as glycolytic shift (Raimundo et al., 2011). In Venomated hosts, proline→glutamate conversion appears to be blocked as proline levels increase, whereas glutamate and α -ketoglutarate levels decrease from day 1 to 5 (Figure 4b). Decreased availability of α -ketoglutarate for oxidation, and the corresponding decrease in proline hydroxylation that is coupled to this reaction, could likely be causing a block in TCA cycle and oxidative phosphorylation. Again venom responses are distinct from the hypoxia response (Table 1b), where proline and glutamate remain unchanged (Feala et al., 2007). Moreover, the increase in fumarate in Venomated hosts contrasts with hypoxia, where fumarate does not change (Feala et al., 2007).

Sarcophaga subjected to low-temperature treatment do not show a significant change in succinate in relation to controls (Teets et al., 2012). While this is also true at day 1 for Venomated hosts compared to Controls (Table 1a), succinate is 0.41 fold higher in Control hosts compared to Venomated by day 5 ($p=0.0081$, MWU). Also, low-temperature treated *Sarcophaga* show increases in fumarate, malate, and phosphate in relation to controls (Teets et al., 2012), whereas in diapausing hosts only fumarate was measured and found to increase (Michaud and Denlinger, 2007). In Venomated hosts, malate and phosphate were significantly higher than time-matched Control hosts at day 5, while there was no difference in fumarate.

From our results, we hypothesize that one of the effects of venom could be to alter mitochondrial function. Differences in regulation of TCA cycle metabolites in Venomated hosts compared to hypoxia, low-temperature treatment, and diapause, suggest that the

mechanism of deactivation of TCA cycle and blocking of oxidative metabolism by venom is unique.

(ii) Glycerol-3-Phosphate (G-3-P) Shuttle: The Glycerol-3-Phosphate (G-3-P) shuttle is an important energy generating pathway in insects (Nation, 2008). Conversion of G-3-P to dihydroxyacetone phosphate (DHAP) in the mitochondria and back to G-3-P in the cytoplasm regenerates NAD⁺ required for cytoplasmic glycolysis (Figure 4a).

In both treatments, G-3-P decreases from day 1 to 5 (Figure 4a), however in 5-day Venomated hosts, G-3-P levels are 1.85 fold higher than in time-matched Controls ($p=1.08E-05$, MWU). Similarly, in both treatments, significant changes in glycerol levels are not detected from day 1 to 5 (One-way ANOVA, $p<0.05$, $q<0.10$), although by 5-day Venomated hosts, glycerol is 1.14 fold higher than in Controls ($p=0.06$, MWU). In both treatments, there is significant day 1 to 5 enrichment for metabolites in the G-3-P shuttle ($Holm-p=0.01$, $Holm-p=6.70E-07$), although the magnitude of enrichment is greater in Controls (>10 fold for Controls versus <5 fold for Venomated) (Figure S5a & b). This suggests that *Nasonia* venom could be blocking the G-3-P shuttle, possibly resulting in the backing up of G-3-P and (to a lesser extent) glycerol.

In a typical hypoxia response, over expression of *glycerol-3-phosphate dehydrogenase* increases conversion of DHAP to G-3-P (Kelly et al., 2011). This response is known to attenuate hypoxia by increasing proline hydroxylation and decreasing the stability of Hypoxia Inducible Factor subunits (Kelly et al., 2011). However, G-3-P decrease from day 1 to 5 in Venomated hosts, could imply that there is no overexpression of *glycerol-3-phosphate dehydrogenase*. Therefore, the regulation of G-3-P shuttle in the venom response appears to be different from the typical hypoxia response.

3.3.4 Arrest in structural carbohydrate metabolism—In Venomated hosts, a suite of classical indicators of chitin metabolism interference, such as lack of structural differentiation, morphogenesis, and locomotory and sensory organ formation, can be easily observed in the gross morphological phenotype (Figure 1). We present biochemical evidence for arrest in chitin biosynthesis and catabolism *in vivo* due to parasitoid venom action (Figure S6a). Chitin precursors, N-acetyl glucosamine-6-phosphate and UDP N-acetyl glucosamine, remain unchanged over time in Venomated hosts, whereas all chitin precursors increase in Control hosts (Figure S6a). Additionally, N-acetyl glucosamine, a by-product of chitin hydrolysis used in structural remodelling, also did not change temporally in Venomated hosts, while in Controls it increased 4.85 fold ($p=4.33E-05$, MWU). Venom could be preventing chitin biosynthesis as well as chitin hydrolysis. We do not yet know whether disruption of chitin metabolism is a cause or a downstream effect of developmental arrest.

