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RESEARCH ARTICLE

Venom gland components of the ectoparasitoid wasp, *Anisopteromalus calandrae*

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ABSTRACT

The wasp *Anisopteromalus calandrae* is a small ectoparasitoid that attacks stored product pest beetle larvae that develop inside grain kernels, and is thus a potential insect control tool. The components of *A. calandrae* venom have not been studied, but venom from other organisms contains proteins with potential applications, such as pest management tools and treatments for human diseases. We dissected female *A. calandrae* and collected venom and associated glands. Using high throughput sequencing, a venom gland transcriptome was assembled that contained 45,432 contigs, 25,726 of which had BLASTx hits. The majority of hits were to *Nasonia vitripennis,* an ectoparasitoid from the same taxonomic family, as well as other bees, wasps, and ants. Gene ontology grouped sequences into eleven molecular functions, among which binding and catalytic activity had the most representatives. In this study, we highlighted the most abundant sequences, including those that are likely the functional components of the venom. Specifically, we focused on genes encoding proteins potentially involved in host developmental arrest, disrupting the host immune system, host paralysis, and transcripts that support these functions. Our report is the first to characterize components of the *A. calandrae* venom gland that may be useful as control tools for insect pests and other applications.

KEYWORDS: *Anisopteromalus calandrae,* venom transcriptome, venom gland, ectoparasitoid, insect pests

INTRODUCTION

Parasitoid wasps are a diverse group of insects with an estimated 150,000–600,000 species that utilize a large variety of host insects, at different developmental stages, and on different parts of the host (Quicke, 1997). Parasitoid wasps are generally divided into two main categories, endoparasitic and ectoparasitic. Endoparasitic wasps deposit an egg or eggs inside the host, while ectoparasitic wasps deposit one or multiple eggs and complete development on the cuticle of the host. It is hypothesized the main effect of endoparasitoid wasp venom is temporary paralysis and immune suppression of the host to protect the wasp's offspring from encapsulation or other host immune responses (Parkinson et al, 2002b; Vincent et al, 2010; Mortimer et al, 2012). The primary survival strategies of ectoparasitoid wasps are to alter host development and metabolism to provide nutritional resources for their offspring (Rivers and Denlinger, 1994; Nakamatsu and Tanaka, 2003). Although wasp strategies differ between types of parasitoid and species, in all cases the parasitoid must manipulate the host to make the environment conducive to its own needs (Rivers and Denlinger, 1995; Richards and Parkinson, 2000; Parkinson et al, 2001; Rivers et al, 2002; Danneels et al, 2010; de Graaf et al, 2010a).

Changes in host physiology after parasitoid attack include specific responses, such as developmental arrest, paralysis, changes in lipid storage, and suppressed immune response (Beckage and Gelman, 2004). The ectoparasitic wasp genus

Euplectrus injects venom that prevents molting and evokes the premature appearance of storage proteins in the hemolymph of the host, presumably for the benefit of parasitoid nutrition (Coudron and Brandt, 1996). The greater wax moth, *Galleria mellonella*, becomes completely paralyzed after attack from *Bracon hebetor*, and paralysis occurs with as little as one part venom to 200 million parts host hemolymph (Weaver et al, 2001). A recent paper followed the transcriptional changes within flesh flies after attack from the ectoparasitoid wasp *Nasonia vitripennis* and found a large group of developmental genes reduced in expression compared to normally developing flies (Martinson et al, 2014). The authors identified a family of genes from the *enhancer of split* complex that may be involved in initiating apoptosis of neural tissue, in turn causing host development arrest.

Studies have also looked at the venom components responsible for the changes in host phenotype. In *N. vitripennis,* 79 venom proteins were identified; over half were novel proteins not yet associated with wasp venom (de Graaf et al, 2010a). The most highly represented proteins were serine proteases and their inhibitors (Danneels et al, 2010; de Graaf et al, 2010a). Similar proteins also were found in the endoparasitoid wasp *Chelonus inanitus* using an expressed sequence tag and proteomic approach (Vincent et al, 2010). Using transcriptomics and proteomics, Colinet et al (2014) identified 16 venom proteins from the aphid parasitoid wasp, *Aphidius ervi*, including three γ-glutamyl transpeptidases that were the most abundant and involved in intracellular redox status, cytosolic iron metabolism, and inflammation. A functional study of the ectoparasitic wasp *Eulophus pennicornis*, a parasitoid of late-instar larvae of *Lacanobia oleracae*, identified a group of metalloproteinases (EpMP1-3). Injecting host larvae with recombinant EpMP3 resulted in host inability to appropriately molt and induced partial mortality (Price et al, 2009).

At the molecular level, most of the available literature is found within the *Drosophila melanogaster*/endoparasitoid system. Injection of a wasp egg into the *D. melanogaster* cuticle elicits a series of immune pathways including Toll, prophenoloxidase (proPO), Imd, JakStat, JNK, etc., ultimately leading to lamellocyte migration, encapsulation, and melanization of the wasp egg (Rizki and Rizki, 1990; Sugumaran, 2002; Sorrentino et al, 2004; Matova and Anderson, 2006; Tang et al, 2006). This specific cascade is not observed in ectoparasitoid wasps, but general host immune responses are likely similar.

Because of their chemical properties, venom proteins can benefit humans via compounds to fight disease. There are several examples of medicinal venom compounds isolated from snakes (Koh et al, 2006), scorpions (Ding et al, 2014), and spiders (Saez et al, 2010). For example, cobra venom is being developed into therapeutics to help those who suffer from arthritis (Gomes et al, 2010), antitumor-analgesic peptide found in the Chinese scorpion has anti-cancer properties (Liu et al, 2003), and GsMtx-4 in the Chilean rose tarantula has properties to reduce pain associated with surgery, labor, inflammation, and burns (Park et al, 2008). In insects, proteins from the ant (*Pachycondyla goeldii*) and the honeybee (*Apis mellifera*) also are being examined for medicinal potential. *P. goeldii* is part of the Ponerinae subfamily, with peptides appropriately named ponericins, exhibiting antibacterial and insecticidal properties (Orivel et al, 2001). Similarly, the honeybee has antibacterial compounds, apidaecins, which are induced in response to infection and are active against a range of human pathogens (Casteels et al, 1989). Honeybee venom also has potential in cancer therapy (Orsolic, 2012). However, in contrast to parasitoid wasps, these organisms use venom to stun or kill prey, and do not deposit an egg on or within the host. Thus, these venoms may be less complex than those of parasitoids.

The ectoparasitoid wasp *Anisopteromalus calandrae* is a cosmopolitan wasp that shares a family and subfamily (Pteromalidae and Pteromalinae, respectively) with *N. vitripennis*, the current wasp model with a sequenced genome and other genetic tools (Werren et al, 2010). *A. calandrae* is a small (2.25 mm) ectoparasitoid that attacks late-instar stored product pest beetle larvae that develop inside grain kernels, such as *Sitophilus zeamais* (maize weevil) and *Rhyzopertha dominica* (lesser grain borer). The female wasp locates a grain kernel that contains a host larva, drills into the kernel with her ovipositor, injects a venom cocktail into the host, and deposits an egg on the larva cuticle covering the injection site. Egg placement is perhaps to give the newly hatched larva a place to attach its mouthparts. The wasp's venom cocktail induces changes within the host to allow for development of the parasitoid offspring. When the parasitoid larva hatches, it uses the host nutrients for the remainder of its development. The most noticeable phenotypic effect of the venom in the host larva is paralysis, important for the placement of the egg on the host cuticle, and in some cases, allows for host feeding by the female wasp as well (Gokhman et al, 1999). The molecular strategy of the wasp venom and the host's molecular reaction is unknown.

A. calandrae is capable of significantly reducing host populations, and is being used as a biological control agent in the U.S. and other countries (Ahmed, 1996; Ngamo et al, 2007). Despite its common agricultural use, the mechanism by which the wasp controls the host and the components of the venom cocktail are unknown. In this study, we used transcriptome sequencing to analyze the venom gland, venom reservoir, Dufour gland, and ovipositor of female *A. calandrae*. This work is the first to describe the venom transcriptome of a stored product insect parasitoid wasp. We highlight transcripts in highest abundance and those most likely to be the functional components of the venom. We mainly focus on transcripts that encode proteins with potential to paralyze, alter developmental time, or inhibit the immune response of the host.

MATERIALS AND METHODS

Insect cultures

A. calandrae and its host were kept in mass culture at the Center for Grain and Animal Health Research, in a chamber maintained at 30 ±1°C, 65% RH, and 16L:8D photoperiod. Wasps were cultured on wheat infested with *Rhyzopertha dominica* as a host. To produce a cohort of beetle larvae, 100 adult beetles were placed in 1 L jars filled with wheat and allowed to oviposit. After 1 week, adults were removed

from the wheat using a sieve and the jars were returned to the chamber. After 2–4 weeks the wheat kernels containing developing beetle larvae (third-fifth instar) were divided into 0.25 L portions and placed in 0.5 L jars. Twenty wasps of mixed sex were added to each of the jars. Wasp progeny emerged after about 12 days.

