The venom gland transcriptome of the parasitoid wasp *Nasonia vitripennis* highlights the importance of novel genes in venom function

Andre D. Sim and David Wheeler*

Abstract

Background: Prior to egg laying the parasitoid wasp *Nasonia vitripennis* envenomates its pupal host with a complex mixture of venom peptides. This venom induces several dramatic changes in the host, including developmental arrest, immunosuppression, and altered metabolism. The diverse and potent bioactivity of *N. vitripennis* venom provides opportunities for the development of novel acting pharmaceuticals based on these molecules. However, currently very little is known about the specific functions of individual venom peptides or what mechanisms underlie the hosts response to envenomation. Many of the venom peptides also lack bioinformatically derived annotations because no homologs can be identified in the sequences databases. The RNA interference system of *N. vitripennis* provides a method for functional characterisation of venom protein encoding genes, however working with the current list of 79 candidates represents a daunting task. For this reason we were interested in determining the expression levels of venom encoding genes in the venom gland, as this information could be used to rank candidates for further study. To do this we carried out deep transcriptome sequencing of the venom gland and ovary tissue and used RNA-seq to rank the venom protein encoding genes by expression level. The generation of a specific venom gland transcriptome dataset also provides further opportunities to investigate novel features of this specialised organ.

Results: RNA-seq revealed that the highest expressed venom encoding gene in the venom gland was 'Venom protein Y'. The highest expressed annotated gene in this tissue was serine protease *Nasvi2EG007167*, which has previously been implicated in the apoptotic activity of *N. vitripennis* venom. As expected the RNA-seq confirmed that venom encoding genes are almost exclusively expressed in the venom gland relative to the neighbouring ovary tissue. Novel genes appear to perform key roles in *N. vitripennis* venom function, with over half of the 15 highest expressed venom encoding loci lacking bioinformatic annotations. The high throughput sequencing data also provided evidence for the existence of an additional 472 previously undescribed transcribed regions in the *N. vitripennis* genome. Finally, metatranscriptomic analysis of the venom gland transcriptome finds little evidence for the role of *Wolbachia* in the venom system.

Conclusions: The expression level information provided here for the *N. vitripennis* venom protein encoding genes represents a valuable dataset that can be used by the research community to rank candidates for further functional characterisation. These candidates represent bioactive peptides valuable in the development of new pharmaceuticals.

Keywords: Ovary, RNA-seq, Transcriptomics, Serine protease, Drug development, Venom gene
Background

Nasonia vitripennis (Hymenoptera: Pteromalidae) (here after Nasonia) is a parasitoid wasp that utilises species of cyclorrhaphous diptera, such as house flies and blow flies, as hosts to support the growth and development of their offspring. Envenomation of the host increases the overall nutritional value of the food source available to the feeding wasp larvae. Some of these venom induced changes include developmental arrest, immune suppression and changes to the overall metabolite profile of the host [1–3]. The diverse and potent bioactivities of Nasonia venom peptides indicate that they may be useful in the development of novel pharmaceuticals [1, 4–7]. The venom peptides themselves are synthesised in the venom gland (VG) before being secreted into the venom reservoir ready for injection into the host via the ovipositor [8]. A recent proteomic study on these venom reservoir extracts was able to identify 79 peptides [4, 9]. Although several of the peptides had similarity to sequences found in the venom of other animals, a majority had either not been associated with venom before or had no homology to sequences in the National Centre for Bioinformatic Information (NCBI) databases. Bioinformatic based annotation categories that could be applied to the venom peptides included immunity, metabolism, esterases, proteases, and recognition proteins [9]. The most prevalent functional annotation was serine proteases, which is one of the largest gene families found in insect genomes [4, 10].

The response to Nasonia envenomation by the common laboratory host Sarcophaga bullata (Diptera: Sarcophagidae) has been extensively studied at both the molecular and physiological level. Experiments show that the respiration rate of S. bullata remains stable for up to 2 weeks following envenomation (although at lower levels than unstung hosts) indicating that the venom is not immediately lethal to this host [11]. Other physiological changes to the host associated with envenomation include an increase in lipids and free amino acids, as well as the initiation of developmental arrest through an as yet unknown mechanism. S. bullata immune processes are also affected, with the abundance and potency of circulating plasmatocytes declining dramatically (due to cell death) 60 min following envenomation by Nasonia [12]. Importantly, Nasonia venom has been shown to be able to interfere with mammalian immunity pathways. For example, experiments using mouse cell lines demonstrated that Nasonia venom has both antimicrobial and anti-inflammatory properties [6]. Reporter assays provided evidence that the observed immune responses were mediated through venom specific modifications to the steroid and Toll pathways [6]. The activity of Nasonia venom in mammalian systems may indicate that venom peptides have evolved to target highly conserved core pathway components in order to limit the ability of the host to escape envenomation. In this situation evolution has already done much of the work in designing highly specialised peptides that target cellular pathways involved in diseases such as hypertension, diabetes, and cancer. Thus given the incredible species richness of the parasitoid wasps their venoms may represent a diverse untapped reservoir of molecules ready for incorporation into drug development pipelines.

In contrast to the extensive body of work exploring the host response to envenomation, relatively little is known about the individual venom peptides responsible for inducing these changes. Based on sequence homology a Nasonia venom metalloproteinase has been proposed to initiate developmental arrest by interfering with Notch signalling [1, 7, 13]. Supporting this proposal is the observation that several notch pathway genes are differentially expressed in the S. bullata hosts following envenomation. However, currently there is no direct evidence showing that this venom metalloprotease is specifically responsible for the observed expression changes in the Notch pathway [1]. Several other venom peptides are thought to be involved in modifying host metabolic pathways based on homology to sequences from model systems. For example, a venom lipase could be responsible for the increase in phospholipid degradation observed in envenomated hosts [2, 14]. A venom trehalase has been proposed to convert the abundant host trehalose sugars into glucose [2]. Additionally, several proteases found in Nasonia venom are homologous to peptides used by the ectoparasitoid Euplectrus separatae and the tick Haemaphysalis longicornis for blood meal digestion [15, 16].

The most direct functional characterisation of a venom encoding gene comes from a recent RNA interference (RNAi) study by Siebert et al. (2015). Knockdown of Nasonia venom calreticulin (Nasvi2EG037342) resulted in an increase in melanisation occurring at the oviposition wound on the host following envenomation [5]. This observation suggested that venom calreticulin functions to interfere with normal wound healing in the host, perhaps by competing with the endogenous calreticulin in the host for binding of cofactors [17].

Several endoparasitoids from the Braconidae and Ichneumonidae wasp families inject viruses or virus like particles along with their venom in order to prevent the host immune system from attacking eggs deposited in the haemolymph. The virus like particles appear to provide protection to the developing parasitoid by induction apoptosis in host hemocytes [18–22]. As yet viral-like particles have not been implicated in Nasonia venom function and viral transcripts could not be identified in cDNA libraries synthesised from envenomated hosts [23]. The intracellular symbiont Wolbachia is also present in high concentrations in the ovaries and host envenomation has been proposed as a possible mechanism for horizontal transfer of strains between parasitoid species [24].
Currently there is little direct experimental evidence supporting the proposed functions of the 79 *Nasonia* venom proteins and many of the bioinformatic annotations are based on homology to sequences from non-venomous model organisms. The RNAi knockdown system of *Nasonia* clearly provides an opportunity to begin to functionally characterise the venom protein encoding genes [5]. However, given the number of genes involved, an important first step would be the identification of high value candidates. For this reason we carried out deep sequencing of the *Nasonia* VG and ovary in order to use the relative gene expression as a proxy for protein abundance in the venom reservoir. The RNA-seq information generated in this study also provides a valuable dataset for future studies into the reproductive system of this important developing model system.

