

Article

# Functional Immunomics of the Squash Bug, Anasa tristis (De Geer) (Heteroptera: Coreidae)

# Kent S. Shelby

Biological Control of Insects Research Laboratory, United States Department of Agriculture, Agricultural Research Service, 1503 S. Providence Road, Columbia, MO 65203, USA; E-Mail: kent.shelby@ars.usda.gov; Tel.: +1-573-876-8308; Fax: +1-573-875-5364

Received: 15 September 2013; in revised form: 7 November 2013 / Accepted: 13 November 2013 / Published: 26 November 2013

Abstract: The Squash bug, Anasa tristis (De Geer), is a major piercing/sucking pest of cucurbits, causing extensive damage to plants and fruits, and transmitting phytopathogens. No genomic resources to facilitate field and laboratory studies of this pest were available; therefore the first de novo exome for this destructive pest was assembled. RNA was extracted from insects challenged with bacterial and fungal immunoelicitors, insects fed on different cucurbit species, and insects from all life stages from egg to adult. All treatments and replicates were separately barcoded for subsequent analyses, then pooled for sequencing in a single lane using the Illumina HiSeq2000 platform. Over 211 million 100-base tags generated in this manner were trimmed, filtered, and cleaned, then assembled into a de novo reference transcriptome using the Broad Institute Trinity assembly algorithm. The assembly was annotated using NCBIx NR, BLAST2GO, KEGG and other databases. Of the >130,000 total assemblies 37,327 were annotated identifying the sequences of candidate gene silencing targets from immune, endocrine, reproductive, cuticle, and other physiological systems. Expression profiling of the adult immune response was accomplished by aligning the 100-base tags from each biological replicate from each treatment and controls to the annotated reference assembly of the A. tristis transcriptome.

**Keywords:** squash bug; cucurbits; expression profiling; RNA-seq; entomopathogen response; PAMPs; PRRs; anacin

#### 1. Introduction

The squash bug, Anasa tristis (De Geer), is a major pest of squash, pumpkin, watermelon, cucumber and cantaloupe production, causing substantial economic losses throughout its range [1]. Squash bug feeding causes extensive damage to stems resulting in wilting, fruit discoloration and pre/postharvest spoilage. Possibly more important, A. tristis vectors the causal bacterial agent of Cucurbit yellow vine disease (CYVD), Serratia marcescens [2]. This serious disease was first recorded in Texas and Oklahoma in 1988 and is now rapidly spreading through the West and Midwest. The epidemiology of CYVD ranges from little impact in some years to regional crop failure in others. Natural enemies of the squash bug include the scelionid wasp egg parasitoid Grvon pennsylvanicum (Ashmead), the tachinid parasitoid of adult squash bugs, Tricopoda pennipes) [3-5], and several predatory arthropods [6]. A. tristis adults enter diapause in the Fall, and in the Midwest A. tristis adults terminate diapause in May then proceed to feed on host plants [7,8]. A. tristis are univoltine exhibiting one generation each summer season beginning and ending with overwintering adults [8]. There are few effective biological agents or cultural practices for controlling this highly destructive pest aside from insecticides, thus development of more effective control measures are much needed. In their natural environments insects are subjected to a high incidence of opportunistic microbial infection [9] and parasitization [10]; thus immunoevasion and immunosuppression of the insect host is a strategy widely in use by parasitoids, nematodes, trypanosomes, bacteria and viruses [11]. Experimental immunosuppression of insects via malnutrition [10], or by dietary deficiencies of selenium [12–15] or of ascorbic acid [16–18] can enhance entomopathogen virulence. Immunosuppression of host larvae by injection of double stranded RNA directed against immune system components also increases susceptibility to microbial entomopathogens [19-23]. Field application of RNAi targeted specifically against insect pests is a promising new control approach [24-26]. Demonstration of field efficacy on a commercial scale has already been demonstrated by in situ oral vaccination of honey bees against the Israeli Acute Paralysis Virus by inclusion of RNAi against the virus in feeding solutions [27,28]. However, delivery of a sufficient quantity of RNAi to adversely impact pest life processes is far more challenging in the case of piercing/sucking insect pests, requiring plant-based expression of RNAi [29-32], although soaking or spraying with RNAi may become feasible in the future for some crops [26].

Deployment of RNAi against pest insects requires the discovery and generation of species specific gene silencing targets which disrupt tissues of targeted pests [31-33], or block central metabolic pathways such as arginine kinase [34] or mitochondrial Rieske iron-sulfur protein [35]. Immunosuppression as a biological control strategy [36] would first require discovery of viable, easily accessible gene silencing targets from among the squash bug immune system. The objective of this study therefore was to identify candidate immunosuppressive gene silencing targets suitable for control of *A. tristis*. The sequences of many inducible and constitutively expressed immune system components orthologous to those of other insect species were identified, and in addition a transcriptome was compiled which contains the vast bulk of *A. tristis* unigenes enabling a wide range of studies using this pest insect.

# 2. Experiment

#### 2.1. Insects, Infections and RNA Isolation

Colonies were founded by collection of adult squash bugs, Anasa tristis (De Geer) (NCBI Taxonomy ID: 236421), from squash and zucchini plants at the USDA Agricultural Research Service Biological Control of Insects Research Laboratory (38.934159°N, -92.336876°W; Boone Co., Missouri, MO, USA) in June-August, 2010-2011. Colonies were maintained on three week old summer squash plants, a non-disease resistant hybrid yellow crookneck cultivar (Cucurbita pepo cv. Dixie; Willhite Seed Co., Poolville, TX, USA) and fruit under standard conditions of 14 h:10 h (L:D) photoperiod, 55% RH, 28 °C with fluorescent, incandescent and LED lighting. Summer squash, butternut squash and zucchini fruit purchased from local organic markets was provided in addition to squash plants. To activate the antibacterial immune response, adults and 5th instar nymphs were subjected to septic puncture with a tungsten needle dipped into a 1 µg/mL suspension of lipopolysaccharide and peptidoglycan (Sigma Chem. Co., St. Louis, MO, USA) in PBS. The antifungal response was activated by puncture with a tungsten needle dipped into a 1 µg/mL suspension of β-glucan, curdlan and laminarin (Sigma Chem. Co., St. Louis, MO, USA) in PBS [37]. Mock controls received a sterile puncture. At each time point 0–18 hours post-inoculation (h.p.i.) animals were flash frozen on dry ice and stored at -85 °C for later use. Three or more complete, separate biological replicates were collected for each treatment time series. Total RNA was extracted by homogenization

with a Tissue Tearor (BioSpec Products, Inc., Bartlesville, OK, USA) in TriReagent (Sigma Chem. Co., St. Louis, MO, USA). Total RNA was further cleaned with RNeasy<sup>™</sup> kits (Qiagen, Valencia, CA, USA). At least three full biological replicates were collected for each time point.

#### 2.2. Illumina Sequence Generation and Assembly Procedures

RNA was extracted from insects challenged with bacterial and fungal immunoelicitors, insects fed on different cucurbit species, and insects from all life stages from egg to adult. All treatments and replicates were separately barcoded for subsequent analyses, then pooled for sequencing in a single lane using the Illumina HiSeq2000 platform. RNA pools were submitted to University of Missouri Bond Life Sciences Center DNA Core for Illumina GAII sequencing Libraries were constructed following the manufacturer's protocol with reagents supplied in Illumina's TruSeq RNA sample preparation kit (#RS-930-2001, Illumina Inc., San Diego, CA, USA). Libraries were constructed according to the standard Illumina RNA-seq protocol (Part# 1004898 Rev. A, rev Sept 08; Illumina Inc., San Diego, CA, USA) from the pooled PCR products except for the fragmentation step as detailed [37,38].