Dissections

Female wasps were aspirated from culture and placed into a small glass vial for dissection within one wk of emerging. Wasps were placed on ice until non-mobile and were dissected in phosphate buffered saline (PBS). Venom glands, venom reservoir, ovipositor, and Dufour gland were removed with fine-tipped forceps by gently pulling on the ovipositor (Howard and Baker, 2003). Approximately 30–50 tissue sets were placed in a 1.5ml centrifuge tube with 100㎖ RNA*later* (Qiagen, Venlo, Limburg). A total of 150 females were dissected. Groups of venom glands and associated tissues were pulled toward the bottom of the tube via low-speed quick centrifugation to ensure all tissues were covered with RNA-*later* and kept at 4°C until processed.

Sample preparation and sequencing

Samples were centrifuged quickly at low speed and excess RNA*later* was removed from the top of the tube. Samples were covered in TRIZOL and cells were disrupted (BulletBlender, Next Advance Inc., Averill Park, NY, USA) for 2 min at setting 8. Tubes were spun briefly and supernatant was collected. Total RNA was recovered using a mini prep kit (ZymoResearch, Irvine, CA, USA).

Samples were pooled and mRNA was collected using Agilent Technology Dynabeads mRNA DIRECT kit (Santa Clara, CA, USA). cDNA libraries were prepared via a 400 bp RNAseq v2 kit according to the standard input protocol (Life Technologies, Grand Island, NY, USA). The resulting sample was divided into two, diluted, and sequenced on a Personal Genome Machine with 318v2 chip (PGM, Life Technologies) as technical replicates.

Data analysis

Sequences were assembled into contigs using DNASTAR SeqMan NGen (Lasergene Genomics Suite v12.0.9, Madison, WI, USA), resulting in 45,432 contigs with an N50 of 848 bases. Raw contigs have been uploaded to NCBI Sequence Read Archive (accession number PRJNA301414). Contigs were submitted to BLAST2GO PRO CloudBlast (B2GO, Valencia, Spain) using a BLAST strategy to maximize resources. Initially, we limited BLASTx to only the arthropod database and hits to the top 5, $<e^{-10}$. A second BLASTx of non-hits to all databases (nr) also was limited to 5 top hits and $<e^{-3}$. Contigs with BLAST hits were mapped to GO terms, and submitted to InterProScan in B2GO PRO. BLAST statistics were obtained in B2GO PRO. Data files were compared to the contig file to calculate the number of transcripts per contig using Q-seq (DNASTAR Lasergene Genomics Suite v12.0.9, Madison, WI, USA). Individual sequences of interest were translated via B2GO PRO, and conserved domains were found with either B2GO PRO or NCBI conserved Domain Search (Marchler-Bauer et al, 2015). Specific translated contigs of interest were submitted to the protein database at NCBI, using BLASTp. Protein alignments were assembled via MUSCLE in either MEGA (Tamura et al, 2013) or DNASTAR MegAlign Pro (DNASTAR Lasergene Genomics Suite v12.0.9, Madison, WI, USA). Maximum likelihood phylogenetic trees were made in MEGA using default parameters and 500 iterations of bootstrapping analysis.

RESULTS AND DISCUSSION

Sequence statistics

Venom glands from *A. calandrae* were pooled and incorporated into one library, and two technical replicates provided almost 8 million reads with average sequence length of 125 base pairs. Sequences were assembled and aligned into 45,432 contigs, and BLASTx analysis found 25,726 contigs with hits to the NCBI database ($>e^{-3}$). Analysis with BLAST2GO PRO (Götz et al, 2008) indicated that 22,412 had conserved domains reported in InterProScan (SIMAP database (Götz et al, 2011)), and 17,000 had annotations (Figure 1A). The top five sequence hits for each contig were most common to *N. vitripennis*, accounting for over 35,000 hits, followed distantly by *Apis mellifera* (western honeybee), *Megachile rotundata* (leafcutter bee), *Microplitis demolitor* (parasitoid wasp), among others (Figure 1B). Level two, gene ontology (GO) grouped sequences into eleven molecular functions (Figure 1C), of which binding and catalytic activity had the most representatives.

Most abundant transcripts

Among the most abundant sequences were those with descriptions and functions related to muscle and muscle contraction, including troponin and myosin (Table 1). Troponin C in insects is found in thick muscle bands that give prolonged contraction without the expenditure of much energy. In the *A. calandrae* venom gland, we suggest that these proteins are used to contract muscles around the gland and are associated with the ovipositor, controlling the injection of venom into the host tissue. The function of troponin C in the venom gland also was hypothesized in Colinet et al, (2014), where sequences with predicted muscle function were predicted for pumping and injecting venom during oviposition. Other highly represented transcripts included cytochrome p450, alpha-amylase, carrier protein, mitochondrial ATP synthase subunit, fructose-bisphosphate aldolase-like, and trypsin, most likely involved in cellular maintenance and general housekeeping. Interestingly, a virus polyprotein also was highly expressed in the dataset. The virus is a positive-sense single-stranded RNA virus from the family *Iflaviridae* and was identified in *N. vitripennis* (NvitV-1) as a persistent commensal infection (Oliveira et al, 2010). Thus, this virus likely also infects the closely related *A. calandrae.*

Venom-related transcripts

A large portion of *A. calandrae* contigs encoded venom proteins (D, F, G, H, I, K, L, N, O, Q, T, U, V, X, Z; Table 2), each letter corresponding to venom proteins of *N. vitripennis* with unidentified functions (de Graaf et al, 2010a). Some transcripts were found in multiple contigs (listed in Table 2), but most were short fragments or fragments of the full-length transcript (see example in Supplemental Figure 1). In this study, the read count we report includes full-length and partial transcripts. Thus, the overall read count may be inflated, and only the most complete contig(s) are described in this section.

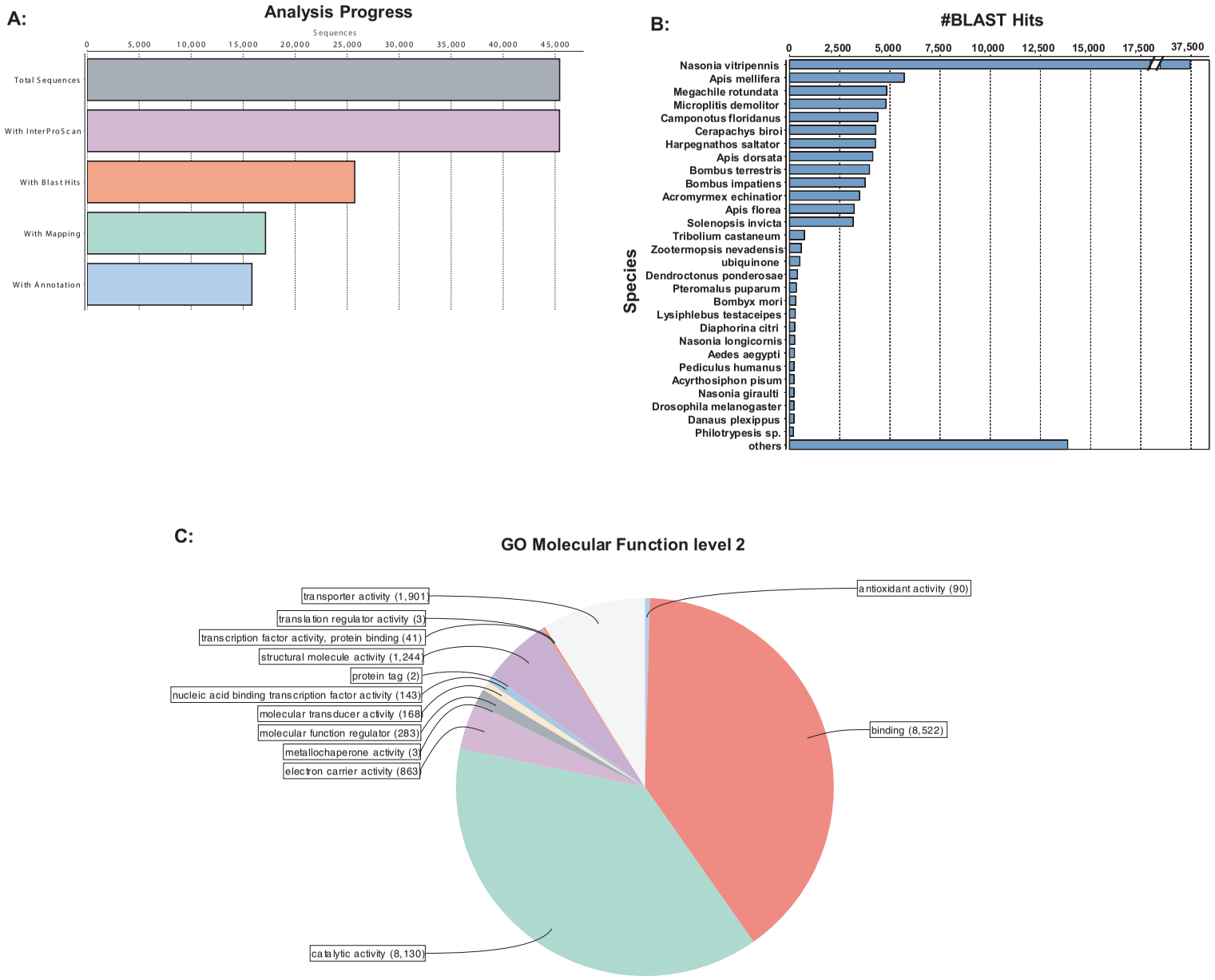

Figure 1. BLASTx statistics for the *A. calandrae* transcriptome assembly. **A.** Total number of contig sequences, InterProScan results (hits solid purple and no hits hashed purple), BLASTx hits, Mapping, and Annotations. **B.** Distribution of species in BLASTx hits. Most sequences from *A. calandrae* hit the closely related wasp *N. vitripennis.* **C.** Pie chart representing the GO term distribution at level 2.

