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A new approach for investigating venom function applied to venom calreticulin in a parasitoid wasp

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Abstract

A new method is developed to investigate functions of venom components, using venom gene RNA interference knockdown in the venomous animal coupled with RNA sequencing in the envenomated host animal. The vRNAi/eRNA-Seq approach is applied to the venom calreticulin component (*v-crc*) of the parasitoid wasp *Nasonia vitripennis*. Parasitoids are common, venomous animals that inject venom proteins into host insects, where they modulate physiology and metabolism to produce a better food resource for the parasitoid larvae. vRNAi/eRNA-Seq indicates that *v-crc* acts to suppress expression of innate immune cell response, enhance expression of clotting genes in the host, and up-regulate cuticle genes. *V-crc* KD also results in an increased melanization reaction immediately following envenomation. We propose that *v-crc* inhibits innate immune response to parasitoid venom and reduces host bleeding during adult and larval parasitoid feeding. Experiments do not support the hypothesis that *v-crc* is required for the developmental arrest phenotype observed in envenomated hosts. We propose that an important role for some venom components is to reduce (modulate) the exaggerated effects of other venom components on target host gene expression, physiology, and survival, and term this venom mitigation. A model is developed that uses vRNAi/eRNA-Seq to quantify the contribution of individual venom components to total venom phenotypes, and to define different categories of mitigation by individual venoms on host gene expression. Mitigating functions likely contribute to the diversity of venom proteins in parasitoids and other venomous organisms.

Keywords

Nasonia; Calreticulin; Clotting factor; vRNAi/eRNA-seq

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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.toxicol.2015.08.012>.

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1. Introduction

Parasitoid wasps are an abundant and diverse group of 100–400 thousand species that parasitize other insects and use venom to alter host physiology to create an improved environment for developing wasp offspring (Whitfield, 1998; Heraty, 2009; Quicke, 1997). The best-characterized parasitoid venom system is that of the small (<2.5 mm) ubiquitous parasitoid *Nasonia vitripennis*, which produces ~100 venom proteins (de Graaf et al., 2010; Werren et al., 2010). Approximately 25% of these proteins have no recognizable homology to proteins found in other organisms. The venom proteins cause developmental arrest and manipulate highly conserved metabolic pathways in several distantly related fly host pupae, including *Sarcophaga bullata* (de Graaf et al., 2010; Mrinalini et al., 2014; Rivers and Denlinger, 1994; Werren et al., 2010). The envenomated fly host remains alive and transcriptionally active for over five days (Rivers and Denlinger, 1995), and exhibits transcriptional changes that correspond to metabolic, immune response, and developmental phenotypes (Danneels et al., 2013; Martinson et al., 2014; Mrinalini et al., 2014).

Attributing functions to individual venom components is challenging because the envenomation phenotype is often driven by synergistic interactions among multiple peptides. Venom proteins from many animals often have redundant functions *in vivo* – comprising “cabals” with overlapping effects on critical host pathways (Jimenez et al., 2003). Functional characterization is further complicated by the observation that venom proteins often share little to no sequence homology with previously characterized proteins in other species (de Graaf et al., 2010). In addition, classical methods that rely on purification of individual proteins followed by biological testing are difficult in parasitoids given the small volume of venom that can be practically isolated from these insects. However, advances in genomic, transcriptomic, and proteomic tools that can use smaller volumes of material now make broad characterization of parasitoid venoms and their effects more practical.

High-throughput genome and transcriptome sequencing is now widely recognized as a powerful tool for both hypothesis testing and hypothesis generation (Egan et al., 2012; Hawkins et al., 2010; Lee et al., 2014b; Mwenifumbo and Marra, 2013; Wittkop et al., 2013). Here we investigate venom function using a combination of genetic knockdown (**KD**) of wasp venom genes by RNA interference (**vRNAi**) followed by high-throughput RNA-sequencing (**eRNA-seq**) of the envenomated host. The method, henceforth called **vRNAi/eRNA-seq**, does not require *a priori* identification of target genes/pathways or purification of biologically active individual venom proteins. Functional redundancy of venom proteins can be addressed by calculating the relative contribution of individual venom components to the total venom phenotype (see Methods Section 2.11).

To demonstrate the efficacy of the vRNAi/eRNA-Seq method, we chose to investigate the function of calreticulin, a calcium-binding protein and molecular chaperone present in both the endoplasmic reticulum and mitochondria, as well as secreted in *N. vitripennis* venom. In other animals calreticulin performs a variety of functions, including regulation of steroid hormone genes, cell adhesion, low-affinity high-capacity calcium sequestration, signal-induced calcium release (Mesaeli et al., 1999), and immune regulation (Pockley, 2003).

The *N. vitripennis* genome contains only a single calreticulin gene that likely serves a dual function as an endogenous physiological regulator and as a venom protein. *N. vitripennis* venom calreticulin has significant sequence homology to the calreticulin protein encoded in other insects, including the fly host *S. bullata* (see Supplementary materials).

Previous *in vitro* studies suggest that in venom, calreticulin may play a role in calcium signaling, initiation of a colloid osmotic lysis mechanism of cell death (oncosis), and developmental arrest in envenomated fly hosts (Rivers and Brogan, 2008). In this study we investigated the function of calreticulin in venom by assessing phenotypic and transcriptional effects on hosts that were envenomated by *N. vitripennis* females with RNAi KD of calreticulin gene expression. This novel approach to remove calreticulin without disrupting other venom components has allowed us to identify a set of venom calreticulin-responsive host genes and develop additional hypotheses about its function. We also develop a general model for categorizing venom effect on host gene transcription, including measuring the proportional contribution of a component to a host expression phenotype, and its potential mitigation of the effects of other venom components.

2. Methods

2.1. Wasp and host rearing conditions

N. vitripennis and *S. bullata* hosts were reared according to the previously published protocols (Werren and Loehlin, 2009a, 2009b). For venom collection experiments, adult *N. vitripennis* females were first hosted for 24-h on *S. bullata* pupae at a ratio of 3:3 per tube to stimulate venom production. Adult *N. vitripennis* females used in the RNA-seq experiments were allowed to host feed for 8 h, hosts were then withheld overnight to encourage stinging during the experimental period.

2.2. Gene identification

The *N. vitripennis* genome contains only a single calreticulin gene. Expression had been detected across the entire lifespan by tiling microarray for five developmental stages (male and female) as well as in the female reproductive tract and the venom apparatus (Werren et al., 2010). This indicates that endogenous and secreted venom calreticulin are expressed from the same primary transcript. The venom calreticulin gene (*v-crc*) model was taken from version 2.0 of the official gene set (Gilbert et al., 2013) with location identified as the peri-centromeric region of chromosome 1 (NV19007/ Nasvi2EG031929). One paralog, calnexin, has not been identified as a venom component in bioinformatics screens of the *N. vitripennis* venom transcriptome (de Graaf et al., 2010). PCR product of the calreticulin primer set (Methods, Section 2.4) yields only a single gene product.