Over 211 million ~100-base long Illumina single end reads were first cleaned of low quality bases at the 3' end, then for low quality overall using the FASTX Toolkit (Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, USA). The remaining reads were screened for homology to contaminants (predominantly rRNA) leaving 115,101,048 reads. After trimming, filtering and cleaning the remaining reads were assembled into a *de novo* reference transcriptome using a local installation of the Broad Institute Trinity assembly algorithm [39]. The assembly was annotated using NCBIx NR, NCBI GVBRL, BLAST2GO [40,41], KEGG and other databases. Of the >130,000 total assemblies 37,327

were annotated including "hypothetical" or "predicted" proteins. Contigs and singletons <80 bases were not analyzed. Annotation of KEGG orthologies (KOs) and metabolic pathway mapping was accomplished using the utilities provided by Kyoto Encyclopedia of Genes and Genomes. All sequence data discussed in this manuscript have been deposited in NCBI GenBank.

## 2.3. Identification of Differentially Regulated Genes

Expression profiling of *A. tristis* immune system responses were analyzed by normalizing the number of times sequence tags aligning with an mRNA was detected in controls [38]. To accomplish expression profiling the 100-base tags from each treatment, controls and replicates were independently aligned to the annotated assembly of the *A. tristis* reference transcriptome. The final assembly output was piped into a tab-delimited file that was imported into an Excel spreadsheet, which includes for each assembled contig the number of reads and the list of unique names for each read, to facilitate counting the contribution of different libraries for the final assembly. Genes those were upregulated or down regulated by a factor of three and that had an e-value less than 0.0001 are shown in Tables.

# 3. Results and Discussion

## 3.1. Anasa Tristis RNA-Seq

A total of 211,532,043 reads were acquired from the 11 barcoded input cDNA libraries. After quality trimming and filtering with FASTx 54.4% of the initial reads, 115,101,048 reads, remained for the assembly (Table 1). These were assembled using the program Trinity [39]. Over 211 million 100-base tags generated in this manner were trimmed, filtered, and cleaned, then assembled into a *de novo* reference transcriptome using the Broad Institute Trinity assembly algorithm. Annotation of this assembly [40] resulted in the identification of 37,327 unigenes (Table 1) of which the majority were genuine orthologs of Arthropoda (Figure 1). The read length of those over 300 bp averaged 1588 bp (range 301 bp to 16,368 bp) (Table 1). In comparison to other insect species this is likely an overestimate of the actual number of genes present in *A. tristis*. However a better estimate will not be available without alignment to a completed *A. tristis* reference genome. Genome size estimates of squash bug adults from this colony were estimated to be  $1726 \pm 29.2$  Mb ( $\mathcal{F}$ ) and  $1782 \pm 14.6$  Mb ( $\mathbb{Q}$ ) [42].

**Table 1.** Assembly and Annotation Summary of Illumina sequence Tags Derived from

 Immune-Stimulated Adult and Nymphal *Anasa tristis*.

Parameter	Output					
Total Sequences	211, 532, 043					
Input Sequences	115, 101, 048 (54.4%)					
Contigs <sup>a</sup>	130,000					
Contigs > 300 bp	96,480					
Annotated <sup>b</sup>						
Contigs >300 bp	37,327					
	(Ave = 1,588 bp; Range 301 to 16,368)					
Contigs $> 5$ kb	858					
Contigs $> 4 \text{ kb} < 5 \text{ kb}$	1,068					

Table 1. Cont.

Parameter	Output	
Contigs $> 3 \text{ kb} < 4 \text{ kb}$	2,450	
Contigs $> 2$ kb $< 3$ kb	5,601	
Contigs $> 1$ kb $< 2$ kb	12,216	
Contigs $> 301$ bp $< 1$ kb	15,135	

<sup>a</sup> Trinity Assembly [40].<sup>b</sup> Significant BLAST score ( $e > 10^{-6}$ ) using BLAST2GO [41].

**Figure 1.** *Anasa tristis* transcriptome assembly Top BLAST hits across insects and other invertebrate species. Highest number of hits was against the insect species most closely related to *A. tristis*, and which has a sequenced genome, *Acyrthosiphon pisum* (green).



Genome Ontology annotation of these contigs demonstrated that the RNA pools constituting the assembly resulted in successful sampling of the major classes of cellular component, molecular function and biological processes. A complete set of cytoplasmic and mitochondrial tRNAs was assembled, and the majority of tRNA-acyltransferases. GO annotation revealed substantial acquisition of transcripts involved in reproduction and reproductive processes (2,043), central metabolism (7,818), cell and organellar proliferation, division and movement (9,580), physiological processes (3,571), and arthropod specific functions such as molting and pupation (911). KEGG maps generated using BLAST2GO demonstrated that major metabolic pathways were reasonably complete, constituting a majority of enzymes required. The experiments were specifically designed to sample *A. tristis* immune system processes and the annotation resulted in the identification of 394 GOs from this category, and in addition 169 immune system regulatory GOs, 76 coagulation, and two respiratory burst GOs were noted (Figure 2).

Contamination with squash and microbial sequences was expected because whole adults and nymphs actively feeding upon live cucurbits were used to generate RNA pools. Also an effort was made to filter and remove contaminating sequences before assembly of the transcriptome. Despite this only a single contaminating sequence annotating as a putative annexin ortholog of *Cucumis melo* was identified. This may be partially explained as consistent with the squash bug piercing/sucking mode of feeding which differs from foliar herbivores that retain large masses of partially digested plant material within their midguts posing a greater risk of contamination with plant sequences.





Trace contamination with *Wolbachia spp., Burkholderia spp., Pseudomonas spp.*, Proteobacteria, phytoplasmodia, mycoplasmodia and other bacterial or fungal species was observed. The sample preparation method would have allowed contamination with midgut floral sequences. Also identified were sequences annotating as *Serratia marcescens*, the squash bug vectored bacterial agent of Cucurbit yellow vine disease [2,43]. Phloem feeding insects are famously dependent upon obligate symbiotic bacteria to compensate for nutritional deficiencies inherent to this diet [44–46], thus it is highly likely that *A. tristis* also may possess symbiotes as these and other data suggest [47]. A substantial number of sequences had significant matches to kinetoplastids (Figure 1), indicating that the very recently established squash bug colony may harbor these parasites. A metagenomic study of *A. tristis* symbionts and other associated flora would confirm and expand on these tantalizing first hits. Finally, the vast bulk of remaining contigs with significant BLAST scores annotated, as expected, within the Arthropoda (Figure 1), Hemiptera, and in particular were orthologous with the only hemipteran insect having a completed/annotated genome, *Acyrthosiphon pisum*.

An explication of all the putative *A. tristis* unigenes identified in this survey would exceed the ambit of a single manuscript, therefore only key categories of identified immunity unigenes will be highlighted below.