Table 1. Top 17 most highly represented sequences in the *A. calandrae* venom gland transcriptome. Sequence description was assigned via BLAST2GO PRO corresponding to contig numbers in our database. Conserved domains were identified via InterProScan. Number of reads includes full-length transcripts as well as sequence fragments. Superscript denotes isoforms and corresponding predicted proteins.

Sequence description	Contig Numbers	Conserved domain or protein family (accession)	Number of reads	Top BLASTx genus/ species	Predicted protein(s)	Potential function/description
Long form-like- myosin tail	105	Myosin tail_1 (pfam01576)	63,530	*Nasonia vitripennis*	XP_008213931.1	Muscle
Tropomyosin-1-like isoform 1	127[1], 16[2]	Tropomyosin (pfam00261)	53,729	*Harpegnathos saltator*[1] *Nasonia vitripennis*[2]	EFN78348.1[1] XP_001599003.1[2]	Muscle
Myosin heavy chain	109	Myosin tail (pfam01576)	52,656	*Camponotus floridanus*	EFN74639.1	Muscle
Hypothetical protein TcasGA2_ Tc010626	1	Retrotransposon (cd01650)	48,750	*Tribolium castaneum*	EFA11647.1	-
Myosin regulatory light chain 2-like	24	EF-hand domain (pfam13405)	43,247	*Nasonia vitripennis*	XP_008207464.1	Muscle
Troponin C-like	470	EF-hand domain (ps50222)	31,029	*Bombus terrestris*	XP_003399334.1	Muscle
Myosin light chain alkali	127	Tropomyosin (pfam00261)	30,910	*Harpegnathos saltator*	EFN78348.1	Muscle
Cytochrome p-450 4g15-like	57	Cytochrome P450 family (pfam00067)	26,448	*Nasonia vitripennis*	CYP4G43	Degradation of environmental toxins
Alpha-amylase-like isoform x1	110	Alpha amylase (pfam00128)	25,751	*Nasonia vitripennis*	XP_001602184.1	Hydrolyse carbohydrates
Beta- partial	168	Actin-related protein (pfam00022)	24,716	*Cerapachys biroi*	EZA62929.1	Muscle
Troponin I isoform x14	13	Troponin (pfam00992)	24,666	*Nasonia vitripennis*	XP_008213406.1	Muscle
Trypsin beta	48	Peptidase S1 (pfam00089)	21,551	*Nasonia vitripennis*	XP_001600807.1	Proteolysis
Carrier protein 2-like	76	Mitochondrial carrier protein (pfam00153)	21,500	*Nasonia vitripennis*	XP_001606673.1	-
ATP synthase subunit mitochondrial	84	ATP-synt_ab (pfam00006)	21,093	*Nasonia vitripennis*	NP_001153366.1	Energy production
Fructose-bisphosphate aldolase-like	35	Glycoytic (pfam00274)	21,077	*Nasonia vitripennis*	NP_001164328.1	Energy metabolism
Cytochrome B	288	Cytochrome b(C-terminal) (pfam00032)	20,805	*Nasonia vitripennis*	ACH81752.1	Electron transport
Polyprotein	219	Serine peptidase domain (SSF20494)	20,636	*Nasonia vitripennis* virus	ACN94442.1	Virus

Table 2. Venom-related transcripts found in *A. calandrae* venom gland. Sequence description was designated by BLAST2GO PRO, corresponding to contig numbers in our database. Conserved domains were identified via InterProScan. Number of reads includes full-length and partial transcripts identified in each sequence description. Superscript denotes isoforms and corresponding predicted proteins.

				Top BLASTx hit		
Sequence description	**Contig Number**	**Conserved domain or protein family (accession)**	**Number of reads**	**Species**	**Predicted Protein(s)**	**Potential function/description**
Small serine proteinase inhibitor-like venom protein	208, 621	Pacifastin I (pfam05375)	9,101	*Nasonia vitripennis*	NP_001155083	Inhibit PO activation
Cysteine-rich pacifastin venom protein 1 and 2	15637, 40965	Pacifastin inhibitor (pfam05375)	35	*Nasonia vitripennis*	XP_003425794	Inhibit PO activation
Serpin 5	10452	Serine protease inhibitor	1,195	*Camponotus floridanus*	EFN72133	Inhibit PO activation
Cysteine-rich ku venom protein 1 and 2	13933, 14273, 21756	BPTI/Kunitz family of serine protease inhibitors (cd00109)	1,099	*Nasonia vitripennis*	NP_001154998	Inhibit PO activation
Kazal type serine protease inhibitor-like venom protein 1	1226, 173	Kazal-type serine protease inhibitor and follistatin-like domain (cd00104)	10,906	*Nasonia vitripennis*	NP_001154995	Interfere with coagulation/ antimicrobial
Kazal type serine protease inhibitor-like venom protein 2	353	Kazal-type serine protease inhibitor and follistatin-like domain (smart00280)	6,384	*Nasonia vitripennis*	NP_001164350	Interfere with coagulation/ antimicrobial
Kazal-type proteinase inhibitor-like protein	3089	Kazal-type serine protease inhibitor and follistatin-like domain (smart00280)	2,743	*Nasonia vitripennis*	XP_001600330	Interfere with coagulation/ antimicrobial
Chymotrypsin inhibitor-like	11459	Trypsin inhibitor like cysteine rich domain (pfam01826)	418	*Nasonia vitripennis*	XP_001607361	-
Venom peptide-2-like	1466	Trypsin inhibiotor like cysteine rich domain (pfam01826)	254	*Nasonia vitripennis*	XP_001607361	-
Venom peptide – like	32786	--NA--	12	*Nasonia vitripennis*	XP_008215348	-
Venom serine protease	29876	Trypsin-like serine protease inhibitor (cd00190)	16	*Nasonia vitripennis*	XP_001604187	-
Venom protease- like	43584	Tryp_SPc; Trypsin-like serine protease (cd00190)	10	*Nasonia vitripennis*	XP_001602546	Inhibit encapsulation
Calreticulin	754, 1607, 2209	Calreticulin/calnexin (pfam00262)	5,713	*Nasonia vitripennis*	NP_001155151	Inhibition of hemocyte spreading factor/ suppression of encapsulation
C1-q-like venom protein	20942, 26203	C1q domain (pfam00386)	111	*Nasonia vitripennis*	NP_001155152	Trace element
Venom serine carboxypeptidase isoform x1 & 2	33255[1], 33448[2]	Serine carboxypeptidase (pfam00450)	31	*Nasonia vitripennis*	XP_003424792[1] XP_001602950[2]	Venom allergen
Venom dipeptidyl peptidase 4 isoform x1-3	1633, 15069, 7588, 38117, 25635	Dipeptidyl peptidatse IV N-terminal region (pfam00930)	3,612	*Nasonia vitripennis*	XP_008204459-63	Venom allergen
Venom acid phosphatase acph-1-like	270	Histidine phosphatase superfamily (pfam00328)	24,002	*Nasonia vitripennis*	NP_001155147	Venom allergen/dephosphorylation of immune proteins
Venom acid phosphatase-like	2473[1], 4597[2],327[1]	Histidine phosphatase superfamily (pfam00328)	2,434	*Nasonia vitripennis* *Camponotus floridanus*	NP_001155147 EFN70392	Venom allergen /dephosphorylation of immune proteins
Venom acid phosphatase acph-1-like isoform 1	26605, 30699	Histidine phosphatase superfamily (pfam00328) & Histidine phosphatase domain (cd07061)	73	*Nasonia vitripennis*	XP_00160542	Venom allergen /dephosphorylation of immune proteins / metabolism, signaling, or regulation