**Results**

**RNA-seq analysis of the *Nasonia* VG and ovary transcriptomes**

We used RNA-seq to measure the expression of venom protein encoding genes in the VG and ovary. We chose the ovary as the comparative tissue because although it is connected to the venom apparatus, it clearly performs a distinct function in *Nasonia* reproduction. High throughput sequencing of VG and ovary RNA-seq libraries (three biological replicates each tissue) generated a total of 92.7 million and 97.9 million of high quality reads (phred quality score >20), respectively. On average 90.7 % of the sequenced reads successfully mapped to the published *Nasonia* genome [4] (Table 1). The lower mapping percentages observed for VG sample three ("VG 3" in Table 1) was due to a higher proportion of rRNA reads in this sample. We predict that this resulted from less effective rRNA removal during library preparation for this replicate. Apart from the higher proportion of *Nasonia* rRNA reads other quality control parameters for this replicate were consistent with those obtained from the other samples (Fig. 1a and Table 1).

Overlap between the mapped reads and annotated features in the *Nasonia* official gene set version 2 (OGS2) is high, with an average of 81 % of mapped reads aligning to at least one gene body (Table 1). Of the 36,329 annotated genes in OGS2, 54 % (19,854) had detectable expression in either the VG or ovary. Based on normalised read counts, tissue specific expression (reads for a gene model detected in only one tissue) was observed for 3475 genes in the ovary and 1424 genes in the VG. Next we utilised the R-package DESeq to identify differentially expressed genes between the VG and ovary RNA-seq datasets [25]. Significantly different expression was observed for 5794 (16 % of OGS2 loci) genes at an adjusted p-value threshold of 0.05 (Benjamini-Hochberg adjusted for multiple testing) (Figs. 1b and c; Additional file 1). Of the differentially expressed genes, 2524 were up and 3270 were down regulated in the VG relative to the ovary. Overall the fold changes of upregulated genes were significantly larger in the VG versus the ovary (Student's t-test p-value < 0.001) (Fig. 1b).

Using Blast2GO we then looked for GO term enrichment amongst the genes either significantly upregulated in the ovary or VG tissues. The reproductive function of the ovary is characterised by several GO terms related to cell division and transcription (Fig. 2a). In contrast, genes significantly upregulated in the VG showed GO enrichment in terms related to translation and protein processing functions (Fig. 2b). The relatively narrow GO functional categorisations of genes upregulated in the VG are likely to reflect that the major model systems are non-venomous.

**The highly expressed venom protein encoding genes**

Previous proteomic studies on *Nasonia* venom reservoir extracts identified 79 venom peptides. Based on our VG RNA-seq data we were able to detect expression from all but one of these venom protein encoding genes (Table 2). *Nasonia* venom genes were significantly more likely to be expressed at higher levels in the VG relative to the ovary (70 of 79 genes; Chi-square p-value <0.0001 with Yates correction). The DESeq analysis showed that six of the venom protein encoding genes were expressed at equal levels in the VG and ovary (Table 2). Two venom

---

**Table 1** RNA-seq statistics of *Nasonia vitripennis* ovary and VG tissue

| Sample | Total Reads | Mapped (%) | Assigned (%)<sup>a</sup> | Multiple Alignments (%)<sup>b</sup> | No Feature (%)<sup>c</sup> |
|--------|-------------|------------|--------------------------|--------------------------------|--------------------------|
| VG 1   | 2.89E + 07  | 95.62      | 88.08                    | 3.63                           | 1.99                     |
| VG 2   | 3.24E + 07  | 96.15      | 87.31                    | 3.37                           | 1.97                     |
| VG 3   | 3.14E + 07  | 67.03      | 59.65                    | 3.54                           | 4.32                     |
| Ovary 1| 3.31E + 07  | 95.17      | 85.51                    | 1.43                           | 5.95                     |
| Ovary 2| 3.42E + 07  | 95.16      | 84.06                    | 1.46                           | 6.34                     |
| Ovary 3| 3.06E + 07  | 95.16      | 84.18                    | 1.43                           | 6.13                     |

<sup>a</sup>Proportion of reads which could be aligned to a gene model

<sup>b</sup>Proportion of reads assigned to multiple gene models

<sup>c</sup>Proportion of aligned reads that did not overlap a gene model
protein encoding genes expressed at higher levels in the ovary than the VG were a aspartylglucosaminidase (Nasvi2EG015708) and trehalase (Nasvi2EG008324). The highest expressed venom encoding gene based on normalised venom gland read counts was the novel gene Venom protein Y (Nasvi2EG013868). A trypsin-like serine protease (Nasvi2EG007167) was the highest expressed annotated gene in the VG (Table 3). The RNA-seq data and semi-quantitative PCR showed that expression of Nasvi2EG007167 was highly tissue specific (Fig. 1d). Only five of the top 15 highest expressed venom protein encoding genes had functional annotations, highlighting the unique functional properties of Nasonia venom (Tables 2 and 3).

Next we used sequence homology based clustering to identify any relationships amongst the highly expressed unannotated genes. Using this approach only two small clusters of unannotated venom encoding genes were identified (V/Z and L/K), suggesting that most of these novel loci have evolved independently, rather than through an expansion of a single gene cluster (Additional file 2). Calreticulin (Nasvi2EG037342) was the highest ovary expressed venom encoding gene, which is not surprising given it also functions as a core immune response gene [26–28]. The single venom encoding gene that was not expressed in our venom dataset, a trypsin-1 serine protease (Nasvi2EG011442), also had very little expression in the ovary (Fig. 3 and Table 2).

**RNA-seq identifies 472 novel genes**

The generation of the official gene set for *Nasonia* did not include evidential sequencing data specifically obtained from VG samples, so we predicted that genes expressed in this relatively small organ might be under-represented in the current gene models. Therefore, we used the Cufflinks software package to identify novel genes based on mapped reads that did not overlap with any current annotations in OGS2 [29] (Additional file 3). This approach identified 472 putative genes from both VG and ovary datasets (Additional file 4). We merged these new gene models into the official gene set and repeated the DESeq differential expression analysis (Additional file 5). This showed that 140 (30 %) of the novel genes were significantly upregulated and 56 (12 %) were significantly down regulated in the VG tissue; the remaining 276 genes (58 %) were not differentially expressed between the tissues (Fig. 4). The highest expressed novel genes in both the VG and ovary were shown to be highly tissue specific. The most differentially expressed novel VG gene (based on fold change) XLOC_013208 was also predicted to have an alternate splice form that truncated exon 3
Unfortunately, the putative proteins encoded by XLOC_013208 splice forms have no homology to any domains in the database so we are not able to predict possible functional consequences of this splicing event. As a result of low homology to sequences in the NCBI database only ten of the 472 novel genes identified here could be annotated with gene ontology terms using Blast2GO (Table 4). Extending the Cufflinks splice form prediction methodology to the currently annotated venom protein encoding genes failed to identify any significant alternative splice forms between the VG and ovary transcriptomes.