2.3. Venom gene knockdown

Genomic DNA extracted from a *N. vitripennis* male of the lab strain AsymCx was used to amplify a single copy of the *v-crc* gene. *N. vitripennis* calreticulin dsRNA was synthesized from genomic DNA using the MEGAScript RNAi Kit [Life Technologies, Grand Island NY]. All kits were used according to the manufacturer protocol. One paralog – calnexin – is present in the 2.0 *N. vitripennis* genome assembly (Nasvi2EG001077+) (Gilbert et al.,

2013). Sequence comparison between calreticulin and calnexin indicates nucleotide sequence identity of 79% (50/63). Micro-RNA target prediction software used to query the *N. vitripennis* transcriptome [Wasp Atlas, RNAi target identification tool; University of Leicester] identified calreticulin as the sole target of double stranded RNA (**dsRNA**) synthesized from the calreticulin amplicon with 100% matching 19-mers. We are therefore confident that *v-crc* KD is specific to calreticulin expressed in *N. vitripennis* and does not target any other venom proteins. Complimentary dsRNA to the *v-crc* transcript was injected into the dorsal posterior end of 6-day instar *N. vitripennis* larvae at a final concentration of 0.36 µg/µl in ddH₂O with 0.5 µl of green food coloring per 10 µl dsRNA solution as a visual marker for microinjection (Werren et al., 2009).

Control wasps were injected with dsRNA complimentary to the *E. coli* LacZ gene (The *N. vitripennis* genome does not contain a Lac operon) to control for possible gene expression changes related to initiation of the RNAi machinery. To compare efficiency of later stage dsRNA KD, pupae were also injected according to the previously published protocol (Lynch and Desplan, 2006) (Supplementary material, siRNA KD of venom calreticulin). All KD *N. vitripennis* were separated by sex at the late pupal stage, prior to eclosion. Adult females were retained for subsequent KD venom experiments. *V-crc* KD females showed a reduction in host stinging and egg laying. Therefore, for host RNA-seq quantification we only utilized hosts that were clearly envenomated based on the presence of a sting site. These hosts showed developmental arrest typical of wild-type envenomated hosts.

2.4. PCR amplification and gel quantification

To confirm *v-crc* KD, RNA was extracted from whole abdomen of dsRNA injected *N. vitripennis* adult females using TRIzol Reagent [Life Technologies, Grand Island NY], followed by chloroform phase extraction, and isopropanol RNA precipitation as per the manufacturer's protocol. Primers were designed to amplify a 245 base pair segment (positions 695–939) of the *v-crc* gene [Forward primer *v-crc* 5'-ATTCGGGATTATCAATCATAGG-3', Reverse primer *v-crc* 5'-CGAATCTGGTCAGCTAGAG-3'] and housekeeping gene RP-49 [Forward primer RP-49 5'-CTTCCGCAAAGTCCTTGTTTC-3', Reverse primer RP-49 5'-TTTATTTCATTCTCCTCAGAACG-3'] as a loading control. Conventional and semi-quantitative (**semi-Q**) RT-PCR was performed using iQ SYBR Green Supermix [BioRad, Hercules CA]. PCR products were detected via UV illumination of samples in a 2% agarose gel stained with Ethidium Bromide.

Semi-Q calreticulin PCR product luminosity was quantified with the ImageJ Gel Analyzer, background was manually subtracted, and signal was normalized to RP-49 loading control visualized on the same gel. Mean normalized luminosity was calculated across five RT-PCR cycle conditions (28 ×, 31 ×, 34 ×, 37 ×, 40 ×) and compared between five injection time points corresponding to various *N. vitripennis* developmental stages (larvae, yellow pupae, black and white pupae, and un-injected control) (n = 15 individuals per group).

2.5. Venom collection

To determine the dose-response effect of total venom on host phenotype, we isolated and injected venom into *S. bullata* pupae at different concentrations. Whole and *v-crc* KD venom was isolated from adult female *N. vitripennis* wasps in 10× ultrapure phosphate buffer solution [Ambion, Grand Island NY] diluted to 1× with UltraPure Distilled Water [Invitrogen, Grand Island NY]. *N. vitripennis* wasps were stunned by placing test tubes containing 3 adult females on ice for approximately 10 min. Fine forceps were used to remove the venom reservoir along with alkaline and acid glands and ovipositor. Minutien pins were used to dissect the reservoir away from venom glands and the ovoposition apparatus. Reservoirs were then washed once and transferred into 1 µL of 1× PBS on dry ice. Aliquots of collected reservoirs were centrifuged at 12,000 × g for 15 min at 4 °C to release contents into solution. Supernatant was then transferred to a clean 1.5 µl tube for NanoDrop Bradford protein quantification. All venom dissection and isolation was conducted under RNase free conditions.

2.6. Venom protein gel

To compare venom protein profiles in *v-crc* KD and control (LacZ) protein content of venom reservoirs was visualized using the NuPAGE SDS-Page gel system as per manufacturer's protocol [Invitrogen, Grand Island NY]. The gel was fixed with 40% ethanol/10% acetic acid and sensitized with 30% ethanol/0.1% sodium thiosulphate (1 mg/mL). The gel was stained with 0.25% Silver Nitrate in water (2.5 mg/mL), developed with 2.5% Sodium Carbonate/0.025% Formaldehyde (25 mg/mL), and stopped with 5% Acetic acid. We observed no significant changes in the overall venom protein-banding pattern, with the exception of a lighter banding pattern in the expected calreticulin size range, and a slight difference in band intensity of two high molecular weight proteins (>160 kD) with light staining for both *crc* KD and control samples. ImageJ, Gel analyzer was used to quantify band intensity of suspected calreticulin band (~50kD) normalized to a control band (~20 kD). Relative intensity of calreticulin protein band in *v-crc* sample was 10% that of the same band in control (LacZ KD) venom (Supplemental Fig. C). This therefore presents convincing evidence that RNAi results in a reduction in the target venom protein (*v-crc*) in the venom reservoir.

2.7. Venom injection

The LC99 (quantity that will kill 99% of the sample population) of whole venom was previously determined to be approximately 1 venom reservoir equivalent per pupae (~1.54 µg/reservoir) (Rivers and Brogan, 2008). Therefore, isolated *N. vitripennis* venom was diluted with 1× PBS to serial concentrations (2.0 µg/µl, 1.0 µg/µl, and 0.5 µg/µl). To assay the dose-response venom phenotype, 1 µl of venom-PBS solution was injected into the anterior end of *S. bullata* pupae using a pulled glass capillary tube connected to a PicoSpritzer III Microinjection Dispenses System [Parker Instrumentation, Huntsville AL]. Pupae were then photographed daily and graded respectively for immune response (melanization reaction), developmental markers (eye pigment deposition and body bristle formation), eclosion, and signs of necrosis and/or death. Host gene expression was not assessed in these manually injected hosts.