Vitellogenin-like	162	Low quality protein	34,806	*Nasonia vitripennis*	XP_001607388	Venom allergen
Endonuclease-like venom protein	1954, 6277	Endonuclease NS (smart00892) DNA/RNA nonspecific	6,605	*Nasonia vitripennis*	NP_001155087	Cleave nucleic acid
Endonuclease domain-containing protein	1904	DNA/RNA non-specific endonuclease (pfam01223)	3,719	*Nasonia vitripennis*	XP_001601224	Cleave nucleic acid
Poly-specific endoribonuclease homolog	6154	Endoribonuclease XendoU (pfam09412)	1,589	*Nasonia vitripennis*	XP_001606738	Cleave nucleic acid
Venom protein a1yi24cm3	529, 9307	SCP_euk (cd05380)	786	*Chelonus inanitus*	CBN72521	endopeptidases
Venom metalloproteinase 2-like	3993	---NA---	534	*Nasonia vitripennis*	XP_008206471	Developmental arrest
Venom metalloproteinase 3-like	308[1], 5633[2]	Zinc –dependent metalloprotease, salivary glands of arthropods (cd04272)	10,340	*Nasonia vitripennis*	XP_003426007[1] XP_008213426[2]	Developmental arrest/ break down gelatin, fibrin, fibronectin
A disintegrin and metalloproteinase with thrombospondin motifs 1	5386	Reprolysin (pfam01421)	1,563	*Acromyrmex echinatior*	EGI57486	Cleaves peptidases, snake venom endopeptidases
γ-glutamyl cyclotransferase-like venom protein isoform x1	34508[1], 36225[2]	GGCT-like domain (cd06661)	34	*Nasonia vitripennis*	NP_001155144[1] NP_001155145[2]	Apoptosis of host germ cells
Venom allergen	13197[1], 3104[2]	SCP-like extracellular protein domain (cd05380)	94	*Nasonia vitripennis*[1] *Megachile rotundata*[2]	NP_001155154[1] XP_003708569[2]	Venom allergen
Venom allergen 3	30907	CAP Cysteine-rich secretory protein (pfam00188) (ant venom)	46	*Tribolium castaneum*	XP_970898	Potent allergens in that mediate allergic reactions
Venom allergen 3-like	2046	SCP_euk (cd05380)	3,376	*Nasonia vitripennis*	XP_008214984	endopeptidases
Venom allergen 5	7323	SCP_euk (cd05380)	24	*Nasonia vitripennis*	XP_008214984	endopeptidases
Venom allergen 5-like	133	---NA--- (probably cd05380)	10,612	*Microplitis demolitor*	XP_008545886	---NA---
Venom carboxylesterase-6	13545[1], 10623[1], 12603[2], 13047[2], 14514[2], 24778[3]	COesterase; carboxylesterase family (pfam00135)	3,033	*Nasonia vitripennis*	NP_001155148[1] XP_001601401[2] XP_001599255[3]	CCE-B21
GOBP-like venom protein	142, 2480, 2519, 8481	PBP/GOBP family; olfactory receptors (pfam01395)	11,338	*Nasonia vitripennis*	NP_001155150	OBP67; odor binding
Chemosensory protein 1	254	Insect odorant-binding protein A10	17,818	*Nasonia vitripennis*	XP_008208395	Odor binding
Corticotropin-releasing factor-binding	528	CRF-BP (pfam05428)	2,012	*Nasonia vitripennis*	XP_008204599	Stress response
Growth hormone-inducible transmembrane	1489	Bax inhibitor 1-related (pfam01027)	1,340	*Nasonia vitripennis*	XP_001608127	Molting and metamorphosis
A chain crystal structure of bee venom hyaluronidase	30340, 27737	Hyaluronidase family 56 (pfam01630)	93	*Nasonia vitripennis*	XP_003426664	Venom spreading factor
Arginine kinase	1396[1], 1658[1] 297[2]	Arginine kinase-like (cd07932)	14,776	*Pteromalus puparum* *Nasonia vitripennis*	ACZ68114[1] XP_008214788[2]	Paralysis

(Continued)

Table 2. Continued

				Top BLASTx hit		
Sequence description	**Contig Number**	**Conserved domain or protein family (accession)**	**Number of reads**	**Species**	**Predicted Protein(s)**	**Potential function/description**
Superoxide dismutases 1 and 3	2375[1], 6075[2]	Copper/zind superoxide dismutase (pfam00080)	2,692	*Nasonia vitripennis*	XP_008216838[1] XP_001608103[2]	Conversion of superoxide radicals to hydrogen peroxide and molecular oxygen
Peroxiredoxin 1	261		1,065	*Apis florea*	XP_003698912	Protect against oxidative stress
Chitin binding venom protein	8670	Chitin-binding domain type 2 (smart00494)	636	*Nasonia vitripennis*	NP_001164343	Interfere with chitin biosynthesis
Low-density lipoprotein receptor-like venom protein	13731	Complement control protein (CCP) modules (cd00033)	110	*Nasonia vitripennis*	NP_001155040	Mediate endocytosis of yolk protein s
Venom protein D	2643, 6422, 6955	--NA--	1,667	*Nasonia vitripennis*	NP_001155171	-
Venom protein F isoform x1	16925, 23693	Drf_FH1; Formin homology region 1 (pfam06346)	99	*Nasonia vitripennis*	XP_008209209	Polymerization of actin?
Venom protein F isoform x4	6406	--NA--	61	*Nasonia vitripennis*	XP_008209212	-
Venom protein F isoform x7	17875	Med15 (pfam09606)	44	*Nasonia vitripennis*	XP_008209215	Transcription regulation of cholesterol and fatty acid homeostasis?
Venom protein F	4393	--NA--	1,578	*Nasonia vitripennis*	XP_001155160	-
Venom protein G isoform x1	14, 33, 493	--NA--	37,500	*Nasonia vitripennis*	XP_008203717	-
Venom protein H isoform x1	6787	--NA--	267	*Nasonia vitripennis*	XP_008206613	-
Venom protein H	1020	--NA--	4,792	*Nasonia vitripennis*	NP_001155027	-
Venom protein I-like protein	11490	--NA--	149	*Nasonia vitripennis*	NP_001164345	-
Venom protein K	1345[1], 1638[2], 3202[2]	--NA--	2,104	*Nasonia vitripennis*	XP_003426464[1] NP_001155028[2]	-
Venom protein L	323, 386	--NA--	2,237	*Nasonia vitripennis*	NP_001155029	-
Venom protein N	214	--NA--	9,309	*Nasonia vitripennis*	NP_001164349	-
Venom protein O	6837	PBP/GOBP (pfam01395)	237	*Nasonia vitripennis*	NP_001155031	OBP19
Venom protein Q	2, 3, 5, 29, 41, 50, 95, 155, 199, 216, 302, 1207, 2718	Seryl-tRNA synthetase (PRK05431)	72,069	*Nasonia vitripennis*	NP_001155161	-
Venom protein T isoform x2	2489	--NA--	164	*Nasonia vitripennis*	XP_008213294	-
Venom protein T	234, 299, 2511	--NA--	15,208	*Nasonia vitripennis*	NP_001155166	-
Venom protein U	1699, 2852	--NA--	6,078	*Nasonia vitripennis*	NP_001155170	-
Venom protein V	20, 22, 777, 1289	Chaperone_ClpB (TIGR003346)	14,053	*Nasonia vitripennis*	NP_001155041	-
Venom protein X	1077	Foldase protein PrsA (PRK01326)	4,338	*Nasonia vitripennis*	NP_001155167	-
Venom protein Z	2, 3, 79, 97, 99, 154, 269	Phosphodiesterase (PRK12704)	83,453	*Nasonia vitripennis*	NP_001155169	Regulator for virulence

The most abundant transcripts encoded venom protein Z (contig 79; 10,715 reads; Table 2), highly similar to *N. vitripennis* venom protein Z (e^{-90}). A PRK12704 conserved domain was found at the 3' end of the molecule, and is described as a phosphodiesterase in the chaperone_C1pB protein family. This type of domain has been described in *Staphylococcus aureus* and is part of a novel regulator required for virulence (Nagata et al, 2008). The most abundant venom protein Q transcript, contig 2 (25,059 reads), was highly similar to *N. vitripennis* venom protein Q (e^{-71}) (Supplemental Figure 1). A PRK05431 conserved domain was identified at the 3' end of the molecule, described as seryl-tRNA synthetase. More work is needed to characterize these venom components, originally found in *N. vitripennis,* and now also identified in *A. calandrae.*

Other highly expressed transcripts in the transcriptome encoded a vitellogenin protein (contig 162, 34,806 reads), venom acid phosphatase acph-1-like (contig 270, 24,002 reads), three arginine kinases (contigs 1396, 1658, 297, 14,776 reads), four GOBP-like venom proteins (contigs 142, 2480, 2519, 8481, 11,338 reads), two metalloproteinases 3-like (contigs 308, 5633, 10,340 reads), and two Kazal-type serine protease inhibitor-like venom proteins (contigs 1226, 173, 10,906 reads, Table 2). Many of the enzymes encoded in the *A. calandrae* transcriptome have been characterized in different systems and/or identified in other insect venoms and we highlight those likely involved in venom function based on the existing literature.

Protease inhibitors

Parasitoid systems have evolved strategies to inhibit host immune pathways. The main host defense against damaged tissue, bacterial infection, or parasitoid attack is melanization. The melanization pathway encapsulates foreign material before it can cause the host further damage (Rivers et al, 2002). One pathway that leads to melanization is the prophenoloxidase (proPO) system (Cerenius and Soderhall, 2004). ProPO is found in insect hemolymph and when activated leads to antimicrobial cascades and melanization of foreign material (Figure 2). A serine proteinase cascade is activated by very small amounts of lipopolysaccharides or peptidoglycan, components of microbes, that in turn cleave prophenoloxidase-activating enzyme (Pro-ppA) to active ppA. This enzyme catalyzes the reaction that converts proPO to active PO, and in the presence of oxygen and phenols, leads to melanization. While this process is necessary for immune defense, it must be held in check, as byproducts (quinones) are highly reactive and damaging. Therefore, several types of serine protease inhibitors (SPI) are employed, including pacifastin, serpins, and Kuntz family inhibitors (Kanost, 1999). SPIs have been identified in crayfish and locust (Simmonet et al, 2002). In the crayfish pacifastin inhibits the proteolytic cascade blocking the activation of proPO (Hergenhahn et al, 1987). In the migratory locust, pacifastin was isolated from many tissues including hemolymph and fat body in both adults and larvae (Clynen et al, 2001; Kromer et al, 1994). Additionally, a *Drosophila melanogaster* mutant lacking a serpin inhibitor resulted in excessive melanization (De Gregorio et al, 2002). SPIs have been identified in parasitoid venoms as well, including *C. inanitus* (Vincent et al, 2010), *Cotesia rubecula* (Asgari et al, 2003), *N. vitripennis* (de Graaf et al, 2010a), *Pimpla hypochondriaca* (Parkinson et al, 2004), *P. puparum* (Zhu et al, 2010), and now *A. calandrae.* Thus, it is likely that