Expression of venom protein encoding genes previously identified as high value candidates

Using our data we were then able to examine the expression pattern of genes previously identified as high value venom candidates based on bioinformatic annotations or results from studies in other venomous species [7, 9]. To differentiate the genes based broadly on their expression level we placed the 79 venom protein encoding genes into high, medium or low expression categories based on three equal divisions of normalised counts across our entire dataset (see methods). These categories were defined by normalised read count ranges of <181700, 181701-1218700, >1218701 for low, medium and high, respectively.

The highly expressed category was dominated by novel venom genes (Venom proteins G, Q, X, Y, and Z), with the single annotated loci being the trypsin-like serine protease Nasvi2EG007167 (Table 2). Serine proteases play broad functional roles across insect physiology, including apoptosis, immunity, and development [30, 31]. The medium expression category includes the gamma-glutamyltranspeptidase and endonuclease-like venom genes, which have been previously implicated in initiating venom induced apoptosis by interfering with normal metabolism of glutathione and by degrading nuclear DNA, respectively [7, 32, 33]. Similarly, the aminotransferase-
Table 2  Summary of VG and ovary RNA-seq data for the 79 *Nasonia* venom protein encoding genes

| Gene ID                | Ovary read counts | VG read counts | Log2 Fold Change | Adj p-val | Classification                  |
|------------------------|-------------------|----------------|------------------|-----------|----------------------------------|
| Nasvi2EG013868         | 141               | 4600307        | 15.00            | 2.33E-127 | Venom protein Y                  |
| Nasvi2EG009648         | 76                | 2859048        | 15.20            | 7.49E-129 | Venom protein G                  |
| Nasvi2EG009662         | 93                | 2701601        | 14.82            | 7.75E-126 | Venom protein Z                  |
| Nasvi2EG009647         | 90                | 2310849        | 14.65            | 2.5E-124  | Venom protein Q                  |
| Nasvi2EG014072         | 75                | 2063715        | 14.75            | 4.41E-85  | Venom protein X                  |
| Nasvi2EG007167         | 43                | 1532232        | 15.11            | 7.49E-129 | Serine protease                  |
| Nasvi2EG003930         | 76                | 1218607        | 14.61            | 5.88E-123 | Serine protease inhibitor 2      |
| Nasvi2EG014069         | 26                | 976928         | 15.20            | 1.2E-106  | Venom protein L                  |
| Nasvi2EG006243         | 25                | 819734         | 15.03            | 5.88E-111 | γ-glutamyltranspeptidase         |
| Nasvi2EG012348         | 23                | 756942         | 15.04            | 1.14E-125 | Venom protein H                  |
| Nasvi2EG009035         | 22                | 666528         | 14.92            | 5.13E-127 | Endonuclease-like                |
| Nasvi2EG036525         | 18                | 660159         | 15.17            | 4.8E-129  | Aminotransferase-like            |
| Nasvi2EG008596         | 25                | 595426         | 14.56            | 7.8E-121  | Venom protein P                  |
| Nasvi2EG009661         | 21                | 590933         | 14.80            | 1.37E-124 | Venom protein V                  |
| Nasvi2EG002524         | 11                | 386873         | 15.11            | 1.45E-74  | Venom protein I                  |
| Nasvi2EG001168         | 68                | 385586         | 12.47            | 3.96E-92  | Immunoglobulin-like              |
| Nasvi2EG014575         | 10                | 312136         | 14.94            | 1.22E-99  | Venom protein K                  |
| Nasvi2EG010245         | 10                | 257730         | 14.72            | 4.26E-84  | Odorant-binding protein          |
| Nasvi2EG019091         | 48                | 245164         | 12.31            | 4.4E-13   | Venom protein M                  |
| Nasvi2EG010351         | 7                 | 197286         | 14.79            | 1.09E-124 | Glucose dehydrogenase-like       |
| Nasvi2EG004342         | 6                 | 181642         | 14.97            | 9.19E-125 | Lipase-like                      |
| Nasvi2EG016543         | 12                | 155729         | 13.63            | 3.31E-110 | Venom protein N                  |
| Nasvi2EG007615         | 4                 | 153498         | 15.08            | 1.03E-125 | Lipase                           |
| Nasvi2EG021024         | 737               | 149042         | 7.66             | 3.33E-50  | Serine protease homologue        |
| Nasvi2EG009991         | 4                 | 145328         | 15.06            | 3.41E-127 | Acid phosphatase                |
| Nasvi2EG020297         | 387               | 141654         | 8.52             | 6.11E-59  | Serine protease/CLIP             |
| Nasvi2EG022918         | 4                 | 130887         | 14.97            | 7.1E-125  | Serine protease                 |
| Nasvi2EG008779         | 4                 | 115742         | 14.82            | 1.21E-124 | Aminotransferase-like            |
| Nasvi2EG000667         | 5                 | 104214         | 14.48            | 1.04E-119 | Serine proteinase inhibitor      |
| Nasvi2EG004628         | 8                 | 98159          | 13.59            | 4.58E-65  | Venom protein D                  |
| Nasvi2EG000354         | 7                 | 91416          | 13.72            | 2.06E-19  | Cysteine-rich/TIL 1              |
| Nasvi2EG016421         | 91                | 90433          | 9.96             | 9.19E-65  | α-Esterase                      |
| Nasvi2EG004144         | 5                 | 81771          | 14.02            | 6.13E-19  | Venom protein T                  |
| Nasvi2EG037101         | 6                 | 77272          | 13.57            | 2.54E-48  | Chitin binding protein-like      |
| Nasvi2EG019611         | 2                 | 75339          | 15.19            | 5.42E-125 | Laccase                          |
| Nasvi2EG020586         | 2                 | 66726          | 15.06            | 9.42E-120 | Serine protease                 |
| Nasvi2EG013736         | 54                | 64975          | 10.24            | 2.62E-45  | Serine protease inhibitor 1      |
| Nasvi2EG020997         | 1                 | 63251          | 15.64            | 7.83E-121 | Venom protein J                  |
| Nasvi2EG026553         | 4                 | 46698          | 13.56            | 3.89E-109 | Lipoprotein receptor-like        |
| Nasvi2EG020296         | 716               | 42847          | 5.90             | 1.43E-32  | Serine protease/CLIP             |
| Nasvi2EG006920         | 3                 | 42299          | 13.91            | 9.42E-113 | Aroylsphatase b                  |
| Nasvi2EG002112         | 224               | 38349          | 7.42             | 1.67E-47  | β-1,3-Glucan recognition         |
| Nasvi2EG022916         | 2                 | 38281          | 14.41            | 4.45E-79  | Serine protease                 |
| Nasvi2EG000112         | 18                | 34599          | 10.87            | 3.97E-83  | Apyrase                          |
like venom gene encodes an enzyme predicted to produce kynurenic acid from kynurenine and this may also be involved in apoptosis [34, 35]. Other medium expression category genes of note include IMPL-L2, enzymes involved in carbohydrate metabolism, and several serine proteases/proteinase inhibitor genes. Insulin binding protein (Nasvi2EG001168) is possibly involved in initiating the developmental arrest by blocking insulin signalling [7, 36].