## 3.2. Immunity Related Transcripts Identified in the A. Tristis Transcriptome

Many developmental, rare or environmental transcripts are not adequately sampled by insect transcriptome projects as the treatments needed to induce transcription or particular life stages were not included. Thus the inducible transcripts of the insect immune system are routinely missing from published transcriptomes. In this report a deliberate effort was made to sample transcripts from a variety of environmental insults, developmental stages and immune activation. As no entomopathogens are currently known for A. tristis, and because the RNA-seq method is extremely sensitive to contaminating entomopathogen nucleic acid sequences, only purified cell wall components of bacteria and fungi were used to elicit an immune response in lieu of live or intact entomopathogens. The immune system of adult squash bugs was activated by septic puncture using either bacterial or fungal elicitors [37]. Control insects were subjected a sterile puncture. Septic puncture of adult squash bugs with bacterial cell wall elicitors resulted in the increased transcription of 3,950 contigs, and decreased levels of 5,242 contigs. Septic puncture with fungal cell wall elicitors resulted in the increased expression of 2,434 transcripts, and decreased transcription of 6,021. Control adults receiving a sterile puncture expressed 1,491 unique transcripts, while 2,088 transcripts were unique to the bacterial elicitation, and 1,941 were unique to the fungal elicitation. An upper bound estimate of the number of genes directly involved in the insect immune response was reported by Brucker et al. [48] who identified 489 putative immune system genes by hidden Markov model search of the completed genome of the jewel wasp, Nasonia vitripennis.

#### 3.3. Pattern Recognition and Signal Transduction

The immune response is triggered by the presence of pathogen associated molecular patterns (PAMPs) such as bacterial cell wall or flagellar components in the presence of host damage associated molecular patterns and chemokines [49]. Pattern recognition receptors located on hemocytes and other tissues bind to the PAMPs activating one or more signal transduction pathways which lead to the synthesis of many antimicrobial enzymes and peptides. Several orthologs of PAMPS and pattern recognition receptors were identified within the *A. tristis* transcriptome. Among these were  $\beta$ -1,3-glucan recognition protein 4a [JQ398676], peptidoglycan recognition protein S2 (JQ398681), C-type lectins, galectins, Dscams, hemolectin, scavenger receptor class B [KF578379], and leucine-rich repeat proteins (Table 2). Elicitation with bacterial or fungal cell wall components via septic puncture led to the upregulation of several PAMPS. Expression of Dscam was upregulated 15-fold by bacterial elicitation, and 10-fold by fungal elicitation. The scavenger receptor ortholog was upregulated 149-fold by bacterial and 124-fold by fungal elicitation of *A. tristis* adults (Table 2).

Three main conserved signal transduction pathways occur in insects: The *toll* pathway responsive to gram-positive bacteria and fungi; the *imd* pathway responsive to gram-negative bacteria; and the JAK/STAT pathway which responds to bacterial or viral infection [50]. Several components of these signal transduction pathways were identified within the assembly. The toll pathway components toll, toll-interacting protein, toll-like receptor 13, dorsal, NF-K-B inhibitor alpha, NF-K-B inhibitor-interacting Ras-like protein, spaetzle, snake, easter, and relish (Table 2). Among the *imd* pathway components identified were dFADD, JNK-interacting protein 1, JNK-interacting protein 3, (Table 2).

Transcript ID	NR Annotation	MeanControl <sup>a</sup>	Mean Bacterial <sup>a</sup>	B/C <sup>b</sup>	Mean Fungal <sup>a</sup>	F/C <sup>b</sup>	NRid	Score	Eval	Best Match
2224c0seq1	15-hydroxyprostaglandin dehydrogenase	325.96	771.19	2.36	508	1.56	ref XP_001943808.2	204	1E-51	Acyrthosiphon pisum
3518c0seq1	β-1,3-glucan recognition protein 4a	306.34	381.82	1.25	363	1.19	gb ADZ45541.1	247	2E-64	Hepialus pui
20931c0seq2	Croquemort	6.74	16.1	2.39	13.17	1.96	gb EFN76142.1	323	2E-87	Harpegnathos saltator
18080c0seq1	C-type Lectin	16.67	5.33	0.32	15	0.9	gb ABD60991.1	192	6E-49	Glossina morsitans
7613c1seq8	Dorsal	29.84	42.47	1.42	33.62	1.13	gb ABU96698.1	555	1E-157	Rhodnius prolixus
17785c0seq3	Dscam	0.96	14.69	15.25	9.83	10.2	ref XP_003395940.1	1423	0	Bombus terrestris
1470c3seq3	FADD	266	313	1.18	134	0.51	ref]NP_001189465.1	61.2	4E-08	Bombyx mori
6326c0seq5	Galectin	28.92	82	2.84	39.5	1.37	ref XP_001656933.1	157	3E-37	Aedes aegypti
12793c0seq5	JNK-interacting protein 3	2.07	1.74	0.84	2	0.96	ref XP_396524.4	428	1E-118	Apis mellifera
10217c0seq1	Leucine-rich repeat- containing protein 4B	225	172	0.77	100	0.45	ref XP_972275.1	526	1E-148	Tribolium castaneum
14846c0seq15	MAP kinase-activating death domain	19.3	13.33	0.69	12.28	0.64	gb EFN64544.1	585	1E-166	Camponotus floridanus
2228c0seq2	NF-κ-B inhibitor alpha	132	130	0.99	118	0.9	ref XP_002427786.1	237	3E-61	Pediculus humanus
255c0seq1	PGRP S2-like protein	2462	2134	0.87	2648	1.08	ref NP_001164436.1	156	2E-37	Nasonia vitripennis
109335c0seq1	Phospholipase A2 inhibitor 31 kDa	2	0	0	0.33	0.16	ref]XP003222879.1	87	1E-15	Anolis carolinensis

**Table 2.** Expression Profiling of Anasa tristisPattern Recognition and Signal Transduction Unigenes in Response to Bacterial or FungalElicitation 18 h post inoculation.

720

Transcript ID	NR Annotation	MeanControl <sup>a</sup>	Mean Bacterial <sup>a</sup>	B/C <sup>b</sup>	Mean Fungal <sup>a</sup>	F/C <sup>b</sup>	NRid	Score	Eval	Best Match
7436c0seq2	Phospholipase A2, iPLA-2	282.73	254	0.89	174	0.61	ref XP_970721.2	631	1E-179	Tribolium castaneum
9865c1seq1	Phospholipase A-2- activating protein	144.90	157	1.08	107	0.74	ref]XP_002423298.1	445	1E-123	Pediculus humanus
5036c1seq1	Phospholipase A2	129.37	117	0.90	141	1.09	ref NP_001191907.1	201	1E-50	Acyrthosiphon pisum
8868c0seq1	Prostaglandin E synthase 2	162	209.3	1.28	125	0.77	ref XP_002432321.1	202	1E-101	Pediculus humanus
28963c0seq2	Prostaglandin E2 receptor EP3 subtype	29	0	0	7.3	0.25	ref XP_001943957.2	237	4E-61	Acyrthosiphon pisum
6943c0seq1	Prostaglandin reductase 1	100	216	2.15	116	1.15	ref XP_001603755.1	373	1E-102	Nasonia vitripennis
111273c0seq1	Relish	0.67	0	0	0	0	gb ACT66912.1	50.8	3E-06	Apis andreniformis
20920c0seq4	Scavenger receptor class B	0.09	12.95	149	10.77	124	ref]XP_001947205.2	173	2E-42	Acyrthosiphon pisum
16467c0seq1	Easter	73.33	18.67	0.25	54	0.74	gb EFN62640.1	176	2E-43	Camponotus floridanus
15721c0seq1	Snake	45	52.67	1.17	11	0.24	ref XP_003247331.1	245	1E-63	Acyrthosiphon pisum
36985c0seq2	Spaetzle 4	1	4.56	4.56	2.12	2.12	ref NP_001153592.1	132	5E-30	Acyrthosiphon pisum
19735c0seq1	toll	84	84	1	69	0.83	ref]XP_002424097.1	1514	0	Pediculus humanus

<sup>a</sup> Average number of reads/contig (three biological replicates); <sup>b</sup> Ratio of reads, Bacterial/Control or Fungal/Control.