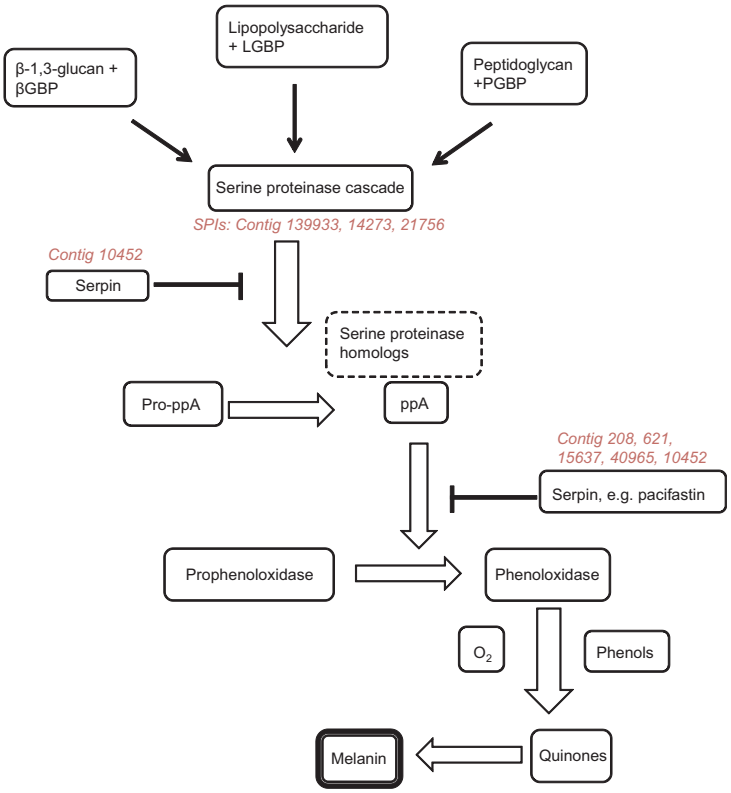

Figure 2. ProPO pathway in an insect host, adapted from Cerenius and Soderhall (2004). The ProPo pathway in the host is depicted with black colored boxes and arrows, and *A. calandrae* contigs encoding proteins with possible inhibitor action at specific points in the pathway are indicated with red text.

parasitoids take advantage of this mechanism by suppressing melanization and encapsulation of wasp material by SPIs at the ovipositor injection site and/or larval feeding site.

Specifically, we identified transcripts that encoded four groups of SPIs that are potential inhibitors of the proPO pathway: a small SPI-like venom protein (contigs 208 and 621), cysteine-rich pacifastin venom protein 1 and 2 (contigs 15637 and 40965), serpin 5 (contig 10452), and cysteine-rich ku protein 1 and 2 (contigs 13933, 14273, and 21756; Table 2). Small SPI-like venom protein transcripts were abundant in *A. calandrae* venom (9,101 total reads) and were highly similar to *N. vitripennis* protein NP_001155083. SPI-like venom protein and cysteine-rich pacifastin venom protein 1 and 2 (35 reads) had a pacifastin I conserved domain, thus likely candidates for proPO suppression. Additionally, *A. calandrae* cysteine-rich ku venom protein transcripts were similar to those in *N. vitripennis,* encoding five cysteine-rich proteins (de Graaf et al, 2010a). InterProScan identified a conserved Kunitz family of serine protease inhibitor domain (cd00109) in these sequences that consists of six conserved cysteine residues linked by three disulphide bonds. A similar protein was identified in *P. hypochondriaca* (Parkinson et al, 2004), but its function has yet to be determined.

We also identified transcripts for three Kazal-type serine protease inhibitor-like venom proteins (KPI, contigs 1226, 173, 353, and 3089, 20,033 total reads, Table 2). KPIs are typically about 40–60 amino acids long in invertebrates and can inhibit more than one protease based on variation in six conserved cysteine residues (Rimphanitchayakit and Tassanakajon, 2010). KPIs have been described in the saliva of blood sucking insects, where they interfere with coagulation factors, prolonging the blood meal (Campos et al, 2004; Friedrich et al, 1993). There is also evidence that KPIs have antimicrobial properties. Two KPIs have been identified in the wax moth (*G. mellonella*), particularly in the cocoon silk (GmSPI 1 and GmSPI2) that inhibit bacterial subtilisin and fungal proteinase K (Nirmala et al, 2001).

Transcripts encoding proteins with conserved trypsin inhibitor domain were also found in *A. calandrae* venom: chymotrypsin inhibitor, venom peptide-2-like, venom peptide-like, venom serine protease, and venom protease-like (contigs 11459, 1466, 32786, 29876, 43584, respectively, Table 2). Contigs 11459 and 1466 were similar to *N. vitripennis* venom peptide SjAPI-2-like that also has a trypsin-conserved domain. Similar proteins were found in *P. hypochondriaca* (cvp1; Parkinson et al. 2004) although function has not been determined in this wasp.

Calreticulin

In addition to melanization, insects have the ability to neutralize large foreign material via encapsulation. The encapsulation process refers to the movement of host hemocytes to a foreign object, surrounding the object, and eventually forming a smooth, melanized capsule around it. A contributor to encapsulation is calreticulin (CRT). CRT is a ubiquitous calcium-binding protein with many properties and functions in the cell. For example, CRT is involved in calcium modulation for homeostasis by binding intercellular calcium, and acts as a molecular chaperon, leading to phagocytosis, cell adhesion, cell spreading and encapsulation. CRT has been identified in a number of parasitoid systems, including *N. vitripennis* (de Graaf et al, 2010a), *Cotesia plutellae* (Cha et al, 2015), *Diadegma semiclausum* (Etebari et al, 2011), and *P. puparum* (Fang et al, 2011; Wang et al, 2013). Two recent studies have suggested CRT works as an antagonist, competing for binding sights with host CRT, thus inhibiting hemocyte encapsulation of wasp material (Cha et al, 2015; Wang et al, 2013). Down regulation of CRT was found in *Tenebrio molitor* larvae challenged with the microbial toxin Cry3Aa from *Bacillus thuringiensis* (Oppert et al, 2012). Our data in *A. calandrae* included three CRT contigs (754, 1607, and 2209) with a combined 5,713 reads (Table 2). Therefore, *A. calandrae*, may use CRT to inhibit hemocyte encapsulation of wasp material, the venom injection site, and/or the larval feeding site.

C1-q-like venom proteins

A protein was identified from *A. mellifera* with a conserved C1-q domain, with similarity to a C1-q venom protein in *N. vitripennis* and *Acyrthosiphon pisum* (de Graaf et al, 2010b). In higher order organisms, C1-q is a major link between innate and acquired immunity. In humans, CRT blocks the C1-q-immunoglobulin interaction, and it is hypothesized that CRT inhibits C1-q-hemolytic activity (Kishore et al, 1997). However, the function in venom is unknown and currently thought to be a venom trace element, or one with little expression and no direct function in venom secretion, largely based on the fact that C1-q protein is expressed in the *N. vitripennis* female and male abdomen as well as in venom, hypopharyngeal, mucus, and salivary glands, and also brain, midgut, hemocyte, and abdomen in *A. mellifera* (de Graaf et al, 2010b). Thus, it is likely that CRT and C1-q perform vital functions in insects as well, although more research is needed (Danneels et al, 2010). In this study, we found two transcripts with C1-q domains with a total of 111 reads in *A. calandrae* venom (contig 20942 and 26203, Table 2).

Peptidases

The *A. calandrae* venom transcriptome included transcripts encoding two types of exopeptidases: venom serine carboxypeptidase isoforms x1 and x2 and venom dipeptidyl peptidase IV isoform x1-3 (contigs 33255, 33448 and 1633, 15069, 7588, 38117, 25635, respectively, Table 2). Both exopeptidases have been described in honeybees as members of the nine major allergens (Kim and Jin, 2014). Venom serine carboxypeptidase is named Api m 9 (Matysiak et al, 2014) and dipeptidyl peptidase IV is termed Api m 5,in *A. mellifera*. The latter enzyme was proposed to process premelittin into its active form, melittin, one of the two most highly expressed allergens in honeybee venom (Blank et al, 2010).

We also identified a transcript encoding a trypsin-like serine peptidase, venom protease-like (contig 43584, Table 2). A similar sequence was identified in the nematode that parasitizes *Galleria mellonella* (Balasubramanian et al, 2010). The authors found that nematode trypsin affected insect hemocyte spreading and disorganized actin filaments. A similar process in *A. calandrae* is conceivable, where venom trypsin affects the host's encapsulation mechanism.