The majority of venom protein encoding genes fit into the low expressed category based on our designations. Notable venom protein encoding genes in the low expression group include apyrase, beta-1-3-Glucan recognition protein, dipeptidyl peptidase, metalloprotease, trehalase, chitinase, and all five of the venom cysteine-rich/Pacifastin protease inhibitors (Table 2). The first three of these aforementioned genes have been proposed

| Gene ID          | Library Size (reads) | Transcript Length (kb) | Fold Change | Function Description                                      |
|------------------|----------------------|------------------------|-------------|----------------------------------------------------------|
| Nasvi2EG022914   | 1                    | 33717                  | 14.50       | Serine protease                                          |
| Nasvi2EG037342   | 39.26                | 33363                  | 3.09        | CaFreticulin                                              |
| Nasvi2EG004152   | 18                   | 26602                  | 10.57       | Venon protein R                                          |
| Nasvi2EG020295   | 1                    | 24624                  | 14.74       | Serine protease/CLIP                                     |
| Nasvi2EG005749   | 1                    | 23005                  | 14.93       | Serine protease                                          |
| Nasvi2EG013838   | 711                  | 22562                  | 4.99        | Venon protein F                                          |
| Nasvi2EG011463   | 1                    | 20535                  | 14.43       | Inositol phosphatase                                     |
| Nasvi2EG004824   | 3                    | 19029                  | 12.49       | Venon proteine E                                         |
| Nasvi2EG006071   | 1                    | 18923                  | 14.14       | Venon protein S                                          |
| Nasvi2EG023753   | 103                  | 12408                  | 6.91        | Dipeptidylessipectidase IV                              |
| Nasvi2EG013885   | 1                    | 9901                   | 13.00       | γ-Glutamyl transpeptidase 2                              |
| Nasvi2EG005790   | 1                    | 8481                   | 13.24       | Antigen-S like                                           |
| Nasvi2EG006543   | 30                   | 7665                   | 8.00        | γ-Glutamyl cyclotransferase                              |
| Nasvi2EG016379   | 0                    | 7408                   | 15.24       | Venon protein U                                          |
| Nasvi2EG005645   | 0                    | 5919                   | 13.75       | Venon protein D                                          |
| Nasvi2EG007282   | 390                  | 4909                   | 3.65        | Serine protease                                          |
| Nasvi2EG000351   | 84                   | 3807                   | 5.51        | Cysteine-rich/TIL 2                                      |
| Nasvi2EG008007   | 5                    | 1805                   | 8.46        | Angiotensin-converting                                   |
| Nasvi2EG022626   | 326                  | 532                    | 0.71        | Serine protease/CUB                                      |
| Nasvi2EG009433   | 47                   | 426                    | 3.17        | Cysteine-rich/KU                                         |
| Nasvi2EG000909   | 93                   | 147                    | 0.66        | C1q-like venom protein                                   |
| Nasvi2EG009664   | 1                    | 110                    | 6.65        | Cysteine-rich/Pacifastin 1                               |
| Nasvi2EG009665   | 4                    | 93                     | 4.62        | Cysteine-rich/Pacifastin 2                               |
| Nasvi2EG012510   | 5                    | 83                     | 4.17        | Chitinase 5                                              |
| Nasvi2EG012285   | 9                    | 77                     | 3.15        | Acid phosphatase                                         |
| Nasvi2EG015708   | 929                  | 68                     | −3.76       | Aspartylglycosaminidase                                  |
| Nasvi2EG008324   | 200                  | 62                     | −1.70       | Trehalase                                                |
| Nasvi2EG007347   | 18                   | 41                     | 1.19        | Serine protease                                          |
| Nasvi2EG005784   | 1                    | 21                     | 4.63        | Antigen S-like protein                                   |
| Nasvi2EG010516   | 3                    | 20                     | 2.88        | Metalloprotease                                          |
| Nasvi2EG007166   | 0                    | 16                     | N/A         | Serine protease                                          |
| Nasvi2EG015589   | 1                    | 8                      | 3.37        | Laccase                                                  |
| Nasvi2EG021414   | 0                    | 3                      | 4.23        | Nucleoside hydrolase                                     |
| Nasvi2EG011314   | 0                    | 1                      | N/A         | Venon protein W                                          |
| Nasvi2EG011442   | 1                    | 0                      | N/A         | Serine protease                                          |

Table 2 Summary of VG and ovary RNA-seq data for the 79 Nasonia venom protein encoding genes (Continued)

Expression category, see methods for details
Library size normalised read counts from DESeq divided by transcript length (in kb) and sorted in descending order based on the venom gland values
Fold changes calculated directly from library size normalised read counts by DESeq
to be involved in modifying the host immune response [7, 37, 38]. The metallopeptase has been suggested to be involved in venom induced developmental arrest by interfering with normal Notch signalling [1]. The trehalase gene important for metabolising carbohydrates is also one of the few such genes expressed at higher levels in the ovary in our dataset. As noted by [7] the observation of proteins normally associated with breakdown of the chitin rich cuticle is somewhat puzzling given Naso-

| Gene ID          | Ovary normalised counts | VG normalised counts | Log2 Fold Change | Adj p-val | Classification                      |
|------------------|-------------------------|----------------------|------------------|----------|-------------------------------------|
| Nasvi2EG013868²  | 141                     | 4600307              | 15.00            | 2.33E-127| Venom protein Y                     |
| Nasvi2EG009648²  | 76                      | 2859048              | 15.20            | 7.49E-129| Venom protein G                     |
| Nasvi2EG009662²  | 93                      | 2701601              | 14.82            | 7.75E-126| Venom protein Z                     |
| Nasvi2EG009647²  | 90                      | 2310849              | 14.65            | 2.50E-124| Venom protein Q                     |
| Nasvi2EG014072²  | 75                      | 2063715              | 14.75            | 4.41E-85 | Venom protein X                     |
| Nasvi2EG021198   | 62                      | 1557446              | 14.61            | 6.16E-55 | Unknown                             |
| Nasvi2EG007167²  | 43                      | 1532232              | 15.11            | 7.49E-129| Serine Protease                    |
| Nasvi2EG003930²  | 49                      | 1218607              | 14.61            | 5.88E-123| Serine protease inhibitor 2         |
| Nasvi2EG009363²  | 54                      | 1217247              | 14.45            | 5.14E-121| Unknown                             |
| Nasvi2EG014069²  | 26                      | 976927               | 15.20            | 1.20E-106| Venom protein L                     |
| Nasvi2EG006243²  | 25                      | 819734               | 15.03            | 5.88E-111| γ-glutamyltranspeptidase            |
| Nasvi2EG012348²  | 23                      | 756942               | 15.04            | 1.14E-125| Venom protein H                     |
| Nasvi2EG011080²  | 20                      | 722740               | 15.15            | 3.50E-126| Unknown                             |
| Nasvi2EG009035²  | 22                      | 666528               | 14.92            | 5.13E-127| Endonuclease-like                   |
| Nasvi2EG036525²  | 18                      | 660159               | 15.17            | 4.80E-129| Aminotransferase-like 2             |