2.8. Gene expression in envenomated hosts

We assessed global changes in gene expression attributable to venom calreticulin by comparing RNA-seq profiles of hosts envenomated by female wasps with *v-crc* KD (**K**) vs. total venom LacZ controls (henceforth referred to as total venom or **V** hosts) (Supplemental material, siRNA KD of venom calreticulin). Envenomation was visually confirmed by presence of a localized melanization reaction at the sting site. Gene expression was quantified by RNA-seq at 4, 24, and 72 h post-envenomation, and across three biological replicates comprised of 5-pooled hosts each (total of $n = 15$ individuals assessed per treatment/time point). These data were then compared with total venom control envenomated hosts to produce a list of host genes specifically responsive to removal of calreticulin from *N. vitripennis* venom. As a benchmark for normal host gene expression we used RNA-seq data from un-envenomated hosts collected at matching time points, number of replicates, sequenced at the same read depth, and processed with the same data cleaning/alignment protocol (Martinson et al., 2014). Two out of three un-envenomated control replicates were collected and sequenced at the same time as envenomated and *v-crc* hosts. As the venom function of *crc* is not yet fully understood, we cannot rule out that the changes we observe in the host following envenomation by KD wasps are not the result of effects of *crc* venom KD on other components of the venom. However, the similarity of banding patterns obtained from KD and control venom reservoir extracts on SDS-page gels does not support this interpretation. Furthermore, the finding that few genes are altered in transcription by *v-crc* KD supports a targeted function for this venom component. Finally, SDS-Page protein gels indicate a reduction in a protein of the expected size of calreticulin, and no qualitatively observable changes in other protein bands.

2.9. RNA isolation and preparation for RNA-seq

For the RNA-seq experiment *N. vitripennis* females were exposed to the anterior end of *S. bullata* pupae for a period of 4 h. After this stinging period the anterior end of the host puparium was removed and any *N. vitripennis* eggs were removed with a paintbrush. RNA was extracted from envenomated *S. bullata* pupae with a visually confirmed sting site. Pupae were manually extracted from the puparium and collected in TRIzol for RNA extraction as per manufacturer's protocol. Residual genomic DNA was removed by incubating the RNA at 37 °C with 1 µl of TURBO DNASE (Life Technologies, Grand Island NY) in 1× TURBO DNase buffer for 30 min. Before Illumina library preparation, the RNA concentration was assessed with the Agilent Bioanalyzer (Agilent, Santa Clara CA). All samples were determined to have an RNA Index of >9.0 prior to sequencing. The TruSeq RNA Sample Preparation Kit V2 (Illumina, San Diego CA) was used for next generation sequencing library construction per manufacturer's protocols. High throughput RNA sequencing was performed on an Illumina HiSeq2500 machine. The library preparation and sequencing were performed by the University of Rochester Functional Genomics Center according to standard Illumina protocols.

2.10. NGS data processing & RNA-seq data analysis

Raw 100 bp reads were de-multiplexed using configure bcl2fastq.pl version 1.8.3. Low complexity reads and vector contamination were removed using sequence cleaner

("seqclean") and the NCBI univec database, respectively. The FASTX toolkit (fastq_quality_trimmer) was used to remove bases with quality scores below $Q = 13$ from the end of each read.

Cleaned reads (Section 2.9) were aligned to the previously published *S. bullata* transcriptome (Martinson et al., 2014) using the Burrows-Wheeler Aligner and default parameters. Read counts were generated with HTSeq-count (version 0.6.1p1) and **differential expression (DE)** analysis was performed using DESeq2 (version 2.1.1) as described in the vignette (Anders et al., 2013; Love et al., 2014). For comparison, differential expression detection was also performed using the cufflinks package using default flags (Trapnell et al., 2012). Command line settings for all bioinformatics methods are provided in supplemental material (Supplemental Material, Bioinformatics Options). Comparisons are made between total venom and *v-crc* KD hosts (referred to as VK), *v-crc* KD and un-envenomated hosts (KU), and total venom and un-envenomated hosts (VU). For the VK comparison, we found a larger number of DE genes by CuffDiff than by DESeq2 ($n = 75$ vs. $n = 30$). We found that CuffDiff also identified all but 2 DE genes identified by DESeq2. For our analysis we focused on the set called by both CuffDiff ($p < 0.001$, $q < 0.01$) and DESeq2 (p -adjusted < 0.05) unless otherwise noted. Significant DE genes identified by DESeq2 did not include several genes with the largest log2 fold estimates produced by CuffDiff. Therefore, for our analysis we also included DE genes with CuffDiff estimated log2 fold-change above the largest estimate amongst genes identified by DESeq2 only. At 72 h we did not identify any DE genes by DESeq2. In order to detect any underlying patterns of gene expression changes at this late time point, we examined genes with log2 fold estimates that qualified for inclusion at the 24-h benchmark (see above).

Gene ontology enrichment analysis was conducted using DAVID (Dennis et al., 2003) and biological pathway enrichment analysis was conducted using KEGG (Kanehisa and Goto, 2000; Kanehisa et al., 2014).

2.11. Estimating contribution of calreticulin to total venom effect

We selected DESeq2 fold change estimates since they were the most conservative (i.e. fewer genes were called as significantly DE, especially those with low expression levels in both samples). Direction of log2 fold change estimates was the same for both methods (DESeq2 and CuffDiff), therefore the contribution phenotype categorization will be the same; however, by using DESeq2 we excluded genes that did not meet the more stringent DESeq2 criteria for significance. To calculate the relative contribution of venom calreticulin to the total venom effect on host gene expression for calreticulin-responsive genes, the DESeq2 log2 fold change difference is compared between un-envenomated (U) hosts and *v-crc* KD (K), as well as between U and total venom (V). VU = total venom relative to un-envenomated hosts; KU = KD venom relative to un-envenomated effects in the host. We then transformed log2 fold change to a linear scale and calculated the relative contribution of *v-crc* to the total venom phenotype. The reciprocal ($-1/\text{linear fold}$) was taken for fold change values < 1 .

$$\text{VenomEffectIndex} = \frac{|(V - U \text{ linear fold}\Delta) - (K - U \text{ linear fold}\Delta)|}{(V - U \text{ linear fold}\Delta)} * 100$$

This percentage provides an estimate of the relative contribution of venom calreticulin to the whole venom phenotype (Supplemental Figure D).

2.12. Quantitative PCR

A subset of differentially expressed (DE) genes identified by computational analysis in the VK comparison were confirmed with Quantitative PCR (**Q-PCR**) using Sybr Green PCR Master Mix (Life Technologies, Carlsbad CA) according to the manufacturer's protocol. The Q-PCR reaction was run on an Applied Biosystems 7300 Real-time PCR System in absolute quantification mode. Log 2-fold expression differences were calculated according to the Pfaffl method (Pfaffl, 2001) (Supplemental Table 2).

3. Results & discussion

The primary goal of this study is to determine whether RNAi KD of a venom component can be used to elucidate its possible function(s) in the host. We chose venom calreticulin due to previous work suggesting that it contributes to targeted cell death, immune modulation, and developmental arrest in the host (Rivers and Brogan, 2008; Rivers et al., 2005; Rivers et al., 1999; Rivers et al., 2006). Calreticulin protein sequence demonstrates a high degree of conservation between *N. vitripennis* and *S. bullata* (Supplemental material, Phylogeny) and it has been proposed that venom calreticulin may be able to interact with endogenous calreticulin targets in the host (Supplemental Figure A). To independently identify venom calreticulin function(s), we assessed host pupae envenomated with *v-crc* KD venom for changes in phenotype and gene expression, compared to total venom (V) envenomated controls. Comparisons are also made to un-envenomated hosts (U) to determine relative contribution to the total venom phenotype.