N. vitripennis venom contains a *chitinase* enzyme (de Graaf et al., 2010), which also appears to be a common component of endoparasitoid wasp venoms (Krishnan et al., 1994). Chitinolytic function of a venom *chitinase* has been demonstrated *in vitro* in the braconid wasp *Chelonus* (Krishnan et al., 1994). Potential applications for venom chitinases exist in several areas of biotechnology such as in agriculture for controlling soil pathogens (e.g.,

Gohel et al., 2006) and insect pests (Herrera-Estrella and Chet, 1999), in epidemiology for controlling mosquito populations, and in pathology for treating fungal infections (Dahiya et al., 2006). Traditionally, chitin synthesis and sequestration studies have used yeast and fungi models (Valdivieso et al., 1999). With the discovery of *N. vitripennis* venom effects on chitin metabolism of *S. bullata*, a novel, sophisticated, and laboratory treatable insect model system is now available.

3.3.5 Effect of venom on amino acid metabolism and protein synthesis—*N.*

vitripennis venom causes increases (day 1 to 5) in ten standard FAAs, as well as α and β -amino acids homoserine and β -alanine (Figure S6b). These results partly agree with previous measurements of FAAs from host hemolymph (Rivers and Denlinger, 1994b).

However, di and tripeptides and γ -glutamyl amino acids, synthesized via glutathione metabolism, largely remain unchanged from day 1 to 5 in Venomated hosts (Table S2b). Moreover, metabolite set enrichment for various amino acid metabolism pathways is generally lower from day 1 to 5 in Venomated hosts (Figure S5a). This suggests that, despite temporal increases in several FAAs, they are possibly not being utilized within amino acid metabolism pathways. Larval parasitoids are known to modulate FAA profiles in hosts, presumably to use as a source of direct nutrition. For example, *Aphidius smithi* changes FAAs in the honey dew of aphids (Cloutier, 1986). FAAs can also be used to produce other sources of energy, such as in *Manduca sexta* parasitized by *Cotesia congregata*, where alanine is used for gluconeogenesis (Thompson and Dahlman, 1998).

N. vitripennis venom contains several proteases and peptidases (de Graaf et al., 2010), and the mechanism of FAA increase could be attributable to hydrolysis of peptide bonds in host proteins via direct enzymatic action of venom serine proteases and/or metalloproteases. Inhibition of protein synthesis could also likely be a mode of venom increasing FAA accumulation. Venom components in other organisms, such as phosphodiesterases and exonucleases in snakes, hydrolyze amino acyl-tRNA which is necessary for protein synthesis (Petrova et al., 1975; Bruns and Philipps, 1970). Among parasitoids, a teratocyte secreted protein in *Microplitis croceipes* inhibits incorporation of methionine into proteins (Dahlman et al., 2003). It seems likely that *N. vitripennis* venom components, such as esterases (de Graaf et al., 2010), could be interfering with protein synthesis. Where in the process this occurs remains unknown. Although there is currently no direct evidence, accumulation of FAAs could serve as a more advantageous food reserve for the developing parasitoid larvae than complex pupal proteins.

The pattern of venom-induced FAA increase has some commonalities with *Sarcophaga* subjected to low-temperature treatment (Table 1a). 10 FAAs are elevated in both low-temperature treated *Sarcophaga* (Teets et al., 2012) and Venomated hosts (Table 1a). It has been proposed that FAA accumulation could perform cryoprotective functions and/or support protein synthesis required for flies to survive cold temperatures (Joplin et al., 1990; Teets et al., 2012). In diapause however, only two FAAs (alanine and leucine) were higher (Michaud and Denlinger, 2007) (Table 1a). Venom-induced increase in FAAs is more similar to flies surviving cold temperatures (Table 1a).

Few FAAs decrease temporally in Venomated hosts including glutamine and glutamate, which are precursors of γ -aminobutyric acid (GABA), a major neurotransmitter in insects. However, GABA levels increase from day 1 to 5. In contrast, tyrosine and L-dihydroxyphenylalanine (L-DOPA), which are precursors for synthesis of the major neurotransmitter dopamine, remain unchanged. High levels of dopamine have been shown experimentally to retard normal growth and delay pupation in armyworms parasitized by *Cotesia kariyai* (Noguchi et al., 1995; Noguchi and Hayakawa, 1996). Rather than killing *S. bullata* entirely, *N. vitripennis* venom is known to act on select neuronal cells by inducing apoptosis (Rivers et al., 2011). Compared to Controls, most metabolites involved in GABA and L-DOPA pathways are significantly lower in Venomated hosts by day 5. Within Venomated hosts however, there are low grade increases or lack of significant reduction in neurotransmitter biosynthesis from day 1 to 5. Our results could be indicative of the living host being maintained for the benefit of wasp larvae.