| Gene ID          | Ovary normalised counts | VG normalised counts | Log2 Fold Change | Adj p-val | Classification                      |
|------------------|-------------------------|----------------------|------------------|----------|-------------------------------------|
| Nasvi2EG004175²  | 35984                   | 37                   | −9.92            | 4.96E-65 | Unknown                             |
| Nasvi2EG002830²  | 34980                   | 10187                | −1.78            | 7.40E-05 | HEAT shock 70                       |
| Nasvi2EG000341²  | 29283                   | 27085                | −0.11            | 1        | CCHC-type zinc finger               |
| Nasvi2EG007353²  | 29093                   | 708.41               | −5.36            | 2.33E-32 | ATP-dependent RNA helicase          |
| Nasvi2EG002390²  | 25623                   | 73530                | 1.52             | 2.81E-3  | 40S ribosomal protein               |
| Nasvi2EG006904²  | 25600                   | 45210                | 0.82             | 2.70E-1  | Elongation factor 1-alpha           |
| Nasvi2EG011214²  | 25321                   | 70701                | 1.48             | 2.69E-2  | 40S ribosomal protein               |
| Nasvi2EG020214²  | 25158                   | 139912               | 2.48             | 1.38E-4  | 60S acidic ribosomal protein        |
| Nasvi2EG004598²  | 25153                   | 72614                | 1.53             | 2.70E-3  | 60S ribosomal protein               |
| Nasvi2EG007380²  | 23250                   | 117959               | 2.34             | 1.28E-3  | 60S acidic ribosomal protein        |
| Nasvi2EG019818²  | 23062                   | 85908                | 1.90             | 1.68E-4  | 40S ribosomal protein               |
| Nasvi2EG003839²  | 22711                   | 103851               | 2.20             | 1.39E-05 | 60S ribosomal protein               |
| Nasvi2EG007413²  | 22602                   | 92995                | 4.11             | 5.36E-05 | 40S ribosomal protein               |
| Nasvi2EG011575²  | 21836                   | 57655                | 2.64             | 2.21E-2  | 60S ribosomal protein               |
| Nasvi2EG014261²  | 21805                   | 219749               | 10.08            | 3.29E-11 | Unknown                             |

¹Indicates venom protein encoding gene
²Library size normalised read counts from DESeq, divided by transcript length (in kb) and sorted in descending order based on the venom gland values
³Fold changes calculated from library size normalised read counts by DESeq

Metatranscriptomic analysis of Nasonia VG and ovary tissue
Finally, although the library preparation method included an mRNA enrichment step, we were interested in looking for any evidence for the involvement of bacteria (such as Wolbachia) or viruses in Nasonia venom function. Therefore, we performed a metatranscriptomic analysis of reads that did not align to the Nasonia genome.
At the phylum level reads from both tissues could be assigned to viruses (42.5 %), Nematoda (17 %), Chordata (18 %) and Proteobacteria (5.5 %). A large proportion of the unmapped reads were assigned to viruses, with most of these being identified as an uncharacterised ‘Nasonia vitripennis virus’ (Taxonomic id #626355) (Fig. 6). The nematode reads were assigned to Brugia malayi and Trichuris trichiura, thus we initially suspected that this result may represent hits to the Wolbachia symbionts that are associated with both of these species [44]. However, subsequent megablast searches against the NCBI nr/nt database with these reads revealed both the Nematoda and Chordata groupings were based on low diversity rRNA sequences that were taxonomically uninformative.

As expected a large proportion (87 %) of the Proteobacteria reads from the ovary were assigned to Wolbachia species (Fig. 7). In contrast relatively few reads could be assigned to Wolbachia species in the Proteobacteria grouping from the VG. Clostridium cellulosi and Streptococcus anginosus were the most common species assignments in the VG, however, relative to the abundance of Wolbachia assigned reads in the ovary, very few reads could be assigned at this narrow taxonomic level in the VG data (Fig. 7). Thus based on this data there is little evidence supporting a role for microorganisms in Nasonia venom function.

Discussion

The expression levels of venom protein encoding genes obtained in this study provides additional information that can be used to select candidates for further functional analysis using either RNAi or recombinant techniques. The ovary RNA-seq also provides a proxy for the tissue specificity of the venom encoding gene expression that can be used to further refine candidate selection. It should be noted that high venom gene expression does
not automatically imply that the encoded peptide is responsible for important envenomation phenotypes. Other biological features, such as translational regulators, post-translational modifications and mRNA/protein stability could also be critical to the function of gene products in the venom context. Indeed, it has been argued that as a result of extensive posttranslational modifications, final venom protein composition is largely independent of transcriptional control [45, 46].

The legitimacy of this view has been challenged by a more recent study showing strong correlations between the venom transcriptomes and proteomes of several snake species [47]. Ultimately, additional high quality quantitative proteomic and transcriptomic data will be required to more fully appreciate the general importance of posttranslational mechanisms on venom composition. Finally, it is also worth considering that highly expressed venom genes may perform accessory functions, such as protecting the venom reservoir of the wasp from being attacked by its own venom, as has been observed in snakes [48].

The highest expressed annotated venom encoding gene was a trypsin-like serine protease. Unfortunately, serine proteases are a very common functional category in insect genomes making it difficult to propose a role for this highly expressed gene (especially in the venom...
context). Previous studies using Sf21 cells have shown that the apoptotic activity of Nasonia venom could be reduced by the addition of serine protease inhibitors [49]. Interestingly, amongst the ten most highly expressed venom protein encoding genes with annotations, all three point to roles in apoptosis [7, 32, 33]. This may highlight the importance of apoptosis for the early envenomation effects, perhaps by assisting with the distribution of venom throughout the host or by suppressing the immune system by destroying circulating hemocytes [49]. Nasonia venom has also been shown to induce apoptosis in S. bullata neural tissue and this has been suggested as a possible explanation for developmental arrest in envenomated this host [50].

Perhaps the most dramatic finding from this study was that over half of the top 15 expressed venom protein encoding genes have no functional annotation, including the highest expressed gene ‘Venom protein Y’. In retrospect maybe this should not be surprising given the complex evolutionary processes that drive specialisation of venom peptides [51]. However, this observation does highlight the value of candidate selection methods (such as RNA-seq) that are not biased towards genes with existing annotation information. Even for those genes