3.1. Calreticulin inhibits melanization response to venom but is not necessary for host developmental arrest

Previous studies attributed the developmental arrest phenotype of envenomated *S. bullata* pupae to venom calreticulin (Rivers and Brogan, 2008). In those studies, venom treatment with anti-calreticulin antibodies prior to injection failed to induce developmental arrest. The same investigation found no developmental effect of a synthetic calreticulin protein when injected into host pupae alone. Those results suggested that venom calreticulin might be necessary but not sufficient to cause developmental arrest. However, antibody treatment of venom could also have inactivated other venom components, leading to an over-estimation of function attributable to *v-crc*.

In contrast to these prior studies, we observed complete developmental arrest in all *S. bullata* pupae envenomated by *v-crc* KD females. Although there is no effect of calreticulin on the timing of visible developmental markers (e.g. eye pigment deposition, body bristle formation), we did observe a marked increase in melanization reaction in host pupae stung

by *v-crc* KD wasps (Fig. 1). In insects, melanization is caused by phenyloxidase activity of innate immune cells in response to foreign proteins, and is inhibited in envenomated fly hosts (Abt and Rivers, 2007). Calreticulin in the venom of several other parasitoid species has been shown to inhibit host encapsulation response (Wang et al., 2013, 2012; Zhang et al., 2006). Our findings suggest that calreticulin is also necessary to limit innate immune response to *Nasonia* venom in the *S. bullata* host pupae.

Next we investigated whether *v-crc* KD in *Nasonia* alters the developmental arrest phenotype of venom when manually injected into the host. Venom was isolated from *v-crc* KD (K) and total venom (V) control *N. vitripennis* females and injected into *S. bullata* pupae at three different protein concentrations (see Methods). As with normal stinging, *v-crc* KD venom caused developmental arrest of host pupae, and *v-crc* KD and control venom had indistinguishable effects on markers of developmental arrest (eye pigment deposition, body bristle formation, eclosion) (Table 1). We found a dose-response relationship between venom protein concentration injected and grading of developmental markers. Specifically, 0.1 µg of venom protein (~0.07 venom reservoirs) is sufficient to inhibit eclosion and 0.2 µg (~0.13 venom reservoirs) inhibits both eye pigment deposition and body bristle formation. These results demonstrate no decrease in the primary developmental arrest phenotype of venom when calreticulin is knocked down.

3.2. Categories of venom effects on host gene expression

Previous research has shown that total venom alters transcription of a well-defined set of host genes (Danneels et al., 2013; Martinson et al., 2014). This study took a novel approach to identify possibly functions of a venom component by examining the effect of *v-crc* KD in the wasp on host gene expression.

Here we present a model for the broad categories of effects that individual venom components can have on host gene expression. A key point is that many venom components will mitigate the effects of other venom components on host transcription and physiology. This can be favored when it is advantageous for the parasitoid to keep the host alive and physiologically stable for a period of time post envenomation, while altering host physiology in ways advantageous for the parasitoid young. It is likely that a major role of some venom proteins is to ameliorate the effect(s) of others. For example, if a particular venom component would induce host death prematurely, then other venom components could have a role in compensating for this “dysregulation”. In fact, we postulate that the role of many venom proteins in parasitoids (and other venomous animals) may be to mitigate the “downside” consequences of other venom proteins, or to modulate their effects.

Based on gene expression, we propose four broad categories listed below and illustrate them in Fig. 2. We are considering the knockdown of a target venom protein, and its effects on expression of a particular host gene. The following abbreviations are used: VU = expression difference of total venom relative to un-envenomated hosts; VK = expression difference of total venom relative to target gene KD effects on the host; KU = expression difference of KD venom relative to un-envenomated effects in the host.

3.2.1. Proportional contribution (PC)—When total venom induces a change in host gene expression, knockdown of the target venom can result in a partial to complete reduction in that effect. This indicates that the target venom protein contributes to the total venom expression phenotype. In this scenario $VU \sim KU$, but both demonstrate the same direction of effect (Fig. 2a): the smaller the KU the larger the contribution of the target venom to the total venom expression phenotype. If $KU \sim 0$, then the target venom is the primary contributor of venom effect on that gene. Later we show the estimated proportional contribution of *v-crc* to total venom effect on specific host genes.

3.2.2. Partial mitigation (PM)—The target venom can actually counteract effects of total venom on host gene expression, and we refer to this as “mitigation”. In terms of gene expression, mitigation is apparent when $KU > VU$, and both have the same direction of effect (Fig. 2b). What this means is that knockdown of the target venom actually increases the magnitude of expression difference relative to total venom. Therefore, the target venom gene must be reducing the magnitude of change induced by other components in the total venom. Partial mitigation occurs if the magnitude of expression deviation from un-venomated hosts is reduced, but not restored to normal levels.

3.2.3. Complete mitigation (CM)—A special case of mitigation occurs when total venom has no detectable effect on an expression phenotype of a host gene ($VU \sim 0$), but knockdown of a venom protein significantly changes its expression from the normal un-venomated state. In this case, total venom shows “normal” gene expression, but a mitigating effect of the venom protein is revealed in the KD. We refer to this as “Complete Mitigation” (CM). CM is revealed when a particular venom gene is knocked down, uncovering a network of compensatory interactions, which normally would result in no change in gene expression. In other words, the effect of total venom on host expression of the target gene is “cryptic” and only revealed when the venom protein that fully compensates for the effect is knocked down.

3.2.4. Overcompensation (OC)—In some cases, the knockdown can actually result in changes in gene expression of the opposite sign of total venom. Here KU and VU are both simultaneously significantly different than zero and in the opposite direction. We refer to this as overcompensation. A likely interpretation is that other venom components actually mitigate the effect of the target venom on the expression phenotype, reducing it in magnitude. The KD uncovers its effect on expression of the gene and reveals that other venom components must be mitigating its exaggerated effect on expression of the gene.

3.3. Venom calreticulin contribution to total venom effects on host transcription

Our subtractive vRNAi/eRNA-seq approach was designed to identify genes differentially expressed as a result of the removal of a venom gene component from whole venom. The relative contribution of calreticulin to host gene expression was estimated by comparing total venom (V) and *v-crc*KD (K) host gene expression to RNA-seq data previously generated from un-venomated (U) hosts (Martinson et al., 2014). In Fig. 3 we show gene expression differences between VU and KU. Those genes showing a suggestive difference in expression (expanded CuffDiff, $FDR < 0.10$) between Total venom and *v-crc* KD (VK) are

highlighted. The identity line (slope = 1, intercept = 0) represents zero contribution of calreticulin to global venom-induced changes in host gene transcription. At 4 h there is a significant deviation from this line (Pearson's correlation coefficient = 0.51, slope = 0.38, $p < 2.2e-16$), which indicates that venom calreticulin causes a general up-regulation of many genes at 4 h. Note that, when *v-crc* KD causes a reduction in host gene expression relative to total venom, this indicates that the *v-crc* venom component up-regulates that gene. Therefore, in Fig. 3 points to the left of the 1:1 line are down-regulated by venom calreticulin, and those to the right are up-regulated. A total of 79 genes are significantly up-regulated and 20 are significantly down regulated by *v-crc*. By 24 h this large deviation has vanished (Pearson's correlation coefficient = 0.96, slope = 0.87, $p < 2.2e-16$), and remains so at 72 h (Pearson's correlation coefficient = 0.93, slope = 0.83, $p < 2.2e-16$). Four-hour correlation coefficient is significantly smaller than those at 24 and 72 h (Fisher z transformation, $p < 0.01$) (Meng et al., 1992). These findings are consistent with our interpretation that venom calreticulin effects are early and transient.