Venom acid phosphatase

We identified 34,386 transcripts with sequence descriptions similar to acid phosphatase (AP, contigs 270, 2473, 4597, 327, 26605 and 30699, Table 2), encoding venom AP-like, venom AP, and venom AP acph-1-like proteins. Venom AP is another major allergen in honeybee venom (Api m3) and was recently characterized via recombinant expression (Kim and Jin 2014). APs are also found in other venomous species, including *Bothrops jararaca* (pit viper, Carneiro et al, 2001), and other parasitoid wasps, including *N. vitripennis* (de Graaf et al. 2010a), *P. hypochondriaca* (Dani et al, 2005), and *P. puparum* where the gene has been cloned (Zhu et al, 2008). However, the function of AP in parasitoid venom is unknown. In the desert locust, *Schistocerca gregaria,* APs from an infections fungus were suggested to target the immune system by dephosphorylation of immune proteins, disabling host defenses (Xia et al, 2000).

Endonuclease

Endonucleases cleave phosphodiester bonds within a polynucleotide chain, including both RNA and DNA, single and double stranded molecules. These nucleases were identified in nematocyst venom of marine invertebrates (Neeman et al, 1980; Neeman et al, 1981) and snake venom (Georgatsos and Laskowski, 1962). A similar endonuclease to those encoded by transcripts in *A. calandrae* (contig 1954, 6277, 1904 and 6154, Table 2) is the *N. vitripennis* venom endonuclease that was the first of its kind to be identified in insects (de Graaf et al, 2010a).

Metalloproteinases

The main goal of a parasitoid wasp is to manipulate a host such that the wasp offspring can use it as a vessel and food source. To accomplish this, the venom must contain properties that stop host development, and subvert the developmental timing to benefit the wasp life cycle. Metalloproteinases (MPs) and have been found in many venomous animals and are highly related in wasp species, in particular *E. pennicornis* and *N. vitripennis* (Figure 3A). MPs also are found in snake venoms, sometimes composing up to 65% of the total proteins in the venom, and are considered highly toxic to mammal hosts (Markland and Swenson, 2013). Snake MPs act on the host in many ways, including hemorrhagic, fibrinolytic, blood coagulation factor X activating, apoptosis, pro-inflammatory, and inactivation of blood serine peptidase inhibitors (Markland and Swenson, 2013). We identified venom MP 2-like (contig 3993, 534 reads) and 3-like (contig 308 and 5633, 10,340 reads) transcripts in *A. calandrae* (Table 2). The sequences in our study identified as MP 2-like were partial sequences and thus not discussed further. However, sequences encoding venom MP 3-like enzymes in *A. calandrae* were almost full length and share a similar zinc-binding motif as those found in snakes HEXXHXXGXXH (Figure 3A, black box and asterisk), except for a single substitution, G for N. This motif is identical to the tomato moth parasitoid, *E. pennicornis*, where three MPs have been identified (EpMP1-3). Injection of EpMP3 into tomato moth fifth-instar larvae resulted in delayed development and growth as well as some mortality due to the inability to molt to the sixth instar (Price et al, 2009). The close sequence similarity of *E. pennicornis* and *A. calandrae* suggests a similar function, delaying host development. MPs have also been identified in the wasp venom of *C. inanitus* (Vincent et al, 2010) and *P. hypochondriaca* (Parkinson et al, 2002a). Wasp, ant and snake MPs group mostly into separate subclades (Figure 3B, purple, blue, and green, respectively). However, there are several examples of MP subclades that have high identity, including *A. echinatior* and *N. vitripennis* (bootstrap = 100), snakes and some ants (bootstrap = 99). Most wasp MPs had low identity to snakes (bootstrap = 50), including *A. calandrae.*

γ-glutamyl cyclotransferase

We identified a small number of transcripts with a GGCT-like domain and annotated as γ-glutamyl cyclotransferase-like venom protein (contigs 34508 and 36225, Table 2) found also in *N. vitripennis* (de Graaf et al, 2010a). γ-glutamyl cyclotransferase participates in glutathione metabolism. The balance of glutathione in the cell is important, and deviation can result in oxidative stress and apoptosis. Falabella et al (2007) and Colinet et al (2014) identified a γ-glutamyl transferase in *A. ervi* that induced apoptosis in the germ cells and ovariole sheath in the host, effectively resulting in castration. It is not known if this effect is a byproduct of oxidative stress, or a direct effect of venom γ-glutamyl transferase on reproductive tissue.

Venom allergen

A large group of venom allergens, including those annotated as venom allergen (contig 13197 and 3104), venom allergen 3 (contig 30907) and 3-like (contig 2046), venom allergen 5 (contig 7323) and 5-like (contig 133), and venom carboxylesterase-6 (contigs 13545, 10623, 12603, 13047, 14514, 24778) transcripts were identified in the *A. calandrae* venom transcriptome (Table 2). A BLASTx of contig 2046 and contig 12782 resulted in similar outcomes: high maximum scores to many predicted allergen proteins in other hymenoptera, mostly other wasps and ants, but few annotated with regards to function in the venom. Alignments with contig 2046 and 7323 highlighted the functional region, conserved domain SCP_euk (cd05380) from the cysteine-rich secretory protein family (CAP, pfam00188; data not shown). This type of allergen protein has been studied mostly in the context of human allergies, and is described in several wasp and ant species. For example, allergen 5 has been studied extensively in *A. mellifera* (Api m 5 or allergen C) and has sequence identity to dipeptidyl peptidase IV (discussed above). Similarly, the *Vesupla vulgaris* homolog (Ves v 3) has been studied, because in humans VES v 3 can cause a serious IgE-medicated allergic reaction (Blank et al, 2010). Another example in *Solenopsis invicta* is allergen 3 (So I 3), which is a very prominent component, composing 15–25% of the total venom cocktail (Hoffman et al, 1988). Additionally, carboxylesterase-6 is one of the main allergens in honeybee venom (Api m8; Matysiak et al, 2014). The sequences found in *A. calandrae* were similar to those in *N. vitripennis, M. rotundata, Tribolium castaneum, and Microplitis demolitor.*

The reproductive-associated vitellogenin-like protein transcript was the fifth highest in abundance in the *A. calandrae* venom transcriptome, with 34,806 reads (contig 162, Table 2). Vitellogenin is a major protein in oocyte development (Tufail et al, 2009) and could be contamination from the ovaries. However, vitellogenin has also been recognized

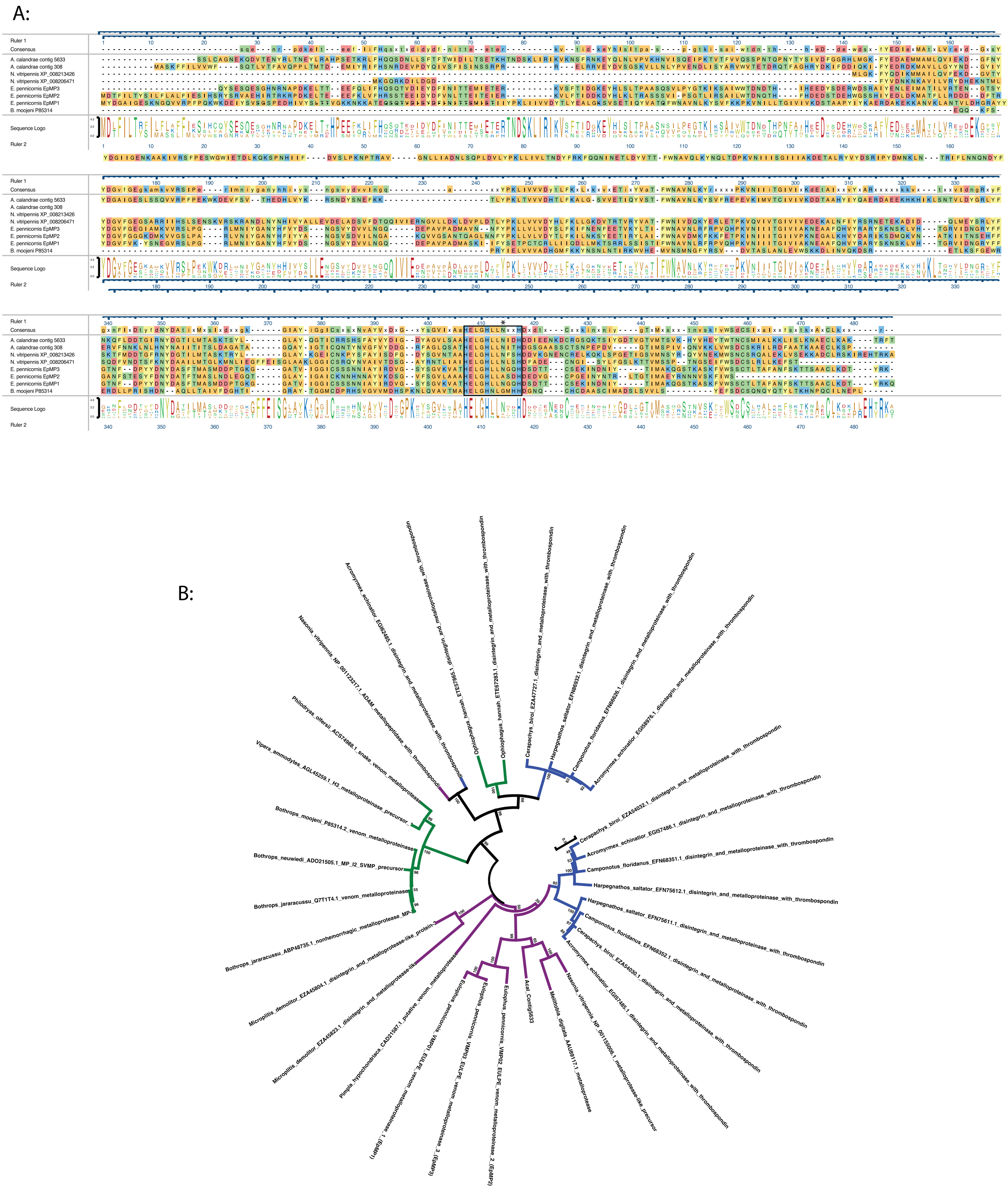

Figure 3. A. Predicted protein alignment of *A. calandrae* metalloproteinases (contigs 5633 and 308), those from two other parasitoid wasp species, *N. vitripennis* (XP_008213426 & XP_008236471) and *E. pennicornis* (EpMP1-3) and from the snake *Bothrops moojeni* (P85314). The black box contains the conserved domain and the asterisk highlights the G to N change found in insects in comparison to snakes. **B.** Phylogenetic analysis of metalloproteinases across taxa. Numbers represent bootstrap values after 500 iterations. Branches are highlighted to show taxa; snake species are in green, ants are in blue, and parasitoid wasps are in purple. *A. calandrae* is highlighted in yellow.