| Gene ID       | Classification                                      | GO terms                                                                                                                                 |
|---------------|-----------------------------------------------------|------------------------------------------------------------------------------------------------------------------------------------------|
| XLOC_005019   | Atp-dependent dna helicase q4                        | metal ion binding, helicase activity, heterocyclic compound binding, organic cyclic compound binding                                    |
| XLOC_010355   | Signal peptidase complex catalytic subunit sec11a   | signal peptide processing, integral to membrane, proteolysis, serine-type peptidase activity                                           |
| XLOC_012101   | N-acetylneuraminic lyase-like                       | lyase activity, metabolic process                                                                                                       |
| XLOC_013961   | NADH dehydrogenase subunit 2                        | mitochondrial electron transport, NADH to ubiquinone, mitochondrial inner membrane, respiratory chain, NADH dehydrogenase (ubiquinone) activity, integral to membrane |
| XLOC_015645   | NADH dehydrogenase subunit partial                  | integral to membrane, mitochondrion, oxidation-reduction process, NADH dehydrogenase (ubiquinone) activity                               |
| XLOC_016115   | Uncharacterized aarf domain-containing protein kinase 1 | protein phosphorylation, oxidation-reduction process, protein kinase activity, ubiquinone biosynthetic process, ATP binding, flavin adenine dinucleotide binding oxidoreductase activity, acting on paired donors, with incorporation or reduction of molecular oxygen, NAD(P)H as one donor, and incorporation of one atom of oxygen |
| XLOC_016465   | 60s ribosomal protein                               | ribosome, structural constituent of ribosome, translation                                                                            |
| XLOC_019886   | 28 s ribosomal protein mitochondrial                | ribosome, structural constituent of ribosome, translation                                                                            |
| XLOC_020689   | Signal peptidase complex subunit 3                 | peptidase activity, signal peptide processing, integral to membrane, signal peptidase complex                                          |
| XLOC_022279   | Inosine-5'-monophosphate dehydrogenase 1b          | oxidation-reduction process, adenyl nucleotide binding, metal ion binding, GMP biosynthetic process, cytoplasm, IMP dehydrogenase activity |

**Table 4** Gene annotations of novel detected transcripts from Nasonia vitripennis VG and Ovary samples

**Fig. 6** Differences in metatranscriptomic composition of the VG and ovary. Distribution of reads that did not map to the Nasonia genome assigned to the phylum level from the VG a and ovary b RNA-seq data by the program Megan
that are bioinformatically annotated, it should be recognised that most of the functional data comes from experiments in model systems that are non-venomous.

Another interesting finding from this study is that several venom peptides that have been previously implicated in venom function were shown to be only lowly expressed in our data [1, 7, 9]. Perhaps the best example is the metalloprotease (Nasvi2EG010516) predicted to be involved in initiating developmental arrest, was one of the lowest expressed of the venom protein encoding genes in our dataset [1, 52]. We also observed that all five cysteine rich protease inhibitors suggested to be involved in disrupting host immunity by inactivating the pro-phenol oxidase cascade were also expressed at low levels [7]. The low expression of chitinase-type venom genes also suggests they may not be directly involved in venom function, especially given Nasonia larvae are able to mechanically disrupt the host integument during feeding.

Cufflink based transcript assembly of the VG and ovary RNA-seq data allowed us to identify 472 previously undescribed transcribed regions in the Nasonia genome. The low expression and lack of homology to other known genes might explain why they have not been previously described. Alternatively these observations may indicate
they represent artefacts of the cufflinks methodology. Re-evaluation of the raw venom proteomic data may enable the identification of additional peptide fragments based on these new gene models. It is worth noting that the OGS2 models were developed based on RNA-seq data from the Wolbachia free Asymcx strain of Nasonia. As some of the new gene models identified here could be induced by Wolbachia infection they represent candidates for being involved in the cytoplasmic incompatibility phenotypes induced by this protobacteria.

Finally, the metatranscriptomic study revealed that viruses represented a large proportion of non-Nasonia reads in the VG and ovary transcripts. Particularly, Nasonia vitripennis virus, which has a positive strand ssRNA genome and is part of the Picornavirales order and the Iflaviridae family but remains unassigned to lower taxonomic ranks [23]. Nasonia vitripennis virus has a ssRNA genome so reads may be derived from either messenger RNA or the ssRNA genome. The latter, opens up the possibility of performing a de novo assembly of the viral genome from our transcriptome data. Studies have shown that this virus causes no observed detrimental effect on Nasonia and it remains an open question as to whether these transcripts end up in the venom itself [23]. As expected the ovary metatranscriptome was dominated by Wolbachia transcripts. In contrast, we identified relatively few Wolbachia sequences in the VG, suggesting this bacteria is unlikely to play an important role in venom function. However, it is important to note that the RNA-seq library preparation methods used in our study enriched for poly A+ reads and thus many bacterial sequences would not have been sequenced in this experiment. Follow up experiments using total RNA library preparation methods are required to conclusively rule out a possible role for Wolbachia in Nasonia venom function.

Animal venoms represent an important source of natural products with pharmaceutical value; as demonstrated by the development of important drugs, such as Exenatide from the Gila monster lizard, and Captopril isolated from the venom of the lancehead viper [53]. More recently molecules isolated from reptile and arthropod venom have been shown to have therapeutic value in the treatment of cancer [54, 55]. With this in mind the venom of parasitoid hymenoptera holds particular promise, given the incredible species richness of this group of animals, a potentially vast and unique source of molecules awaits discovery. We also predict that venoms target highly conserved core cellular pathways, as a mechanism to limit the ability of the host to evolve detoxification strategies. Indeed, Nasonia venom has been shown to have bioactivity against mammalian immune pathways, further highlighting the pharmaceutical value of these molecules [6]. The results reported here point to an important role for novel genes in venom function, and this information should assist with the selection of candidates in future functional studies.

Conclusions
In this study we used RNA-seq to generate a comprehensive dataset of expression information for VG and ovary in the developing model system Nasonia. Information on the expression level of the 79 venom protein encoding genes provides an unbiased approach for selecting RNAi candidates, especially as bioinformatic annotations are likely to be unreliable. As our knowledge of venom gene function increases our ability to understand the fundamental molecular mechanisms underlying envenomation will also be improved. This latter knowledge will be important in future efforts to use Nasonia venom in drug development pipelines.

Methods
Nasonia and tissue collection
The Nasonia (LABI strain) was a gift from Prof. Jack Werren, University of Rochester, USA. The Nasonia were hosted on Lucilia sericata pupae obtained from a commercial insectary (Biosupplies Ltd). Venom gland (VG) and ovary tissue from mated, host exposed, 1–3 day old females, was dissected under aseptic conditions and placed immediately into ice cold Trizol (Ambion). Each replicate contained VG or ovaries tissue pooled from between 40 and 50 individuals.

RNA-seq and functional annotation
RNA was extracted using the modified RNeasy (Qiagen) protocol. Briefly, tissue was initially homogenised in 1 ml of Trizol before the addition of 0.2 ml chloroform (Merck). The sample was centrifuged at 10,000 rpm for 10 min at room temperature and the supernatant was transferred to a Qiagen RNeasy column. The column was centrifuged at 10,000 rpm for 15 s before the bound RNA was washed twice with 0.5 ml Qiagen RPE buffer. The RNA was eluted in 30 ul of RNase free water as described in the RNeasy protocol. The quality of the purified RNA was verified using a Bioanalyzer (Agilent) with all samples having a RNA integrity score >7. Poly-T beads were used to enrich for mRNA and TruSeq (Illumina) stranded cDNA sequencing libraries were constructed by the Otago Genome Service (NZGL). The 100 bp paired end sequencing run was performed on the Illumina HiSeq2500 platform.