Several orthologs of yet another major insect signal transduction pathway, the eicosanoid pathway [51], were recognized within the *A. tristis* transcriptome assembly. Release of arachidonic acid by phospholipase  $A_2$  allows the C20 fatty acid to enter a series of oxidative reactions yielding second messenger prostaglandins. These prostaglandins bind to receptors that in turn activate hemocytes, among other actions [52]. Within the Heteroptera, the classic model insect *Rhodnius prolixus* has been documented to possess several components of the eicosanoid pathway interacting with trypanosomal infection [53]. Contigs within the *A. tristis* assembly that annotated as orthologs of several enzymes within this pathway were 15-hydroxyprostaglandin dehydrogenase [NAD<sup>+</sup>], peroxidasin-like, phospholipase A2 [JQ398686], Phospholipase A-2-activating protein, prostaglandin E synthase, prostaglandin reductase, prostaglandin E2 receptor (Table 2). The transcript levels of these enzymes, however, remain unchanged by bacterial or fungal elicitation of adult squash bugs.

## 3.4. Melanization, Coagulation and Antimicrobial Activities

Upon infection and activation *via* the *toll* and *imd* signal transduction pathways insects produce a suite of antimicrobial peptides, enzymes and enzyme inhibitors which act to limit growth of the pathogen [50]. Analysis of immune-induced transcripts demonstrated that this is also the case with *A. tristis* (Table 3). Several contigs orthologous to antibacterial peptides of other insects induced by infection were identified. These included transcripts orthologous to a *Triatoma brasiliensis* defensin-like peptide. In accordance with accepted nomenclature I propose the name "Anasin" [KF578378] for this defensin-like peptide induced in *Anasa tristis* by bacterial elicitation. A second transcript encoding an antimicrobial peptide orthologous to hemiptericin of *Pyrrhocoris apterus* was identified which was elevated 16.6-fold by bacterial elicitation. I propose that this peptide be named "Anacin". Additional antimicrobial enzymes and protease inhibitors were noted, including lysozymes and short pacifastin-like inhibitors (Table 3).

Melanization reactions catalysed by phenoloxidase are primary immune responses to microbial incursion and parasitization which shroud invaders with melanin, crosslink proteins and directly generate microbicidal free radicals [54–56]. Contigs annotating as the major insect melanization enzyme prophenoloxidase, as well as putative serpins and serine proteases within the prophenoloxidase regulatory cascade [50] were recognized (Table 3). Hemolymph coagulation is a well-known defence pathway [57] requiring the action of phenoloxidase, as well as other components. Several orthologs of putative coagulation pathways were noted in the assembly (Table 3).

Generation of antimicrobial reactive oxygen and nitrogen species by hemocytes and other tissues acts to limit microbial growth [58]. *A. tristis* orthologs of the plasma membrane reactive oxygen generator NADPH oxidase driving the respiratory burst phenomenon [59] were identified (Table 2). Also orthologs of the antimicrobial free nitrogen radical generator nitric oxide synthase [19] significantly upregulated 3.4-fold by bacterial elicitation were identified within the assembly. A dual oxidase ortholog of the *Drosophila* midgut which generates reactive oxygen species also was noted. Enzymes responsible for inactivation of reactive oxygen species also were noted including superoxide dismutase, catalase, peroxidase, glutathione peroxidase, and others. Finally, the orthologs of iron storage and transporting proteins transferrin and ferritin were observed. While transcript levels of the ferritin ortholog were significantly upregulated 5.3-fold by bacterial and 3.7-fold by fungal elicitation the transcript levels of transferrin appeared to be unaffected by these treatments (Table 3).

## Insects 2013, 4

14386c0seq4

254c0seq1

Mean Mean Mean B/C<sup>b</sup> F/C<sup>b</sup> Transcript NR Annotation NRid Score Eval **Best Match** Fungal<sup>a</sup> Bacterial<sup>a</sup> Contro l<sup>a</sup> 693c0seq4 Alaserpin-like 196.48 246.79 0.81 ref|XP003399187.1 3E-62 Bombus terrestris 1.26 159.80 240 3354c0seq1 Amyloid beta A4 precursor 144.59 304.18 2.10 161.73 1.12 ref|XP001943320.1 406 1E-111 Acyrthosiphon pisum 1603c0seq10 CLIP-associating protein ref|XP003246351.1 Acyrthosiphon pisum 307.15 372.83 1.21 305.06 0.99 404 1E-111 83136c0seq1 Coagulation factor IX 1.67 5.33 3.20 1.40 ref]XP001943624.2 2E-67 Acyrthosiphon pisum 2.33 256 23755c0seq2 Coagulation factor X 1.49 1.87 gb|EFN83879.1 Harpegnathos saltator 1.26 4.02 2.70150 2E-35 Complement C1r-B 4923c0seq1 366.47 ref]XP001120594.2 Apis mellifera 492.67 2E-68 1.34 100.31 0.27 261 subcomponent Triatoma brasiliensis 393c1seq1 Defensin "Anasin" 1014.84 533.22 0.53 sp|P80407.1 85.5 2E-16 1665.55 1.64 15509c0seq1 Dual oxidase 82.33 102.67 1.25 45.67 0.55 ref|XP 001951113.2 2294 0 Acyrthosiphon pisum 8c0seq1 ferritin 48316 56283 1.19 gb|ABR27877.1 291 3E-77 Triatoma infestans 1.16 57436 82370c0seq1 Ferritin, heavy subunit 2.67 0.00 0.00 1.67 0.63 gb|ACO15170.1 130 2E-30 Caligus clemensi 20023c0seq1 Ferritin, middle subunit 39.93 gb|ACO10415.1 Caligus rogercressevi 10.67 56.72 5.32 3.74 201 7E-51 Gelsolin precursor Aedes aegypti 344c0seq1 7089.52 4998.67 0.71 1494.70 0.21 ref|XP001657431.1 743 0 33115c0seq1 Hemiptericin "Anacin" 1.33 22.07 16.55 3.00 2.25 gb|ABW16857.1 79.7 8E-17 *Pvrrhocoris apterus* 2804c0seq1 Hemolectin 61.16 38.67 0.63 16 0.26 ref[XP 002430354.1 52 7E-06 Pediculus humanus 167.25 144.80 0.87 gb|ABX11554.1 159 7E-38 Rhodnius prolixus 533c2seq8 Lysozyme 118.97 0.71 Multiple coagulation factor 2227c0seq2 304.36 245.79 0.81 0.85 gb|EGI57786.1 2E-37 Acromyrmex echinatior 258.63 157 deficiency protein 17478c0seq1 NADPH oxidase 184.00 130.33 0.71 146.67 0.80 tpd|FAA00348.1 58.2 5E-07 Apis mellifera Nitric oxide synthase Apis mellifera 5121c0seq1 342.29 1154.75 3.37 160.71 0.47 sp|Q26240.1 1784 0 8767c1seq1 Plasma kallikrein 11.66 26.19 2.25 17.23 1.48 gb|EFN62475.1 54.3 7E-07 Camponotus floridanus Platelet-activating factor Pediculus humanus 6324c0seq4 62.28 75.57 1.21 111.93 1.80 ref|XP002427586.1 311 9E-84 4507c0seq2 Proclotting enzyme 219.76 357.26 1.63 177.83 0.81 gb|EFN62552.1 296 5E-79 Camponotus floridanus Prophenoloxidase subunit 2 107.67 103.33 53.33 ref|XP967179.2 10065c0seq1 0.96 0.50 688 0 Tribolium castaneum Prophenoloxidase subunit A3 16.00 ref|NP001011627.1 3E-80 15584c0seq1 8.00 0.50 8.00 0.50 298 Apis mellifera

Table 3. Expression profiling of Anasa tristis antimicrobial and melanization unigenes in response to bacterial or fungal elicitation 18 h post inoculation.