as another venom allergen in *A. mellifera* (Api m 12) and *V. vulgaris* (Ves v6; Blank et al, 2013).

Odorant Binding

Odorant binding proteins (OBP) and chemosensory proteins (CSP) are involved in odorant reception by binding, solubilizing, and delivering odorant molecules to olfactory receptors or chemoreceptors (Deng et al, 2013). In the *A. calandrae* venom transcriptome, we identified many OBPs and CSPs, and two with high sequence counts: general odorant binding (GOBP)-like venom protein and chemosensory protein 1 (contigs 142, 2480, 2519, 8481 and 254, Table 2).

The predicted *A. calandrae* GOBP-like venom protein was similar to *N. vitripennis* OBP67 (NP_001155150), a member of the pheromone binding proteins (PBP) that are specific to males and associated with pheromone-sensitive neurons and GOBP conserved domains. Finding GOBP conserved domains in *A. calandrae* was perplexing, since we only used female wasps in our study. However, OBPs are a dynamic and highly specific group of proteins, and have low similarity between species (Vieira and Rozas, 2011). Historically, OBPs were assumed only to be in chemosensory tissues, but recent work has identified OBPs throughout the insect body, including an OBP (PpOBP) gene expressed and localized in the venom apparatus of *P. puparum* (Wang et al, 2014). The specific function of OBPs and CSPs in the venom and venom-associated glands has not been established, but is hypothesized to be part of the parasitoid-host interaction, such as finding an appropriate (i.e. not already parasitized) host for oviposition.

Hormones

In vertebrates, corticotropin-releasing factor binding protein (CRH-BP) is a 322-amino acid soluble protein. CRH binds to CRH-BP with high affinity in humans, protecting the former from degradation and acting as a delivery system. However, it is believed CRH-BP has an antagonistic role in regulating CRH (Woods et al, 1994). In bony fish, CRH-BP regulates the stress response (Huising et al, 2004), a process that has been well conserved across taxa. A study with *A. mellifera* indicated a striking similarity between honeybee CRH-BP sequence and the vertebrate homolog, indicating the CRH signaling system in both phyla share a common ancestor (Huising and Flik, 2005). In insects, it is thought that CRH-BP binds diuretic hormone-I (DH-I), which is released by endocrine glands that receive input from the insect brain (Kataoka et al, 1989; Reagan, 1994; Schoofs et al, 1997). DH-I, as the name suggests, is involved in water regulation via the Malpighian tubules (Clottens et al, 1994). A similar sequence was identified in the Chinese honeybee (*A. cerana cerana),* and authors found the expression of CRH-BP increased following environmental stressors, such as cold, heat, and UV treatment. Expression was higher during adult and pupal stages, and in particular the pupal head (Liu et al, 2011). The function of CRH-BP in parasitoid wasps is unknown, and finding this transcript in *A. calandrae* is the first report in the venom apparatus (contig 528, Table 2). We hypothesize that CRH-BP in wasp venom could be used to manipulate the host CRH and in turn its response to stress. Additionally, it may be used to regulate DH-I, which affects the amount of water secreted by the host Malpighian tubules, and sustain the nutritional value of the host for the wasp offspring. Alternatively, it may be associated with the Dufour gland, which was included in the dissections.

We also found a sequence encoding a Bax inhibitor 1-related domain called growth hormone-inducible transmembrane (Ghitm, contig 1489, Table 2). In mammals, this type of protein inhibits apoptosis, and Ghitm expression has been proposed to function in tumorigenesis and adipose tissues (Li et al, 2001; Nagel et al, 2004; Reimers et al, 2007). An ortholog of Ghitm was identified in *B. mori* and named prothoracic gland-derived receptor (Pgdr) in insects (Nagata et al, 2006; Yoshida et al, 2006). The function in insects is crucial for the regulation of insect molting and metamorphosis (Gilbert et al, 2002; Gilbert et al, 1997). The function of Pgdr in parasitoid venom may influence the timing of host molting to be more conducive to its own developmental timing. Since this is the first report of Pgdr in transcripts from the venom gland and associated tissues, more research is needed on the potential role in venom mode-of-action.

Hyaluronidase

Hyaluronidase has been found in the venoms of most reptiles (Girish et al, 2004; Pukrittayakamee et al, 1988; Tu and Hendon, 1983) and insects including honeybees (Skov et al, 2006) and wasps (Baek et al, 2013; King et al, 1996; Skov et al, 2006; Vincent et al, 2010). Hyaluronidase depolymerizes hyaluronic acid, one of the major components of animal connective tissue, and is referred to as a venom spreading factor (Humphrey and Jaques, 1953). *A. calandrae* contig30340 and 27737 encoded a protein that was 94% similar to *N. vitripennis* hyaluronidase-like predicted protein (Table 2). We propose that *A. calandrae* uses hyaluronidase to move components of the venom through the host tissues more quickly.

Arginine kinase

As previously discussed, a major task of parasitoid venom is to paralyze the host. Paralysis may be necessary for proper attachment of the wasp egg to the cuticle of the host, or a side effect of other processes. We identified two types of arginine kinase (AK; contigs 1396 and 1658, 297) transcripts in *A. calandrae* (Table 2). In general, AKs are made up of an N-terminal domain involved in substrate specificity and a C-terminus catalytic domain, and catalyze the buffering of ATP in cells required for high and fluctuating energy requirements (Fritz-Wolf et al, 1996), including the muscles involved in insect flight and jumping. A study with the paralytic spider wasp, *Cyphononyx dorsalis,* identified two forms of AK, a full length and truncated version, lacking the N-terminal domain (Yamamoto et al, 2007). The authors made recombinant forms of AK in bacteria and found both paralyzed spiders, via relaxing of the legs similar to the effect of the crude venom. However, the crude venom caused a prolonged paralysis, up to 40 days, whereas the paralysis caused by the truncated protein lasted only 15 days, but had stronger paralytic activity, achieving paralysis with only 1–3 µg in contrast to 10 µg of the full-length enzyme. Similarly, two forms were identified in two solitary wasp species, *Eumenes pomiformis* and *Orancistrocerus drewseni*, comprising 31.9% and 43.5% of total venom components, respectively (Baek and Lee, 2010). The AK transcripts identified in *A. calandrae* are much shorter in length than that of *C. dorsalis* and may represent fragments of the full transcript. We speculate involvement of *A. calandrae* AK in host paralyzation and immobilization.

Superoxide dismutases

Superoxide dismutases (SODs) have been described as a virulence strategy in bacterial and fungal pathogens, and are one of the most important cellular enzymatic defenses against detrimental reactive oxygen species (ROS) generated by aerobic metabolism. In the context of parasitoidism, the wasp may use SODs to prevent tissue damage and ultimately death of the host while the wasp offspring develops. This is important for both endoparasitoids that need to maintain the host for development, as well as ectoparasi-

toids that need reliable nutrition after offspring emerge as larva. Three types of SODs (SOD1-3) have been identified in two *Leptopilina* species (*L. boulardi* and *L. heterotoma)* venom (Colinet et al, 2011). SOD1 was primarily located in the cytoplasm, SOD2 was restricted to the inner matrix of mitochondria, and SOD3 has an N-terminus signal peptide for extracellular matrix binding. In *A. calandrae*, we found SOD-associated contigs with sequence similarity to SOD1 and SOD3 (contigs 2375 and 6075, Table 2). A BLASTx of contig 2375 and the protein database at NCBI resulted in sequence similarity of 71% or greater with all top 100 hits, including insect orders Hymenoptera, Coleoptera, Diptera, Orthoptera, Hemiptera, Lepidoptera, Isoptera, and Ixodida, as well as sequences from mammals to plants to polychaetes, suggesting SOD 1 is highly conserved across all taxa (Figure 4A). However, the orthology is maintained within orders, as indicated by higher bootstrap values for *A. calandrae* to other wasps. *A. calandrae* contig 2375 was similar to *L. boulardi* and *L. heterotoma* SOD1 (Figure 4B), while *A. calandrae* contig 6075 was similar to *L. boulardi* and *L. heterotoma* SOD3, with a similar signal peptide (Figure 4B, black box). An alignment of the *A. calandrae* sequences with other parasitoid wasp SODs, including *L. boulardi*, *L. heterotoma*, and *N. vitripennis* demonstrated high sequence similarity, with seven identical residues scattered throughout the enzymes (Figure 4B, asterisks).