The raw RNA-seq data was processed with fastq-mcf to remove sequencing adapters and primers [56]. The reads were quality trimmed to a Phred score of >20 using SolexaQA v3.1.3 [57]. The paired-end reads were then mapped to the N. vitripennis genome [4] using TopHat2 (version 2.1) with the default settings, except for library type set as “fr-firststrand” based on the sequencing
chemistry used during the library preparation [58]. Read counts for gene feature in the *N. vitripennis* official gene set version 2 were generated using HTSeq-count (version 0.6.1p) with ‘union mode’ on exon features and incorporating strand information [59].

Differential expression between the ovary and VG read counts was determined using DESeq as described in the package vignette [25, 60]. A gene was considered differentially expressed if it had an adjusted *p*-value < 0.05. Principal component analysis was performed using R with the pcomp function. The heatmaps were generated using heatmap.2 (ggplots CRAN package) and clustered based on euclidean distance. A one-tailed enrichment analysis for enrichment of functional categories was carried out with Blast2GO using both the newly annotated venom and ovary expressed genes as the target and the entire gene set as the reference [61]. Solely for the purposes of ranking genes based on expression level, we divided the DESeq normalised read counts by the transcript length (in kilobases) to compensate for the effects of gene length on counts. These length normalised counts are shown in Table 2 and Table 3 and also provided in Additional files 1, 2, 3 and 4. The expression level boundaries of high, medium, and lowly expressed venom genes were determined by binning all genes into windows of expression of 100. A bin was classified as low expressed if the value of the bin was multiplied by its number of containing genes and this value was less than a third of the sum of these values, high if equal or greater than two thirds, and medium elsewhere.

The Cufflinks pipeline was used to identify putative alternative transcripts as well as unannotated genes based on comparisons to the current *Nasonia* gene models as described in the package documentation [29]. Novel transcripts were annotated using Blast2GO v2.8 with the default parameters [61]. Normalised read counts were generated for the new gene models by merging the cufflinks models with those of OGS version 2 and repeating the DESeq based analysis described above.

**Metatranscriptomics**

Reads that did not align with the *Nasonia* genome using Tophat2 were used in a Diamond BlastX search against the NCBI non-redundant (nr) database. Results presented do not include data from unmapped reads that were discarded by Tophat2 during the generation of non-redundant splice junctions. The high number of unmapped reads in the third VG sample necessitated that only a random sample of 10 % of the reads from this particular sample were used due to memory limitations. Megan5 was used for visualisation of the taxonomic distribution of reads in the data [42].

**Semi-quantitative PCR**

Complementary DNA was generated from 1 ug of RNA extracted from dissected ovary and VG tissue using the transcriptor first strand cDNA synthesis kit (Roche) as described by the manufacturer. The PCR was then performed using primers for the housekeeping gene *RP49*: 5'-CTTCGGCAAGTCTCCTTTCC-3', 5'-TTATTCTCATTCCTCAGAACG-3'. The primers specific for *Nasi*- vi2EG007167 were: 5'-TGGCTGTCATCAGATTGACG-3', 5'-TATCCCGAGGACGATGTAG-3'. Reaction tubes were removed at 24 and 30 cycles, at a denaturing temperature of 94 °C for 30 s, annealing temperature of 55 °C for 30 s, and extension temperature of 72 °C for 45 s with one unit of *taq* polymerase (Roche).

**Additional files**

| Additional file 1: | DESeq results from the VG and Ovary RNA-seq data for all *Nasonia* 2.0 gene models. (XLSX 3172 kb) |
| Additional file 2: | Sequence based clustering of venom encoding genes using BLASTClust. (DOCX 18 kb) |
| Additional file 3: | Novel venom gland and ovary transcribed regions predicted by cufflinks. (TXT 406 kb) |
| Additional file 4: | Genomic locations of 472 new gene predictions based on the ovary and VG RNA-seq data. (XLSX 50 kb) |
| Additional file 5: | DESeq based differential expression data for all *Nasonia* 2.0 gene models merged with the new gene models identified in this study. (XLSX 3636 kb) |

**Acknowledgements**

We would like to thank Dr Jack Werren (University of Rochester) and the Werren lab for the gift of the *Nasonia* LabII strain. We also wish to thank Drs Patrick Biggs and Matthew Campbell for discussions and comments that improved the draft manuscript. We are grateful for the valuable feedback received from two anonymous reviewers. We also wish to thank Dr Helen Fitzsimons, Dr Natisha Magan, and James Connell for vital assistance with the import of *Nasonia* into New Zealand.

**Funding**

This work was supported by Massey Honours Scholarship to AS, Massey University start-up and MURF grants (RM17937) to DW.

**Availability of data and materials**

The raw RNA-seq reads are available at the SRA under accession: SRP067692. The RNA-seq data supporting the results of this article is available in the GEO repository under accession number GSE76257.

**Authors’ contributions**

AS performed the bioinformatics and the molecular biology and helped in the preparation of this manuscript. DW conceived of the study, assisted with the bioinformatic analysis, and wrote the manuscript. Both authors read and approved the final manuscript.

**Competing interests**

The authors declare that they have no competing interests.

**Consent for publication**

Not applicable.

**Ethics approval and consent to participate**

Not applicable.
Animal Ethics

This invertebrates research was exempt from animal ethics requirements by section 2 of the New Zealand Animal Welfare Act 1999.