4. Conclusions

Our investigations have revealed a number of metabolic cascades that *Nasonia vitripennis* venom induces in *Sarcophaga bullata*, opening several avenues for future investigation of this untapped potential pharmacopeia. In summary, venom up regulates polyol and amino acid biosynthesis, down regulates glycolysis and oxidative metabolism, and arrests chitin biosynthesis. A major venom effect is the dramatic increase in sorbitol synthesis while maintaining stable glucose levels. This is an unusual mechanism of sorbitol regulation and could make the *Nasonia-Sarcophaga* system a useful tool for studying the sorbitol pathway, with possible implications for diabetes research. Although sorbitol has also been found to be higher in low-temperature treatment, the stability of glucose is unique to venom treatment. Moreover, venom does not entirely mimic low-temperature treatment in the regulation of other polyols.

A second major venom effect appears to be blocking of TCA cycle activity, which could cause the shift to anaerobic metabolism. Although, some metabolite patterns are similar to the hypoxia response in flies, the breakdown of glucose homeostasis observed in hypoxia is not seen in the venom response. Venom effects also differ from low-temperature treatment and diapause, where oxidative metabolism does not appear to be affected.

Venom appears to stop chitin biosynthesis and degradation, reflecting the developmental arrest phenotype of Venomated hosts. Whether blocking of chitin synthesis is a proximate cause or a downstream consequence of developmental arrest is currently unresolved. Venom increases FAAs in hosts, and this could be a way to increase host nutritional value to benefit the parasitoid young. It is inconclusive whether hydrolysis of pupal proteins, decrease in protein synthesis, or both, lead to FAA increases. Metabolic flux analyses could be informative. Our investigations have generated a number of hypotheses about *Nasonia* venom action, and provide baseline information that can aid future parasitoid venom research.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig. 1. Developmental arrest in *S. bullata* pupae following venom injection by *N. vitripennis*
 The top row shows progression of normal development and metamorphosis in Control *S. bullata* over 7 days. In Venomated hosts (bottom row), development is arrested. Failure of eye pigmentation is visible from day four and bristle pigmentation by day 6. The black arrow indicates the sting site where melanization occurs.

Fig. 2. Treatment and time based clustering of Control (C) and Venomated (V) samples using 249 metabolites

A Principal Component Analysis (PCA) plot, with total variance of 51.2% explained by PC1 & PC2. Venomated samples separate along PC1, signifying that Venomated host metabolism is distinct from Control hosts. There is clear temporal differentiation of Control hosts along the PC2. In Venomated hosts however, there is a greater degree of overlap between 3 day and 5 day samples. This indicates that Venomated host metabolism at these time points are similar to each other.

Fig. 3. Temporal regulation of functionally relevant metabolites in Control and Venomated hosts Shown are the total number of metabolites in each functional category that significantly increase or decrease from day 1 to 5 in Control and Venomated hosts (One-way ANOVA & FDR cut off of $p < 0.05$ and $q < 0.10$ respectively). **(a) Carbohydrate, energy, and lipid metabolism** and **(b) Amino acid and peptide metabolism**. The direction of change and fold change for each metabolite is provided in Supplementary Table S5. Results are interpreted in the text.

Fig. 4. Temporal regulation of intermediary metabolism in Control and Venomated hosts
 All metabolites measured in our study for intermediary metabolism are shown in black boxes. Significantly changing ($p < 0.05$, $q < 0.05$) metabolites from day 1 to 5 were identified by a one-way ANOVA. The direction of change is denoted by: Red=Increasing, Light Blue=Decreasing, and Black=No significant change. Similarly temporally regulated metabolites, but those that show significant difference between Control & Venomated at day 5, are indicated by *. (a) Glycolysis/gluconeogenesis and polyol biosynthetic pathway: Although polyols increase in both treatments, sorbitol levels are significantly higher in Venomated hosts. In Venomated hosts, glycolysis is dysregulated, and the increase in lactate and alanine indicates a switch to anaerobic respiration. (b) **TCA cycle:** Synthesis of metabolites involved in succinate turnover (italicized) is under control of enzymes that are found only in the mitochondria. Hence the decrease in α -ketoglutarate and succinate in Venomated hosts may indicate altered mitochondrial function. Enzyme isoforms for fumarate and malate synthesis exist in the cytoplasm, and this could account for increases of these metabolites in Venomated hosts.