Similarly, transcripts encoding peroxiredoxin were found in the *A. calandrae* wasp transcriptome (Table 2). Peroxiredoxins are enzymes that protect against oxidative stress, like SODs, but specialize in trapping nitrogen reactive oxidative species (NROS). Peroxiredoxins have been identified in many other insects, such as *D. melanogaster* (Radyuk et al, 2001), *Gryllatalpa orientalis* (Kim et al, 2005), *Anopheles* sp. (Peterson and Luckhart, 2006), *Bombyx mori* (Shi et al, 2012; Wang et al, 2008), *Bombus ignitus* (Hu et al, 2010), *Papilio xuthus* (Zhu et al, 2009) and *Tenebrio molitor* (Zhu et al, 2014). However, the literature describes peroxiredoxin in terms of the host, and our report is the first of peroxiredoxin in the venom of a parasitoid.

Chitin binding venom protein

Transcripts were identified in this study encoding proteins with a conserved chitin binding and peritrophin-A domain (contig 8670, Table 2), found in the peritrophic matrix of insect chitinases (Elvin et al, 1996). In *N. vitripennis,* a similar protein is part of the cuticular lining of the venom reservoir (Bridges and Owen, 2005; Ratcliffe and King, 1969). Some chitin binding proteins (CBP) also exhibit antifungal properties. For example, many CBPs in plants bind to chitin in pathogenic fungi, and chitinases degrade the foreign chitin (Mehmood et al, 2011). A similar process also has been found in the sweet potato hornworm, *Agrius convilvuli* (Chae et al, 1999). Additionally, CBPs degrade the larval gut between developmental instars and metamorphosis (Chen et al, 2014).

Low-density lipoprotein receptor-like venom protein

We identified a small group of transcripts from *A. calandrae* annotated as low-density lipoprotein receptor-like (LpR) venom protein (contig 13731, Table 2). Similar proteins have been isolated in *N. vitripennis* (de Graaf et al, 2010a). The function of this protein in venom remains unknown, but insect LpR is part of a distinct group of low-density lipoproteins with function different than those in mammals (Rodenburg et al, 2006). Generally, LpRs are involved in mediating endocytosis of the major yolk proteins vitellogenin and lipophorin. LpRs have been isolated in many tissues of the female insect, including ovary, fat body, midgut, brain, Malpighian tubules, and muscles (Tufail and Takeda, 2009).

CONCLUSIONS

We have identified 64 different types of transcripts in the venom gland, venom reservoir, ovipositor, and Dufour gland of the agriculturally important wasp *A. calandrae*. Seven transcripts encode proteins that are likely involved in suppressing host encapsulation/wound healing at the ovipositor injection site and/or larval feeding site (small serine proteinase inhibitor-like venom protein, cysteine-rich pacifastin, serpin 5, cysteine-rich ku venom protein, venom protease-like, and calreticulin), nine transcripts likely encode venom allergens (venom serine carboxypeptidase, vitellogenin, venom dipeptidyl peptidase, three venom acid phosphatases, and venom allergen, venom allergen 3 and 5), and two encode SODs. Two groups of hormones were identified; one involved in a general stress response, and the other with potential function in modifying the host's molting and metamorphosis (corticotropin-releasing hormone and growth hormone, respectively). We also found evidence of a hyaluronidase that may promote the spreading of venom throughout the host body, an arginine kinase that likely causes host paralysis, and two metalloproteinase 3-like transcripts for inducing host developmental arrest, among others. This is the first work to characterize components of *A. calandrae* venom, and validation of the speculated function of venom proteins and host responses are needed. Future work will lead to a more in-depth understanding of host-parasite interactions as well as the potential to develop novel pest control strategies.

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COMPETING INTEREST

None declared.

ABBREVIATIONS

proPO; prophenoloxidase
Pro-ppA; prophenoloxidase-activating enzyme
SPI; serine protease inhibitors
KPI; Kazal-type serine protease inhibitor
GmSPI1-2; KPIs in the wax moth
CRT; calreticulin
AP; acid phosphatase
MP; metalloproteinases
OBP; odorant binding protein
CSP; chemosensory protein

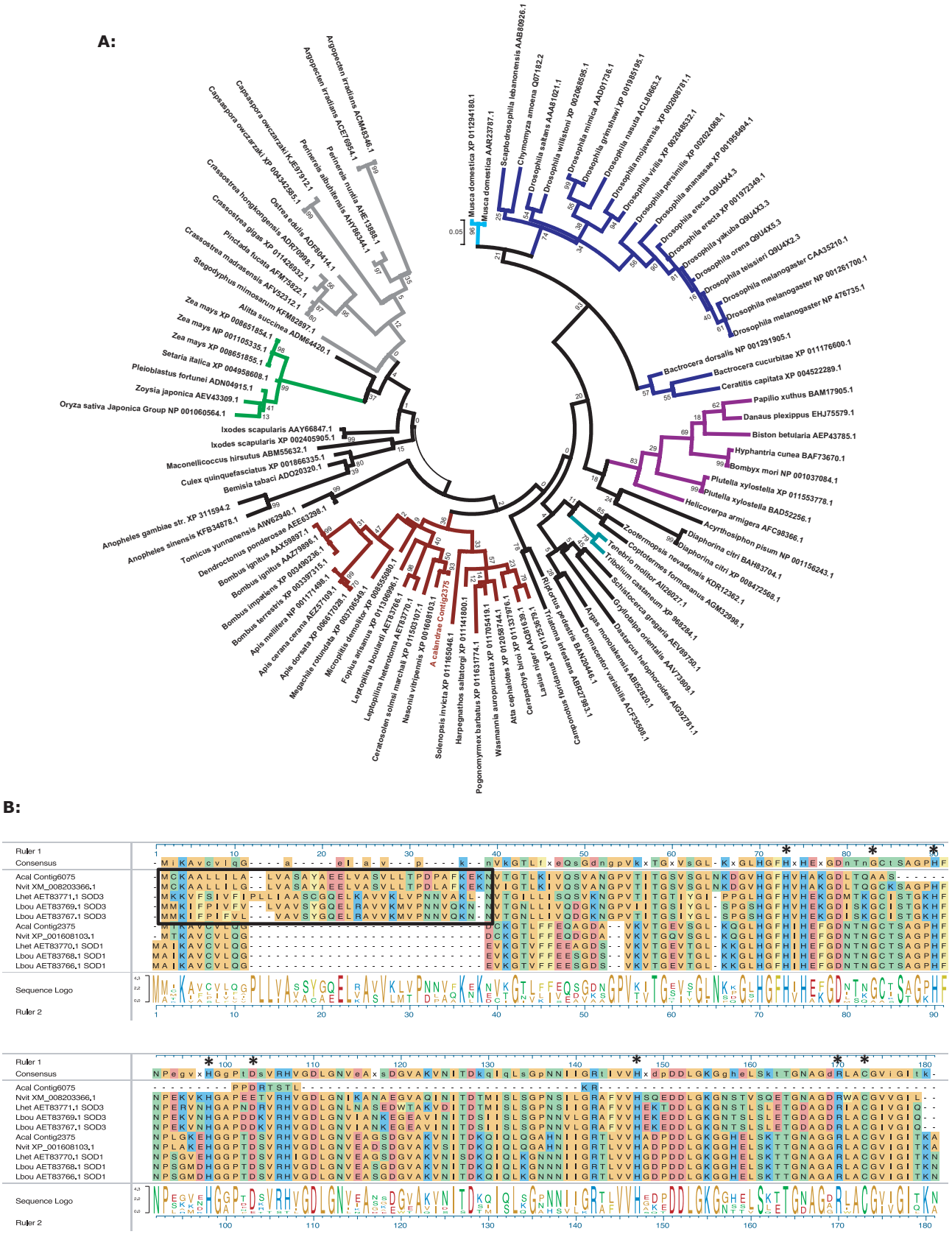

Figure 4. A. Phylogenetic analysis of highly conserved superoxide dismutases. Species names are followed by the protein used in the alignment. Branches are colored to highlight different groups of organisms; Ostreoida in grey, plants in green, Hymenoptera in maroon, Lepidoptera in purple, Diptera in blue, and mammals in light blue. *A. calandrae* is highlighted with maroon text. Numbers represent bootstrap values after 500 iterations. **B.** Protein alignment of *A. calandrae* (Acal) SODs with SODs 1 and 3 from wasps *Leptopilina boulardi* (Lbou), *L. heteratoma* (Lhet), and *Nasonia vitripennis* (Nvit). The box indicates the signal peptide and the asterisk shows the conserved functional residues.

GOBP; general odorant binding protein
CRH-BP; corticotropin-releasing factor bindng protein
DH-1; diuretic hormone-I
Ghitm; growth hormone-inducible transmembrane
Pgdr; prothoracic gland-derived receptor
AK; arginine kinase
SOD; superoxide dismutases
ROS; reactive oxygen species
NROS; nitrogen reactive oxidative species
CBP; chitin binding protein
LpR; low-density lipoprotein receptor

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