Received: 26 January 2016 Accepted: 7 July 2016

References

1. Martinson EO, Wheeler D, Wright J, Minalini, Siebert AL, Werren JH. Nasonia vitripennis venom causes targeted gene expression changes in its fly host. Mol Ecol. 2014;23:918–30.
2. Minalini, Siebert AL, Wright J, Martinson E, Wheeler D, Werren JH. Parasitoid venom induces metabolic cascades in fly hosts. Metabolomics. 2014;11:350–66.
3. Rivers DB, Denlinger DL. Venom-induced alterations in Fly lipid metabolism and its impact on larval development of the ectoparasitoid nasonia vitripennis (Walker) (Hymenoptera: Pteromalidae). J Invertebr Pathol. 1995;66:104–10.
4. Werren JH, Richards S, Desjardins CA, Niehuis O, Gadou J, Colbourne JK, et al. Functional and evolutionary insights from the genomes of three parasitoid Nasonia species. Science. 2010;332:343–8.
5. Aisha LS, Wheeler D, Werren JH. Combined parasitoid wasp RNA interference knockdown / fly host RNA sequencing to test and generate hypotheses about the function of venom calreticulin. Toxicon. 2015;100:304–16.
6. Danneels EL, Gerlo S, Heyninck K, Van Craenenbroeck K, De Bosscher K, Haegeman G, et al. How the venom from the ectoparasitoid Wasp nasonia vitripennis exhibits anti-inflammatory properties on mammalian cell lines. PLoS One. 2014;9:e86225.
7. Danneels EL, Rivers DB, De Graaf DC. Venom proteins of the parasitoid wasp Nasonia vitripennis: recent discovery of an untapped pharmacopee. Toxins. 2012;4:94–516.
8. Ratcliffe NA, King PE. The "venom" system of Nasonia vitripennis (Walker) (Hymenoptera: Pteromalidae). Proceedings of the Royal Entomological Society of London Series A, General Entomology Blackwell Publishing Ltd. 1967;42:49–61.
9. De Graaf DC, Aerts M, Brumain M, Desjardins CA, Jacobs FJ, Werren JH, et al. Insights into the venom composition of the ectoparasitoid Nasonia vitripennis from bioinformatic and proteomic studies. Insect Mol Biol. 2010;19:11–26.
10. Ross J, Jiang H, Kanost MR, Wang Y. Serine proteases and their homologs in the Drosophila melanogaster genome: an initial analysis of sequence conservation and phylogenetic relationships. Gene. 2003;304:117–31.
11. Rivers DB, Denlinger DL. Redirection of metabolism in the flesh fly, Sarcophaga bullata, following envenomation by the ectoparasitoid Nasonia vitripennis and correlation of metabolic effects with the diapause status of the host. J Insect Physiol. 1994;40:207–15.
12. Rivers DB, Ruggiero L, Hayes M. The ectoparasitoid Wasp nasonia vitripennis (Walker) (Hymenoptera: Pteromalidae) differentially affects cells mediating the immune response of its flesh fly host, Sarcophaga bullata Parker (Diptera: Sarcophagidae). Ecol Entomol. 2007:35:453–65.
13. Price DRG, Bell HA, Hinchliffe G, Fitches E, Weaver R, Gatehouse JA. A venom metalloproteinase from the parasitic wasp Eulophus pennicornis is toxic towards its host, tomato moth (Lacanobia oleracea). Insect Mol Biol. 2009;18:195–202.
14. Rivers DB, Rocco MM, Frayha AR. Venom from the ectoparasitoid Wasp Nasonia vitripennis increases Na+ influx and activates phospholipase C and phosphatidylserine A 2 dependent signal transduction pathways in cultured insect cells. Toxicol. 2002;40:9–21.
15. Miyoshi T, Tsuji N, Islam KM, Kamio T, Fujisaki K. Enzymatic characterization of a cubulin-related serine proteinase from the hard tick Haemaphysalis longicornis. J Vet Med Sci. 2004;66:1195–8.
16. Nakamatsu Y, Tanaka T. The function of a trypsin-like enzyme in the saliva of Euplectus separates larvae. J Insect Physiol. 2004;50:847–54.
17. Abt M, Rivers DB. Characterization of phenoloxidase activity in venom from the ectoparasitoid Nasonia vitripennis (Walker) (Hymenoptera: Pteromalidae). J Invertebr Pathol. 2007;94:108–18.
18. Hayakawa Y, Yazeri K. Envelope protein of parasitic wasp symbiotic virus, polydnavirus, protects the wasp eggs from cellular immune reactions by the host insect. Eur J Biochem. 1997;246:820–6.
19. Huw Davies D, Strand MR, Vinson SB. Changes in differential haemocyte count and in vitro behaviour of plasmatocytes from host Heliothis virescens caused by Campbell's sionoensy polydnavirus. J Insect Physiol. 1987;33:143–53.
20. Labrosse C, Carton Y, Dubuffet A, Dreuze JM, Poire M. Active suppression of D. melanogaster immune response by long gland products of the parasitic wasp Leptopilina boulardi. J. Insect Physiol. 2003;49:513–22.
44. Sironi M, Bandi C, Sacchi L, Di Sacco B, Damiani G, Genchi C. Molecular evidence for a close relative of the arthropod endosymbiont Wolbachia in a filarial worm. Mol Biochem Parasitol. 1995;74:223–7.

45. Casewell NR, Wagstaff SC, Wüster W, Cook DAN, Bolton FMS, King SI, Davinia P, Sanz L, Calvete JJ, Harrison RA. Medically important differences in snake venom composition are dictated by distinct postgenomic mechanisms. Proc Natl Acad Sci U S A. 2014;111:9205–10.

46. Durban J, Pérez A, Sanz L, Gómez A, Bonilla F, Rodríguez S, Chacón D, Sasa M, Angulo Y, Gutérrrez JM, Calvete JJ. Integrated "omics" profiling indicates that miRNAs are modulators of the ontogenetic venom composition shift in the Central American rattlesnake, Crotalus simus simus. BMC Genomics. 2013;14:294.

47. Rokyta DR, Marges MJ, Calvin K. Post-transcriptional Mechanisms Contribute Little to Phenotypic Variation in Snake Venoms. G3. 2015;5:2375–82.

48. Fox JW. A brief review of the scientific history of several lesser-known snake venom proteins: l-amino acid oxidases, hyaluronidases and phosphodiesterases. Toxicon. 2013;62:75–82.

49. Formesyn EM, Heyninck K, de Graaf DC. The role of serine- and metallotripeptidases in Nasonia vitripennis venom in cell death related processes towards a Spodoptera frugiperda Sf21 cell line. J Insect Physiol. 2013;59:795–803.

50. Rivers DB, Keefer DA, Ergin E, Uçkan F. Morphology and Ultrastructure of Brain Tissue and Fat Body from the Flesh Fly, Sarcophaga bullata Parker (Diptera: Sarcophagidae), Envenomated by the Ectoparasitic Wasp Nasonia vitripennis (Walker) (Hymenoptera: Pteromalidae). Psyche 2011; Article ID 520875.

51. Kordis D, Gubensek F. Adaptive evolution of animal toxin multigene families. Gene. 2000;261:43–52.

52. Jia LG, Shimokawa K, Bjarnason JB, Fox JW. Snake venom metalloproteinases: structure, function and relationship to the ADAMs family of proteins. Toxicon. 1996;34:1269–76.

53. Vetter I, Davis JL, Rash LD, Anangi R, Mobli M, Aelwood PF, Lewis RL, King GF. Venomics: a new paradigm for natural products-based drug discovery. Amino Acids. 2011;40(1):15–28.

54. Heinen TE, da Veiga ABG. Arthropod venoms and cancer. Toxicon. 2011;57(4):497–511.

55. Zambelli VO, Pasqualoto KFM, Picolo G, Chudzinski-Tavassi AM, Cury Y. Harnessing the knowledge of animal toxins to generate drugs. Pharmacol Res. 2016. doi:10.1016/j.phram.2016.01.009.

56. Aronesty E. Comparison of Sequencing Utility Programs. Open Biotechnol J. 2013;7:1–8.

57. Cox MP, Peterson DA, Biggs PJ, SolexaQA: At-a-glance quality assessment of Illumina second-generation sequencing data. BMC Bioinformatics. 2010;11:485.

58. Kim D, Perteza G, Trapnell C, Pimentel H, Kelley R, Salzberg SL. TopHat2: accurate alignment of transcriptomes in the presence of insertions, deletions and gene fusions. Genome Biol. 2013;14:R36.

59. Anders S, Pyl PT, Huber W. HTSeq--a Python framework to work with high-throughput sequencing data. Bioinformatics. 2015;31:166–9.

60. Gentleman RC, Carey VJ, Bates DM, Bolstad B, Dettling M, Dudoit S, et al. Bioconductor: open software development for computational biology and bioinformatics. Genome Biol. 2004;5:R80.

61. Götz S, García-Gómez JM, Terol J, Williams TD, Nagaraj SH, Nueda MJ, et al. High-throughput functional annotation and data mining with the Blast2GO suite. Nucleic Acids Res. 2008;36:3420–35.