Fig. 5. Temporal changes in glucose and sorbitol in Control and Venomated hosts

Scaled intensity (on Y axis) represents the raw area counts of metabolite peaks scaled to a median of 1.0. Median values (horizontal lines) and 1st quartiles to either side are shown by black boxes. The lower and upper whiskers represent the minimum and maximum values excluding outliers, which are represented by a dot. Significant temporal change (One-way ANOVA, $p < 0.05$, $q < 0.10$) in each treatment is indicated by *. **(a) Sorbitol:** In Venomated hosts, sorbitol increases dramatically over 5 days (medians ranging from 2.45 at day 1 to 21.52 at day 5). **(b) Glucose:** Levels remain comparable in both Control and Venomated hosts with no significant change within or between treatments. In Venomated hosts, sorbitol increase in the absence of a concomitant increase in glucose reflects an unusual activation of the sorbitol pathway.

Table 1

Comparison of metabolic responses to venom, low-temperature treatment¹, diapause², and hypoxia³

(a) Metabolite regulation in Venomated, low-temperature treated ¹ , and diapausing ² flies				
Metabolic Categories	Metabolites	Venomated	Low-temperature Treatment ¹	Diapause ²
Polyol biosynthesis	Sorbitol	Up	Up	Down
	Ribitol	Up	Up	N/A
	Glycerol	No change	Up	Up
	Erythritol, Arabitol	No change	Up	N/A
	Threitol, Mannitol	No change	N/A	N/A
	Xylitol	Down	Up	N/A
Glycolysis	Glucose	No change	Up	Up
	Glucose-6-Phosphate	Up	Up	N/A
	Pyruvate	No change	N/A	Up
	Lactate	Up	N/A	N/A
Anaerobic respiration	Fructose	Up	Up	N/A
	Mannose	Up	Up	Down
TCA cycle	Ribose, Maltose	No change	No change	N/A
	Succinate	No change	No change	N/A
	α -ketoglutarate	Down	N/A	N/A
	Malate, Phosphate	Up	Up	N/A
	Fumarate	No change	Up	Down
	Glycerol-3-phosphate	Up	Up	N/A
G-3-P shuttle				
Free Amino Acids (FAAs)				
	No change	Tyr, Cys, Arg	N/A	N/A
	Decreasing	Gln	N/A	Asp, Gly, Phe, Pro, Tyr

b) Venom response and hypoxia response ³			
Metabolic Categories	Metabolites	Venom Response at Day 1	Hypoxia Response ³ at 4 hrs
Polyols biosynthesis	Sorbitol, Ribitol	Up	N/A
	Glycerol, Erythritol, Mannitol, Arabitol, Threitol	No change	N/A
	Xylitol	Down	N/A
	Glucose	No change	Up
Glycolysis	Lactate	Up	Up
Anaerobic respiration	Glycerate	Down	No change
Sugar acids	Succinate	No change	No change
	α -ketoglutarate	Down	N/A
TCA cycle	Malate, Phosphate	Up	N/A
	Fumarate	No change	No change
	Increasing	Ala, Thr, Met, Phe, Leu, Val, Ser, Ile, Asp, Glu, Lys, Pro, His, Gly, Asn, Try	Ala, Thr, Arg
Free Amino Acids (FAAs)	No change	Tyr, Cys, Arg	Gln, Glu, Pro, Ile, Leu, Val
	Decreasing	Gln	N/A

Regulation of metabolites for each of the above conditions is indicated. We used 1 day Venomated & Control hosts for this analysis and $p < 0.05$ and $q < 0.10$ (One-way ANOVA) to represent significant change. Metabolic responses that are distinctly due to venom injection by *Nasoria* are shaded in grey. N/A denotes information not available. (a) In both Venomated and low-temperature treated *Sarcophaga*, some polyols and several free amino acids (FAAs) increase. The regulation of glucose and TCA metabolites is different between the two treatments. Fewer similarities are observed between Venomated & diapausing *Sarcophaga*, and (b) Lactate and alanine increase in both treatments, indicating anaerobic respiration. Large increases in glucose during hypoxia are not observed in Venomated hosts.

¹Teets et al. (2012),

²Michaud and Denlinger (2007),

³Feala et al. (2007),