Amongst the calreticulin-responsive DE gene set, DAVID (Dennis et al., 2003) analysis of functional annotation enrichment revealed significant enrichment for innate immune response (Benjamini = $4.9E-2$), chitin binding/metabolism (Benjamini = $3.2E-2$), and defense response to bacteria (Benjamini = $6.6E-2$); and specific localization to the extracellular region (Benjamini = $1.2E-2$).

3.4. Venom effect categories reflected in *v-crc* KD

We applied our categorization model to host genes identified as differentially expressed between envenomated and *v-crc* KD (expanded CuffDiff FDR < 0.10). The criteria for defining each of the categories are presented in Section 3.2. Basically, genes showing Proportional contribution (PC) will show reduced effect with *v-crc* KD and as compared to total venom. Partial mitigation (PM) is similar to PC, but with *v-crc* KD (K) shows a larger magnitude of effect than total venom (V). Complete mitigation (CM) is similar to PM, but with no significant effect of total venom (V) on expression of the gene. Overcompensation has significant effects of both venom and *v-crc* KD relative to un-envenomated (U), but they are of opposite sign.

Using these criteria we have defined the expression changes at 4 h (Fig. 4) and reveal genes in each of these major categories including 113 in PC, 173 OC, 13 CM, and 4 PM. The VU to VK plot clearly demarcates genes up-regulated by *v-crc* ($y > 0$) from those down-regulated by *v-crc* ($y < 0$). The large number of OC genes reflects homeostatic function of other venom components that are regulating *v-crc* effects. The second largest category include PC of genes for which calreticulin contributes to the total venom phenotype. PM and CM categories contain the fewest genes, which suggest *v-crc* provides relatively little compensation for side effects of other venom components.

At 24 h there is a similar distribution of genes per category, with fewer genes overall: 36 in PC, 18 OC, 6 CM, and 4 PM. No genes were identified as DE at 72 h using CuffDiff at FDR < 0.10 . However, when we include genes with the largest log 2 fold change (see methods Section 2.9) we uncovered a pattern of CNS-specific changes.

3.5. Closer examination of specific gene alterations

Host gene expression was assayed by RNA-seq at 4, 24, and 72 h post envenomation by either *v-crc* KD or total venom control female wasps. Here we consider in more detail specific genes that show statistically significant alterations in gene expression due to *v-crc* (Table 2) (Cuffdiff FDR < 0.01, DESeq FDR < 0.05).

Of the complete list of statistically significant DE genes, only a subset was annotated in the *S. bullata* transcriptome (n = 38), including 15 at 4 h, 14 at 24 h and 10 at 72 h (Table 2). The complete list of annotated and un-annotated DE genes is presented in Supplemental Table 1. This includes 23 at 4 h, 39 at 24 h and 12 at 72 h post-envenomation.

Amongst the annotated genes we found no significant ontology enrichment at 4 and 24 h, but we did so at 72 h. DAVID analysis (Dennis et al., 2003) revealed significant enrichment in ribosomal structure (Benjamini = $2.8E-3$) and mitotic spindle (Benjamini = $1.2E-1$) biological function and KEGG pathway analysis (Kanehisa and Goto, 2000; Kanehisa et al., 2014) indicated significant enrichment in Ribosomal protein pathway (Benjamini = $1.5E-4$).

To validate our computationally generated data, we confirmed DE by quantitative RT-PCR (qRT-PCR) for three genes (yellow-b, Sapecin, and Hairy B). All three genes were also detected as being differentially expressed by the qRT-PCR, although the magnitudes of change were smaller than those reported using RNA-seq (Supplemental Table 2). Next we analyzed in more detail the specific gene effects at each time point.

3.6. Early (4 h) immune response, clotting, and brain development genes

The greatest number of unique, annotated, DE genes was identified at 4 h (Table 2). This includes two genes implicated in immune response, which along with the observed melanization reaction suggests *v-crc* acts as a specific immune modulator immediately following envenomation. *V-crc* contributed proportionally to up-regulation of Yellow-b in envenomated *S. bullata*, accounting for 51% of the total venom effect (Table 2), as calculated by our formula for estimating contribution of calreticulin to total venom phenotype (see Methods). Several yellow family proteins have been identified as catalyzers of the dopachrome to 5,6-dihydroxyindole conversion in the melanization pathway in *Drosophila*. Although yellow-f and -f2 were identified as primarily responsible for this conversion during development (Han et al., 2002), other yellow family proteins including yellow-b may also alter the melanization pathway as observed in hosts stung with *v-crc* KD venom. No other yellow family genes were found to be DE in our data set. *Lethal (2) 34Fc*, an immune response to infection gene (Felix et al., 2012), experiences over-compensatory up-regulation by *v-crc* (Table 2). Venom calreticulin also proportionally up-regulates Sapecin B in envenomated hosts - an antimicrobial peptide active against both gram-negative and gram-positive bacteria (Yamada and Natori, 1994). Sapecin B-like venom protein identified in scorpion venom have been found to block calcium-activated potassium channels (Suzuki et al., 1997). Two chitin-binding proteins are up-regulated by *v-crc*. Both demonstrate over compensation of *v-crc* to the total venom effect on these genes (Table 2). Effect on the latter chitin-binding domain persists to 24 h. This suggests that *v-crc* is not only primarily responsible for up-regulation of these chitin-binding proteins, but also

mitigates the effects of other venoms that suppress their production *in vivo*. In insects these proteins are known to have anti-nutrient and anti-fungal properties (Raikhel et al., 1993). The latter function comes from synergy with secreted chitinases (Mehmood et al., 2015). Therefor one possible function of venom calreticulin may be to suppress fungal and bacterial growth within stung hosts. Together these findings suggest venom calreticulin may promote synthesis of bacterial response genes and antimicrobial peptides in envenomated hosts to prevent overgrowth of bacteria and fungi.

Other parasitoid species that lay their eggs inside the insect host (endoparasitoids) produce immune modulating venoms, which act by sequestering calcium and inhibiting immune cell activation and destruction of the parasitoid eggs (Mortimer et al., 2013). Although it acts as a calcium-binding protein, *v-crc* does not appear to play this role in envenomated fly hosts since it up-regulates several immune response genes. The venom of *Nasonia*, an ectoparasitoid (one that lays eggs on the surface of the host) experiences less stringent selective pressure to inhibit a cellular immune reaction. However, for both ecto- and endoparasitoids adult host feeding involves wounding of the host, and inhibition of some immediate immune responses, such as melanization and clotting would facilitate this process.