<sup>a</sup> Average number of reads/contig (three biological replicates); <sup>b</sup> Ratio of reads, Bacterial/Control, or Fungal/Control.

0.00

10554.93

0.02

1.91

0.00

1.44

gb|EFA12666.1

gb|AAD02419.1

112

939

8E-24

0

5.50

7350.06

0.10

14070.74

Serpin

Transferrin

Tribolium castaneum

Riptortus clavatus

## 3.5. RNAi Pathways

Although gene silencing *via* injected or *per os* RNAi has been demonstrated in several Hemipteran species few examples of successful Heteropteran gene silencing are published. Here the *A. tristis* orthologs of proteins required for RNAi are presented. Within the *A. tristis* assembly several orthologs of the miRNA processing pathways were identified (Table 4). An ortholog of the nuclear microprocessor complex subunit, DGCR8 was observed, while cytoplasmic subunits argonaute-1, and -2, aubergine, piwi, dicer-1, dicer-2, and a RISC-loading complex subunit were identified. None of the identified orthologs within these pathways appeared to be differentially regulated by microbial elicitation (Table 4). As there are no known viral entomopathogens of *A. tristis*, no viral elicitation was attempted in this report. It should be noted however that viruses were present within the assembly and thus the insects used for the RNA pools may have had active viral infections (see below).

## 3.6. Viral Sequences

Next generation sequencing approaches such as RNA-seq seem ideal methodologies to delineate and to sample the plant-pathogenic virome of plant feeding insect vectors such as squash bugs. Aphids and whiteflies are the major vectors of cucurbit viruses. Squash bugs are known vectors of *Serratia marcescens*, the bacterial pathogen of Cucurbit yellow vine disease [42,60,61]. The newly founded laboratory squash bug colony from which RNA pools used in this study were isolated was comprised of animals recently collected from local squash and zucchini fields. The animals also were occasionally fed with squash and zucchini cuttings brought in from the field or purchased at local organic outlets. Thus it was not entirely unexpected that RNA-seq revealed tags homologous to many RNA and DNA viruses known to infect insects, or to be vectored to plants by insect species.

To obtain a preliminary survey of the A. tristis virome all reads from the combined RNA pools that did not align to the Trinity assembly were BLASTx screened against the NCBI gbvrl database resulting in 915 significant hits logged. Major taxa of insect viruses detected within these hits included ascoviridae. iridoviridae, granuloviridae, entomopoxviridae, baculoviridae, nudiviridae. even ichnoviridae and bracoviridae. Among these were sequences of plant infecting viruses sequences were detected of viroids, bromoviridae, closteroviridae, genimiviridae, luteoviridae, potyviridae, secoviridae, tombusviridae and virgaviridae. The presence of several of the 17 cucurbit associated viruses such as Cucurbit aphid-borne yellows virus, Melon yellow spot virus, and Papaya ringspot virus within the squash bug colony was indicated [62]. Transcripts from DNA viruses also would have been sampled by this approach and thus even RNA/DNA viruses of vertebrates also were observed. These findings set the stage for a more comprehensive survey of plant pathogens harbored by squash bugs in the laboratory and the field. Confirmation that these viruses occur within wild populations of squash bugs would indicate that further prospecting for entomopathogenic viruses effective as biological control agents of A. tristis is warranted. Next generation deep sequencing of small RNAs extracted from infected cells or insects yields short interfering RNAs, which are products of the Dicer-dependent antiviral pathway, could enable the in silico reconstruction of viral transcripts or entire RNA/DNA virus genomes [63-67]. Phloem/xylem feeding insects can also be used to sample the viral diversity of plant populations on which they are feeding ("vector-enabled metagenomics") [68,69]. Viromes of insects [67,70,71], their food plants [72,73], their predators [74], and even their entomopathogens could be sampled simultaneously in toto. Transcriptomes and genomes have recently been completed for several cucurbits [75] which would allow concurrent pathogenomic monitoring of squash bug, virus and cucurbit host transcription during insect feeding, e.g., [76].

# Insects 2013, 4

Transcript	NR Annotation	Mean Control <sup>a</sup>	Mean Bacterial <sup>a</sup>	B/C <sup>b</sup>	Mean Fungal <sup>a</sup>	F/C <sup>b</sup>	NRid	Score	Eval	Best Match
10966c0seq13	argonaute-1	33	34.96	1.06	43	1.3	gb ACO40482.1	133	5E-29	Nasonia vitripennis
1860c0seq1	argonaute-2	1233.65	1107.58	0.9	634.29	0.51	ref XP001607164.1	721	0	Nasonia vitripennis
108824c0seq1	aubergine	0.67	0	0	0	0	ref NP001159378.1	221	2E-57	Apis mellifera
44766c0seq1	dicer-1	9	14	1.56	11.67	1.3	emb CAX68236.1	754	0	Blattella germanica
12963c0seq2	dicer-2	11.08	1.69	0.15	12.46	1.12	ref NP001107840.1	793	0	Tribolium castaneum
12963c0seq3	Endoribonuclease Dcr-1	94.37	110.64	1.17	75.62	0.8	gb EFN62420.1	432	1E-120	Camponotus floridanus
17917c0seq1	microprocessor subunit DGCR8	91.33	130.33	1.43	69	0.76	ref XP003397039.1	575	1E-162	Bombus terrestris
7746c0seq1	PIWI	235.33	304.67	1.29	125	0.53	ref XP001652945.1	722	0	Aedes aegypti
12734c0seq1	RISC-loading complex subunit	92.92	51.74	0.56	58.93	0.63	ref XP001601132.2	380	1E-104	Nasonia vitripennis
2817c0seq1	Translin-like	327.0	373.67	1.14	213.0	0.65	dbj BAG65665.1	611	1E-174	Nasonia vitripennis

Table 4. Expression profiling of Anasa tristis RNA interference pathway genes in response to bacterial or fungal elicitation 18 h post inoculation.

<sup>a</sup> Average number of reads/contig (three biological replicates); <sup>b</sup> Ratio of reads, Bacterial/Control, or Fungal/Control.

#### 4. Conclusions

To construct a reasonably complete *A. tristis* immunotranscriptome, adults were subjected to microbial elicitation of the immune response. The resulting *de novo* assembly contained many *A. tristis* orthologs of immune system proteins known from other insect species, including those of phylogenetically related hemipterans. Key components of the entomopathogen recognition system, humoral and cellular immune responses, and second messenger regulatory networks were identified. Interestingly, a partial virome of *A. tristis* was noted within the *de novo* assembly, along with the presence of known insect transmitted bacterial species which will become the subject of future studies. Novel approaches are needed to target the unique mode of *A. tristis* feeding on the phloem of its cucurbit hosts. The *de novo* transcriptome generated in this report consists of the vast majority of

transcript categories synthesized by this insect to support life processes, such as olfaction, neuroendocrinology, reproduction, digestion, and the immune response against bacterial and fungal elicitation. Targeted disruption of one or more of these transcripts, or the proteins that they encode, could reduce the severe economic impact of *A. tristis* on horticultural production.