Calreticulin proportionally up-regulates two clotting factor genes (hemoclectin and fibrinogen, 64% and 46% respectively), and possibly prevents excess bleeding at the time of envenomation and wasp feeding. This finding is consistent with an increase in bleeding noted upon handling of *S. bullata* envenomated with *v-crc* KD venom (effect not quantified). The venom-responsive host gene Hemoclectin is specifically up-regulated by *v-crc*, accounting for 64% of the total venom effect (Table 2). Hemoclectin is a hemocyte specific protein identified in *Drosophila* with sequence similarity to clotting factors, including von Willebrand factor (VWF), coagulation factor V and VIII, as well as complement factors (Goto et al., 2001). Additionally, KD of hemoclectin in *Drosophila* causes bleeding defects (Goto et al., 2001) similar to those observed in hosts stung with *v-crc* KD venom. The clot-forming glycoprotein Fibrinogen is also up-regulated by *v-crc*, accounting for 46% of the total venom effect. Fibrinogen is a glycoprotein that is converted to fibrin and is essential to clot formation. Taken together, these findings suggest that venom calreticulin plays an important and early role in preventing excess bleeding in envenomated hosts at the time when adult and larval parasitoid feeding occurs. A possible mechanism may be via cell surface binding of calreticulin to B β chain of fibrinogen which has been shown to have mitogenic effects in human fibroblast cells (Gray et al., 1995).

Venom calreticulin also contributes proportionally to up-regulation of several neuronal genes during envenomation, perhaps contributing to targeted effects in the developing host brain. This includes Anoctamin, a calcium activated chlorine channel that has been implicated in augmenting neuronal excitability (Lee et al., 2014a) – at 4 h *v-crc* accounts for 46% of total venom effect on this gene (Table 2). Additionally, a sodium neurotransmitter symporter is affected by *v-crc* accounting for 48% of the total venom effect.

Notably, knockdown of the calreticulin-encoding gene is not sufficient to alter developmental arrest of the host, however changes in two excitatory ion channels,

Anoctamin and the sodium neurotransmitter symporter, imply calreticulin functions in early cell signaling within the nervous system. Alternatively, these may be secondary effects of the excess bleeding combined with a subsequently cellular dysregulation in the head region.

Venom calreticulin induces expression of two transcriptional regulatory genes – Ets at 21C and hairy B. Hairy acts as a developmental regulator and Ets at 21C exhibits a DNA binding domain. Their function in the envenomated host is not clear, but differential expression of these transcriptional regulators may partially explain later changes in venom-induced transcription.

3.7. Middle (24 h) brain/development associated stress-response genes

The majority of DE genes (annotated, and un-annotated) were identified at 24 h, including several heat shock proteins (Hsp). We identified 3 DE paralogs of these small stress proteins: one Hsp23 and two Hsp27-like. Expression of all three Hsp genes are proportionally up-regulated by *v-crc*, which contributes 33–53% of total venom effect (Table 2). Therefore, by 24 h *v-crc* mediates a stress response in the context of envenomation – either by directly stressing cells or by initiating this protective response. Hsp27 confers resistance to apoptosis induced by protease activation and protein kinase C inhibition (Mehlen et al., 1996). This could be significant, as a number of serine proteases are present in *N. vitripennis* venom. Hsp27 is expressed highly in fly neuronal tissue where it increases resistance to oxidative stress (Liao et al., 2008). It is essential for development and specifically acts as a molecular chaperone to refold denatured proteins generated under starvation conditions (Hao et al., 2007). Transcription of the other stress protein, Hsp23 is regulated during development by binding of the ecdysone-receptor complex to the promoter region of the Hsp23 gene (Mestrl et al., 1986). Hsp23 is also induced by heat shock through an independent regulatory mechanism. Both Hsp genes appear to confer stress-resistance to temperature, oxidative stress, and starvation in a neuronal/developmental specific manner. Finally, the m-alpha subunit of the Enhancer of Split Complex is completely mitigated by *v-crc*, meaning that knockdown uncovers its role in regulating this gene back toward normal expression levels in the absence of venom. In *Drosophila*, the gene is essential for targeting pro-neural cells for death as part of the reorganization process that takes place during brain development (Liu and Posakony, 2012). Together these changes indicate that by 24 h, presence of venom calreticulin supports a neuronal-specific oxidative stress response while maintaining expression of a neuronal development gene. These gene expression changes may be downstream effects of venoms now acting un-opposed by calreticulin, or it may result from widespread bleeding now unchecked by venom calreticulin.

Venom calreticulin up-regulates Rrt15p, a regulator of rDNA transcription proteins described in the yeast species *Saccharomyces cerevisiae*. *V-crc* again provides a complete mitigating effect on homeostatic regulating of this gene (Table 2). This effect precedes later regulation of several ribosomal protein genes (see 72 h genes).

At 24 h post-envenomation *v-crc* down-regulates several genes including two functionally un-annotated Osiris family genes (Shah et al., 2012), the actin cytoskeleton binding protein shavenoid (Ren et al., 2006), a protease inhibitor, and a carbonic anhydrase. The carbonic

anhydrase contributes >100% to the total venom phenotype, further supporting the hypothesis that calreticulin mitigates the effects of other venom proteins *in vivo*.

3.8. Late (72 h) notch associated genes

At this late time point, *v-crc* down-regulates genes involved in cell growth, division, Notch signaling, and neurogenesis. Although DESeq2 does not identify any DE genes at 72 h, eight of the nine strongly DE genes identified (>log2 fold DE) with CuffDiff (9 genes) (see Results Section 3.3) were all down-regulated by *v-crc* in the context of envenomation. The first is an Epidermal growth factor (EGF) that functions as the ligand for a trans-membrane glyco-protein receptor. EGF causes receptor-mediated protein-tyrosine kinase activation with downstream signaling mediated by intra-cellular calcium release, resulting in cell proliferation, differentiation, and survival (Roy and Jacobson, 2004). Venom calreticulin also down-regulates the expression of the downstream receptor activated protein kinase C1, which is activated by EGF, accounting for 75% of the venom effect. These results demonstrate significant targeting of the EGF-EGFR signaling cascade by *v-crc*. Given the timing of these observations, this is potentially a downstream effect of earlier *v-crc* action rather than a direct effect.