# Acknowledgments

This work was funded by United States Department of Agriculture CRIS Project 3622-22000-036. I thank Nathan Bivens and Karen Bromert of the University of Missouri DNA Core Facility, and Bill Spollen and Christopher Bottoms of the University of Missouri Informatics Research Core Facility. I thank Larry Brown and Joseph Ringbauer of the USDA Agricultural Research Service Biological Control of Insects Research Laboratory for technical assistance.

#### **Conflicts of Interest**

The author declares no competing interests.

## Disclaimer

The U.S. Department of Agriculture (USDA) prohibits discrimination in all of its programs and activities on the basis of race, color, national origin, age, disability, and where applicable, sex, marital status, familial status, parental status, religion, sexual orientation, genetic information, political beliefs, reprisal, or because all or part of an individual's income is derived from any public assistance program. (Not all prohibited bases apply to all programs.) Persons with disabilities who require alternative means for communication of program information (Braille, large print, audiotape, *etc.*) should contact USDA's TARGET Center at (202) 720-2600 (voice and TDD). To file a complaint of discrimination, write to USDA, Director, Office of Civil Rights, 1400 Independence Avenue, S.W., Washington, D.C. 20250-9410, or call (800) 795-3272 (voice) or (202) 720-6382 (TDD). USDA is an equal opportunity provider and employer.

## References

- 1. United States Department of Agriculture, National Agricultural Statistics Service (USDA-NASS). *Agriculture Statistics 2012*; United States Government Printing Office: Washington, DC, USA, 2012.
- Pair, S.D.; Bruton, B.D.; Mitchell, F.; Fletcher, J.; Wayadande, A.; Melcher, U. Overwintering squash bugs harbor and transmit the causal agent of Cucurbit Yellow Vine Disease. *J. Econ. Entomol.* 2009, 97, 74–78.
- Nechols, J.R.; Tracy, J.L.; Vogt, E.A. Comparative ecological studies of indiginous egg parastioids (Hymenoptera: Scelionidae; Encyrtidae) of the squash bug *Anasa tristis* (Hemiptera: Coreidae). *J. Kans. Entomol. Soc.* 1989, 62, 177–188.
- 4. Olson, D.L.; Nechols, J.R. Effects of squash leaf trichome exudates and honey on adult feeding, survival, and fecundity of the squash bug (Heteroptera: Coreidae) egg parasitoid *Gryon pennsylvanicum* (Hymenoptera: Scelionidae). *Envion. Entomol.* **1995**, *24*, 454–458.
- 5. Vogt, E.A.; Nechols, J.R. Responses of the squash bug (Hemiptera: Coreidae) and its egg parasitoid, *Gryon pennsylvanicum (*Hymenoptera: Scelionidae) to three *Curcurbita* cultivars. *Environ. Entomol.* **1993**, *22*, 238–245.
- 6. Decker, K.B.; Yeargan, K.V. Seasonal phenology and natural enemies of the squash bug (Hemiptera: Coreidae) in Kentucky. *Environ. Entomol.* **2009**, *37*, 670–678.
- 7. Nechols, J.R. Photoperiodic responses of the squash bug (Heteroptera: Coreidae): Diapause induction and maintenance. *Envion. Entomol.* **1988**, *17*, 427–431.
- 8. Nechols, J.R. Voltinism, seasonal reproduction, and diapause in the squash bug (Heteroptera: Coreidae) in Kansas. *Envion. Entomol.* **1987**, *16*, 269–273.
- 9. Tunaz, H.; Stanley, D.W. An immunological axis of biocontrol: Infections in field-trapped insects. *Naturwissenschaften* **2009**, *96*, 1115–1119.
- 10. Smilanich, A.M.; Dyer, L.A.; Gentry, G.L. The insect immune response and other putative defenses as effective predictors of parasitism. *Ecology* **2009**, *90*, 1434–1440.
- Strand, M.R. The interactions between polydnavirus-carrying parasitoids and their Lepidopteran hosts. In *Molecular Biology and Genetics of the Lepidoptera*; Goldsmith, M.R., Marec, F., Eds.; CRC Press: Boca Raton, FL, USA, 2009; pp. 321–336.
- 12. Shelby, K.S.; Popham, H.J.R. Dietary selenium supplementation elevates HzSNPV resistance of *Heliothis virescens* larvae. J. Invertebrate Pathol. 2007, 95, 77–83.
- 13. Popham, H.J.R.; Shelby, K.S. Effect of inorganic and organic forms of selenium supplementation on development of larval *Heliothis virescens*. *Entomol. Exp. Appl.* **2007**, *125*, 171–178.
- 14. Popham, H.J.R.; Shelby, K.S. Uptake of dietary micronutrients from artificial diets by larval *Heliothis virescens. J. Insect Physiol.* **2006**, *52*, 771–777.
- 15. Popham, H.J.R.; Shelby, K.S.; Popham, T.W. Effect of dietary selenium supplementation on resistance to baculovirus infection. *Biol. Control.* **2005**, *32*, 419–426.
- 16. Popham, H.J.R.; Shelby, K.S. Ascorbic acid influences the development and immunocompetence of larval *Heliothis virescens*. *Entomol. Exp. Appl.* **2009**, *133*, 57–64.
- 17. Coudron, T.A.; Shelby, K.S.; Ellersieck, M.R.; Winston, B.R.; Popham, H.J.R. Developmental response of the beneficial predator *Podisus maculiventris* to change in dietary ascorbic acid concentration. *Entomol. Exp. Appl.* **2011**, *139*, 235–241.

- Coudron, T.A.; Shelby, K.S.; Ellersieck, M.R.; Odoom, E.D.; Lim, E.E.; Popham, H.J.R. Developmental response of *Euplectrus comstockii* (Hymenoptera: Eulophidae) to ascorbic acid in the diet of the larval host, *Heliothis virescens* (Lepidoptera: Noctuidae). *BioControl* 2009, 54, 175–182.
- 19. Eleftherianos, I.; Felfoldi, G.; ffrench-Constant, R.H.; Reynolds, S.E. Induced nitric oxide synthesis in the gut of *Manduca sexta* protects against oral infection by the bacterial pathogen *Photorhabdus luminescens. Insect Mol. Biol.* **2009**, *18*, 507–516.
- 20. Zhou, X.; Wheeler, M.M.; Oi, F.M.; Scharf, M.E. RNA interference in the termite *Reticulitermes flavipes* through ingestion of double-stranded RNA. *Insect Biochem. Mol. Biol.* **2008**, *38*, 805–815.
- 21. Zhuang, S.; Kelo, L.; Nardi, J.B.; Kanost, M.R. Multiple alpha subunits of integrin are involved in cell-mediated responses of the *Manduca* immune system. *Dev. Comp. Immunol.* **2008**, *32*, 365–379.
- Zhuang, S.; Kelo, L.; Nardi, J.B.; Kanost, M.R. An integrin-tetraspanin interaction required for cellular innate immune response of an insect, *Manduca sexta. J. Biol. Chem.* 2007, 282, 22563–22572.
- 23. Terenius, O.; Popham, H.J.R.; Shelby, K.S. Bacterial, but not baculoviral infections stimulate Hemolin expression in noctuid moths. *Dev. Comp. Immunol.* **2009**, *33*, 1176–1185.
- 24. Zhang, H.; Li, H.-C.; Miao, X.-X. Feasibility, limitation and possible solutions of RNAi-based technology for insect pest control. *Insect Sci.* **2013**, *20*, 15–30.
- Aronstein, K.; Oppert, B.; Lorenzen, M.D. RNAi in agriculturaly-important arthropods. In *RNA Processing*; Grabowski, P., Ed.; InTech Publisher: Rijeka, Croatia, 2011; pp. 157–180.
- 26. Burand J.P.; Hunter, W.B. RNAi: Future in insect management. *J. Invertebrate Pathol.* **2013**, *112*, S68–S74.
- Hunter, W.B.; van Engelsdorp, D.; Hayes, J.; Westervelt, D.; Glick, E.; Williams, M.; Sela, I.; Maori, E.; Pettis, J.; Cox-Foster, D.; *et al.* Large-scale field application of RNAi technology reducing Israeli Acute Paralysis Virus disease in Honey Bees (*Apis mellifera*, Hymenoptera: Apidae). *PLoS Pathog.* 2010, *6*, e1001160.
- Paldi, N.; Glick, E.; Oliva, M.; Zilberberg, Y.; Aubin, L.; Pettis, J.; Evans, J.D. Effective gene silencing in a microsporidian parasite associated with honeybee (*Apis mellifera*) colony declines. *Appl. Environ. Microbiol.* 2010, *76*, 5960–5964.
- Zha, W.; Peng, X.; Chen, R.; Du, B.; Zhu, L.; He, G. Knockdown of midgut genes by dsRNA-transgenic plant-mediated RNA interference in the hemipteran insect *Nilaparvata lugens*. *PLoS One* 2011, 6, e20504.
- 30. Pitino, M.; Coleman, A.D.; Maffei, M.E.; Ridout, C.J.; Hogenhout, S.A. Silencing of aphid genes by dsRNA feeding from plants. *PLoS One* **2011**, *6*, e25709.
- Price, D.R.G.; Gatehouse, J.A. RNAi-mediated crop protection against insects. *Trends Biotechnol.* 2008, 26, 393–400.
- Baum, J.A.; Bogaert, T.; Clinton, W.; Heck, G.R.; Feldmann, P.; Ilagan, O.; Johnson, S.; Plaetinck, G.; Munyikwa, T.; Pleau, M.; *et al.* Control of coleopteran insect pests through RNA interference. *Nat. Biotechnol.* 2007, *25*, 1322–1326.
- Xue, X.-Y.; Mao, Y.-B.; Tao, X.-Y.; Huang, Y.-P.; Chen, X.-Y. New approaches to agricultural insect pest control based on RNA interference. In *Advances in Insect Physiology*; Jockusch, E.L., Ed.; Academic Press: New York, NY, USA, 2012; pp. 73–118.

728

- 34. Zhao, Y.; Yang, G.; Wang-Pruski, G.; You, M. *Phyllotreta striolata* (Coleoptera: Chrysomelidae): Arginine kinase cloning and RNAi-based pest control. *Eur. J. Entomol.* **2008**, *105*, 815–822.
- Gong, L.; Yang, X.; Zhang, B.; Zhong, G.; Hu, M.-Y. Silencing of Rieske iron-sulfur protein using chemically synthesised siRNA as a potential biopesticide against *Plutella xylostella*. *Pest Manag. Sci.* 2011, 67, 514–520.
- Bulmer, M.S.; Bachelet, I.; Raman, R.; Rosengaus, R.B.; Sasisekharan, R. Targeting an antimicrobial effector function in insect immunity as a pest control strategy. *Proc. Natl. Acad. Sci. USA* 2009, *106*, 12652–12657.
- 37. Shelby, K.S.; Popham, H.J.R. RNA-seq study of microbially induced hemocyte transcripts from larval *Heliothis virescens* (Lepidoptera: Noctuidae). *Insects* **2012**, *3*, 743–762.
- 38. Breitenbach, J.E.; Shelby, K.S.; Popham, H.J.R. Baculovirus induced transcripts in hemocytes from the noctuid moth, *Heliothis virescens*. *Viruses* **2011**, *3*, 2047–2064.
- Grabherr, M.G.; Haas, B.J.; Yassour, M.; Levin, J.Z.; Thompson, D.A.; Amit, I.; Adiconis, X.; Fan, L.; Raychowdhury, R.; Zeng, Q.; *et al.* Full-length transcriptome assembly from RNA-Seq data without a reference genome. *Nat. Biotechnol.* 2011, *8*, 469–477.
- Gotz, S.; Garcia-Gomez, J.M.; Terol, J.; Williams, T.D.; Naagaraj, S.H.; Nueda, M.J.; Robles, M.; Talon, M.; Dopazo, J.; Conesa, A. High-throughput functional annotation and data mining with the Blast2GO suite. *Nucleic Acids Res.* 2008, *36*, 3420–3435.
- 41. Conesa, A.; Gotz, S. Blast2GO: A comprehensive suite for functional analysis in plant genomics. *Int. J. Plant Genomics* **2008**, *2008*, doi:10.1155/2008/619832.
- 42. Johnston, S. Texas A & M University, College Station, TX, USA, personal communication, 2011.
- Wayadande, A.; Bruton, B.; Fletcher, J.; Pair, S.; Mitchell, F. Retention of cucurbit yellow vine disease bacterium *Serratia marcescens* through transstadial molt of vector *Anasa tristis* (Hemiptera: Coreidae). *Ann. Entomol. Soc. Am.* 2009, *98*, 770–774.
- 44. Boucias, D.G.; Garcia-Maruniak, A.; Cherry, R.; Lu, H.; Maruniak, J.E.; Lietze, V.-U. Detection and characterization of bacterial symbionts in the Heteropteran, *Blissus insularis*. *FEMS Microbiol. Ecol.* **2012**, *82*, 629–641.
- Hail, D.; Lauziere, I.; Dowd, S.E.; Bextine, B. Culture independent survey of the microbiota of the Glassy-winted sharpshooter (*Homalodisca vitripennis*) using 454 pyrosequencing. *Environ. Entomol.* 2011, 40, 23–29.
- Koga, R.; Meng, X.-Y.; Tsuchida, T.; Fukatsu, T. Cellular mechanism for selective vertical transmission of an obligate insect symbiont at the bacterocyte-embryo interface. *Proc. Natl. Acad. Sci. USA* 2012, *109*, 7697–7598.
- 47. Boucias, D. University of Florida, Gainesville, FL, USA, personal communication, 2012.
- Brucker, R.M.; Funkhouser, L.J.; Setia, S.; Pauly, R.; Bordenstein, S.R. Insect innate immunity database (IIID): An annotation tool for identifying immune genes in insect genomes. *PLoS One* 2012, 7, e45125.
- 49. Blander, J.M.; Sander, L.E. Beyond pattern recognition: Five immune checkpoints for scaling the microbial threat. *Nat. Revs. Immunol.* **2012**, *12*, 215–225.
- 50. Jiang, H.; Vilcinskas, A.; Kanost, M.R. Immunity in Lepidopteran insects. In *Invertebrate Immunity*; Springer: New York, NY, USA, 2011; pp. 163–180.