Venom calreticulin also down-regulates four ribosomal proteins (Rsp) including S6 isoform B, S3A isoform A, L7A isoform D, L6 isoform B – accounting for 51–218% of total venom effect (Table 2). These genes cluster significantly in ribosomal structure and mitotic spindle biological function gene ontology terms [Blast2GO, DAVID (Dennis et al., 2003)] and the Ribosomal protein pathway [KEGG (Kanehisa and Goto, 2000; Kanehisa et al., 2014)]. The greater than 100% contribution suggests that *v-crc* antagonizes the effect of other venom components *in vivo*, providing some homeostatic effect on protein synthesis. RspS6 demonstrates insulin-induced phosphorylation and is involved in anabolic response of the cell regulating cell cycle, growth and cell division (Radimerski et al., 2000). RspS3A is associated with the 40S ribosomal subunit and its expression is essential for oogenesis in Diptera (Reynaud et al., 1997). RspL7A comprises the 6A region of the gene Deltex (*dx*), which is involved in endosomal trafficking of Notch in a thermo-resistant manner (Shimizu et al., 2014; Wilkin et al., 2008). Suppressor of deltex antagonizes these effects *in vivo* (Cornell et al., 1999; Fostier et al., 1998; Mazaleyrat et al., 2003). Deltex interacts with key neurogenic genes including Notch, Delta, and mastermind (Busseau et al., 1994; Cornell et al., 1999; Xu and Artavanis-Tsakonas, 1990). RspL6 is thought to preferentially translate DDP1, a suppressor of heterochromatin-mediated gene silencing PEV (Batlle et al., 2011). Therefore, venom calreticulin may contribute to maintenance of heterochromatin gene silencing in envenomated hosts, while suppressing neurogenesis by maintaining Notch expression (i.e. suppressing E(spl), a negative regulator of transcription downstream of Notch). However, it is clear that these particular effects are not required for developmental arrest of the host, since hosts envenomated by *v-crc* KD wasps still show developmental arrest.

Venom calreticulin down-regulates an imaginal disc-derived wing morphogenesis gene, known to be involved in developmental timing in a temperature-independent manner in *Drosophila* (Carreira et al., 2013; Mensch et al., 2008), and which may play a role in wing

sexual dimorphism in Diptera (Carreira et al., 2011). Finally, a non-specific DNA/RNA endonuclease is also down-regulated by calreticulin.

Cuticular protein 30B is the only gene up-regulated by *v-crc* at the late 72-h point (Table 2). It may therefore play a role in stabilizing the host cuticle development post-envenomation. Our anecdotal observations indicate that hosts envenomated by *v-crc* KD *Nasonia* were more susceptible to cuticle rupture during handling. Therefore, a function of *v-crc* may be to induce toughening of the cuticle to reduce host bleeding during wasp larval feeding.

4. Conclusions

The vRNAi/eRNA-seq method is an effective means of investigating function of individual venom proteins. Using vRNAi/ eRNA-seq we also tested the hypothesis that wasp *v-crc* is required for developmental arrest of the host. Venom calreticulin is neither sufficient (Rivers and Brogan, 2008) nor necessary (see Results) for venom to cause developmental arrest in the fly host. We were unable to alter or even delay the developmental arrest phenotype by KD of *v-crc*, suggesting that other venom components serve this function *in vivo*.

This genome-wide approach is a useful exploratory tool for hypothesis generation. Using this method we uncovered several previously unknown *N. vitripennis* venom phenotypes attributable to *v-crc*. We were able to knockdown the *v-crc* gene and demonstrated a 90% decrease in *v-crc* protein abundance. Specific hypotheses generated about venom calreticulin are: (1) reducing bleeding during early parasitoid feeding and adult host feeding (2) targeted alteration of melanization response (yellow-b, melanization phenotype), (3) promoting synthesis of specific anti-bacterial (lethal 2 34Fc, Sapecin) and anti-fungal (chitin-binding) peptides (4) altering brain development/neurotransmission in the host, and (5) altering protein synthesis via the rDNA regulatory gene Rrt15p. In particular, we hypothesize that *v-crc* functions as an immediate modulator of immune response, which is supported by both phenotypic evidence (melanization) and altered expression of immune response genes. A likely function for this could be reducing immune response to venom proteins and reducing bleeding due to adult and larval parasitoid feeding. It is possible that the up-regulation of cuticular protein is causally associated with the apparent toughening of the cuticle in parasitized hosts, which was reduced in *v-crc* KD.

Venom calreticulin also appears to play a role in targeting the host brain. These data suggest that *v-crc* may target neurons as early as 4 h post-envenomation and play a role in selective maintenance of cell survival by suppression of apoptosis at 24 h, followed by inhibition of protein synthesis and maintenance of Notch signaling at 72 h. Notch signaling promotes neurogenesis but in *Drosophila*, is later antagonized by Numb to promote differentiation and brain development (Gulino et al., 2010; Kandachar and Roegiers, 2012). Venom calreticulin may act as a signal to maintain neurons in an immature state by suppressing the Notch degradation gene Deltex, thereby preventing brain development. Venom cytotoxicity has previously been proposed to be cell-type specific, and global (whole pupae) gene expression changes induced by whole venom support targeted effects on the host brain (Martinson et al., 2014). Nevertheless, tissue specific studies are needed to determine if these changes in gene expression are specific to the host brain.

What are the mechanisms by which venom alters gene expression? We can imagine four broad categories. First, venom peptides may directly target the regulation of specific sets of genes, for example by binding to chromatin or altering gene regulatory networks. Second, they may alter metabolic signaling pathways within cells, thus causing downstream changes in gene expression through feedback. Third, they may act through protein–protein interactions, which then have downstream consequences for gene expression due to changes in protein abundances or interactions that impact gene expression. Fourth, they may target specific cell types or tissues for loss or proliferation, thus causing changes in gene expression profiles specific to those cell types. The studies described here use whole body RNA-seq on host fly pupae. To better understand the mechanisms, future work can combine vRNAi KD with tissue specific eRNA-seq and cyto-histological analysis to identify more specifically the targets of venom components.

By comparing parasitized hosts exposed to KD vs. total venom (V) one is able to estimate the relative contribution of individual venom proteins to the venom phenotype. Furthermore, we have developed a categorization scheme by which venom effects can be grouped according to the role they play in the context of envenomation. In this way, homeostatic effects can be identified – a potentially important function of many venom proteins. This encompasses Partial and Complete Mitigation, and Over-compensatory venom effects, all of which reveal phenotypes otherwise obscured in the basic total venom (V) to un-envenomated (U) comparison. Therefore, there appears to be web of compensatory interactions altering gene expression in envenomated hosts. This is analogous to the “drug treadmill” where additional medications are added to offset the side effects of previous medications. This may in part explain the richness and diversity of parasitoid venoms, and those of other venomous animals.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig. 1. Venom calreticulin KD in *N. vitripennis* results in marked increase in melanization reaction in stung *S. bullata* pupae. Developmental markers scored over a period of 10 days in n = 10 pupae per treatment.

Using RNAi KD to reveal categories of venom effects

Fig. 2. The pattern of gene expression in total venom (V) exposed, knockdown venom (K), and un-venomated (U) hosts are shown. Illustration depicts an increase in gene expression; reductions in gene expression would simply flip the image.

Fig. 3.

Log2 fold change between venom (x-axis: total venom, y-axis: calreticulin KD venom) and un-venomated controls at estimated by DESeq at (A) 4 h, (B) 24 h, and (C) 72 h. Identity line represents 0% contribution of calreticulin to venom phenotype. DE genes identified likely DE by CuffDiff comparison (FDR < 0.10, more inclusive) of total venom (LacZ) to calreticulin KD highlighted in black. Dotted line is line of best fit.