- 51. Stanley, D.; Haas, E.; Miller, J. Eicosanoids: Exploiting insect immunity to improve biological control programs. *Insects* **2012**, *3*, 492–510.
- 52. Stanley, D.W. Eicosanoids: Progress towards manipulating insect immunity. J. Appl. Entomol. 2011, 135, 534–545.
- Garcia, E.S.; Castro, D.P.; Figueiredo, M.B.; Genta, F.A.; Azambuja, P. *Trypanosoma rangeli*: A new perspective for studying the modulation of immune reactions of *Rhodnius prolixus*. *Parasites Vectors* 2009, 2, doi:10.1186/1756-3305-2-33.
- 54. Kanost, M.R.; Gorman, M.J. Phenoloxidases in insect immunity. In *Insect Immunology*, 1st ed.; Beckage, N., Ed.; Academic Press: New York, NY, USA, 2008; pp. 69–96.
- 55. Nappi, A.J.; Christensen, B.M. Melanogenesis and associated cytotoxic reactions: Applications to insect innate immunity. *Insect Biochem. Mol. Biol.* **2005**, *35*, 443–449.
- 56. Shelby, K.S.; Popham, H.J.R. Plasma phenoloxidase of the larval tobacco budworm, *Heliothis virescens*, is virucidal. J. Insect Sci. 2006, 6, doi:10.1673/2006\_06\_13.1.
- 57. Schmidt, O.; Soderhall, K.; Theopold, U.; Faye, I. Role of adhesion in arthropod immune recognition. *Ann. Rev. Entomol.* **2010**, *54*, 485–504.
- Broderick, N.A.; Welchman, D.P.; Lemaitre, B. Recognition and response to microbial infection in *Drosophila*. In *Insect Infection and Immunity: Evolution, Ecology, and Mechanisms*; Rolff, J., Reynolds, S.E., Eds.; Oxford University Press: New York, NY, USA, 2009; pp. 13–33.
- 59. Renwick, J.; Reeves, E.P.; Wientjes, F.B.; Kavanagh, K. Translocation of proteins homologous to human neutrophil p47phox and p67phox to the cell membrane in activated hemocytes of *Galleria mellonella*. *Dev. Comp. Immunol.* **2007**, *31*, 347–359.
- Bruton, B.D.; Mitchell, F.; Fletcher, J.; Pair, S.D.; Wayadande, A.; Melcher, U.; Brady, J.; Bextine, B.; Popham, T.W. *Serratia marcescens*, a phloem-colonizing squash bug-transmitted bacterium: Causal agent of cucurbit yellow vine disease. *Plant Dis.* 2003, 87, 937–944.
- 61. Rascoe, J.B.M.; Melcher, U.; Mithcell, F.L.; Bruton, B.D.; Pair, S.D.; Fletcher, J. Identification, phylogenetic analysis, and biological characterization of *Serratia marcescens* strains causing curcurbit yellow vine disease. *Phytopathology* **2003**, *93*, 1233–1239.
- Ali, A.; Abdalla, O.; Bruton, B.; Fish, W.; Sikora, E.; Zhang, S.; Taylor, M. Occurence of viruses infecting watermelon, other cucurbits and weeds in the parts of Southern United States. *Plant Health Progress* 2012, doi:10.1094/PHP-2012-0824-01-RS.
- Perera, O.P.; Snodgrass, G.L.; Allen, K.C.; Jackson, R.E.; Becnel, J.J.; O'Leary, P.F.; Luttrell, R.G. The complete genome sequence of a single-stranded RNA virus from the tarnished plant bug, *Lygus lineolaris* (Palisot de Beauvois). *J. Invertebrate Pathol.* 2012, *109*, 11–19.
- 64. Liu, S.; Vijayendran, D.; Bonning, B.C. Next generation sequencing technologies for insect virus discovery. *Viruses* **2011**, *3*, 1849–1869.
- Wu, Q.; Luo, Y.; Lu, R.; Lau, N.; Lai, E.C.; Li, W.-X.; Ding, S.-W. Virus discovery by deep sequencing and assembly of virus-derived small silencing RNAs. *Proc. Natl. Acad. Sci. USA* 2010, 107, 1606–1611.
- Vodovar, N.; Goic, B.; Blanc, H.; Saleh, M.-C. *In silico* reconstruction of viral genomes from small RNAs improves virus-derived small interfering RNA profiling. *J. Virol.* 2011, *85*, 11016–11021.

- 67. Ma, M.; Huang, Y.; Gong, Z.; Zhuang, L.; Li, C.; Yang, H.; Tong, Y.; Liu, W.; Cao, W. Discovery of DNA viruses in wild-caught mosquitoes using small RNA high throughput sequencing. *PLoS One* **2011**, *6*, e24758.
- Rosario, K.; Breitbart, M. Exploring the viral world through metagenomics. *Curr. Opin. Virol.* 2011, 1, 289–297.
- 69. Ng, T.F.F.; Duffy, S.; Polston, J.E.; Bixby, E.; Vallad, G.E.; Breitbart, M. Exploring the diversity of plant DNA viruses and their satellites using vector-enabled metagenomics on Whiteflies. *PLoS One* **2011**, *6*, e19050.
- Cox-Foster, D.L.; Conlan, S.; Holmes, E.C.; Palacios, G.; Evans, J.D.; Moran, N.A.; Quan, P.L.; Briese, T.; Hornig, M.; Geiser, D.M.; *et al.* A metagenomic survey of microbes in honey bee colony collapse disorder. *Science* 2007, *318*, 283–287.
- Ng, T.F.F.; Willner, D.L.; Lim, Y.W.; Schmieder, R.; Chau, B.; Nilsson, C.; Anthony, S.; Ruan, Y.; Rohwer, F.; Breitbart, M. Broad surveys of DNA viral diversity obtained through viral metagenomics of mosquitoes. *PLoS One* 2011, *6*, e20579.
- Al Rwahnih, M.; Daubert, S.; Urbez-Torres, J.R.; Cordero, F.; Rowhani, A. Deep sequencing evidence from single grapevine plants reveals a virome dominated by mycoviruses. *Arch. Virol.* 2011, *156*, 397–403.
- Coaetzee, B.; Freeborough, M.-J.; Maree, H.J.; Celton, J.-M.; Reese, D.J.G.; Burger, J.T. Deep sequencing analysis of viruses infecting grapevines: Virome of a vineyard. *Virology* 2010, 400, 157–163.
- Li, L.; Victoria, J.G.; Wang, C.; Jones, M.; Fellers, G.M.; Kunz, T.H.; Delwart, E. Bat guano virome: Predominance of dietary viruses from insects and plants plus novel mammalian viruses. *J. Virol.* 2010, 84, 6955–6965.
- 75. Li, Z.; Zhang, Z.; Yan, P.; Huang, S.; Fei, Z.; Lin, K. RNA-Seq improves annotation of proteincoding genes in the cucumber genome. *BMC Genomics* **2011**, *12*, doi:10.1186/1471-2164-12-540.
- Sattar, S.; Song, Y.; Anstead, J.A.; Sunkar, R.; Thompson, G.A. *Cucumis melo* microRNA expression profile during herbivory in a resistant and susceptible interaction. *Mol. Plant Microbe Interact.* 2012, 25, 839–848.

© 2013 by the authors; licensee MDPI, Basel, Switzerland. This article is an open access article distributed under the terms and conditions of the Creative Commons Attribution license (http://creativecommons.org/licenses/by/3.0/).