Fig. 4.

4-hours post-venomation *v-crc* responsive host genes cluster into distinct categories of venom effect: open circles, PC = proportional contribution; filled circles, OC = overcompensation; triangles, CM = complete mitigation; open diamonds, PM = partial mitigation. Genes with $y > 0$ are up-regulated by *v-crc* and those with $y < 0$ are down-regulated by *v-crc*. Note: the majority of PC effects are on genes up-regulated by *v-crc*, and the majority of PM changes are on genes down-regulated *v-crc*.

Table 1

Development of pharate adult hosts (*Sarcophaga bullata*) is impaired following manual injection of three doses of *v-crc* KD and control (LacZ KD) venom extracted from adult *N. vitripennis* females (n = 10 per group).

	ug protein	Melanization	Eye darkening	Bristles	Eclosed
Venom	0.2	+++	0	0	0
	0.1	++	0	0	0
	0.05	+	5	1	1
<i>crc</i> KD	0.2	+++	0	0	0
	0.1	+++	1	1	0
	0.05	+++	6	2	1
LacZ KD	0.2	+	1	0	0
	0.1	++	4	0	0
	0.05	++	8	6	2
Control	-		10	10	10
Pricked	+		10	10	10
Vehicle	+		10	10	10
Stung	+		0	0	0

Table 2

Significantly differentially expressed (DE) genes identified computationally from RNA-seq data (Cuffdiff FDR < 0.01, DESeq FDR < 0.05). Greyed out genes identified only by CuffDiff. Log2 fold change estimates listed for DE genes as calculated by each method. Venom Effect Index represents relative contribution of venom calreticulin to total venom phenotype (see Methods Section 2.1). VK = total venom relative to target gene KD effects on the host; VU = total venom relative to un-envenomed hosts; KU = KD venom relative to un-envenomed effects in the host. Categories of venom effect: PC = proportional contribution, OC = overcompensation, CM = complete mitigation, PM = partial mitigation.

Significant <i>v-crc</i> responsive host genes									
VK									
Function	Annotation	4 Hours			Log2 Fold			Log2 Fold	
		DESeq	CuffDiff	Venom Effect Index	Total venom	<i>v-crc</i> KD	Category of venom effect	VU	KU
Innate Immunity	yellow-b	1.30	2.06	50.69	2.22*	1.20*	PC		
	lethal (2) 34Fc [Drosophila melanogaster]	0.43	2.48	201.25	0.12	-0.14	OC		
Anti-fungal/ cuticle	Chitin-binding protein; CG11570	0.53	2.22	217.80	0.15	-0.39	OC		
	Chitin-binding domain; CG3348	0.42	2.23	201.18	0.12	-0.13	OC		
Anti-bacterial	Sapacin; CG14052	1.16	2.37	24.21	0.44	0.04	PC		
Transcription	Ets at 21C, isoform A	1.19	1.59	75.34	3.65*	1.63*	PC		
	hairy, isoform B	1.15	1.75	47.67	1.30*	0.37	PC		
Clotting factor	hemolectin	1.18	1.94	64.38	2.60*	1.11	PC		
	Fibrinogen; CG10359, isoform E	1.14	1.74	45.91	1.46*	0.57	PC		
Neuronal	Na+ neurotransmitter symporter; CG13794	1.06	1.47	47.88	2.01*	1.07*	PC		
	Anoctamin; CG10353, isoform D	0.99	1.27	46.43	0.97*	0.07	PC		
Development	Haemolymph juvenile hormone binding protein; CG16820	0.82	2.25	182.45	0.46	-0.19	OC		
	CG42807	1.12	1.41	69.55	3.24	1.52*	PC		
Unknown	Hypothetical protein [Mus musculus]	-0.33	-2.40	188.78	-0.27	0.09	OC		
	CG13081	-0.55	-3.78	9.12	-0.16	-0.02	PC		
Function	Annotation	24 Hours			Log2 Fold			Log2 Fold	
		DESeq	CuffDiff	Venom Effect Index	Total venom	<i>v-crc</i> KD	Category of venom effect	VU	KU

Significant *v-crc* responsive host genes

Function		Annotation	4 Hours		VK		VU	KU
			DESeq	CuffDiff				
			Log2 Fold	Log2 Fold	Venom Effect Index	Total venom	v-crc KD	Category of venom effect
Anti-fungal/cuticle		Chitin-binding domain; CG3348	0.18	1.50	33.46	-0.08	-0.50	CM
Stress-response		heat shock protein 27	0.80	1.09	51.37	2.50*	1.46*	PC
		heat shock protein 27	0.74	1.17	52.70	3.28*	2.20*	PC
		heat shock protein 23	0.51	0.59	32.94	1.06*	0.48	PC
Development		E(spl) region transcript ml	0.54	2.08	255.32	0.01	-0.65	CM
Ribosomal		Rrt15p	0.38	1.37	119.91	-0.07	-1.21*	CM
Structural		shavenoid, isoform A	-0.57	-1.34	29.31	-0.90	-0.40	PC
Metabolic		Carbonic anhydrase; CG5379	-0.15	-1.47	128.68	0.43	1.62*	PM
		Proteinase inhibitor; CG8560	-0.62	-1.35	28.81	-1.15	-0.66	PC
Unknown		CG10570	0.78	1.01	46.78	1.85*	0.94*	PC
		osiris 11	-0.35	-1.81	13.54	-0.81	-0.60	PC
		CG14292	-0.43	-1.73	226.50	-0.38	0.72	OC
		osiris 8	-0.43	-1.30	12.45	-0.78	-0.59	PC
		CG14395, isoform C	-0.72	-1.27	27.37	-0.97	-0.51	PC
72 Hours								
		Annotation	DESeq	CuffDiff				
			Log2 Fold	Log2 Fold	Venom Effect Index	Total venom	v-crc KD	
Ribosomal		ribosomal protein S3A, isoform A	-0.42	-2.83	50.69	0.48	1.82	PM
		ribosomal protein L7A, isoform D	-0.34	-3.07	201.25	0.01	1.45	CM
		ribosomal protein L6, isoform B	-0.39	-3.53	217.80	-0.47	1.50	OC
		ribosomal protein S6, isoform B	-0.35	-3.82	201.18	0.96	1.94	PM
EGF-EGFR		Epidermal growth factor; CG42255	-0.60	-1.54	24.21	-0.55	0.75	OC
		receptor of activated protein kinase C1	-0.32	-2.88	75.34	-0.54	1.08	OC

Significant *v-crc* responsive host genes

Function	Annotation	VK				Total venom	Log2 Fold <i>v-crc</i> KD	Category of venom effect
		4 Hours	DESeq Log2 Fold	CuffDiff Log2 Fold	Venom Effect Index			
Development	Imaginal disc-derived wing morphogenesis; CG11382		-0.19	-1.64	47.67	-1.18	-0.20	PC
Transcription	DNA/RNA non-specific endonuclease; CG14062		-0.58	-2.04	64.38	-0.69	0.86	OC
Cuticle	cuticular protein 30B		0.34	2.22	45.91	-1.26	-3.05	PM