The parthenogenesis mechanism and venom complement of the parasitoid wasp *Microctonus hyperodae*, a declining biocontrol agent.

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Abstract

A biocontrol system in New Zealand using the endoparasitoid *Microctonus hyperodae* is failing, despite once being one of the most successful examples of classical biocontrol worldwide. In this study, RNA-seq was used to characterise two key traits of *M. hyperodae* in this system, the venom complement, critical for the initial success of biocontrol, and the asexual reproduction, which influenced the decline. Full characterisation of *M. hyperodae* venom revealed 82 candidate venom transcripts with both signal peptides and significantly higher expression in venom. Among these were many involved in manipulating the host environment to source nutrition for the parasitoid egg, preventing a host immune response against the egg, as well as two components that may stimulate the host's innate immune system. Notably lacking from this list was calreticulin, as it also had high expression in the ovaries. *In-situ* hybridisation revealed expression was localised to the follicle cells, which

may result in the deposition of calreticulin into the egg exochorion. Investigating the asexual reproduction of *M. hyperodae* revealed core meiosis-specific genes had conserved expression patterns with the highest expression in the ovaries, suggesting *M. hyperodae* parthenogenesis involves meiosis and the potential for sexual reproduction may have been retained. Upregulation of genes involved in endoreduplication provides a potential mechanism for the restoration of diploidy in eggs after meiosis.

Introduction

Microctonus hyperodae Loan (Hymenoptera: Braconidae) is an endoparasitoid wasp of significant economic importance in New Zealand (NZ) due to its use as a classical biocontrol agent. *M. hyperodae* was released in the 1990s to combat the Argentine stem weevil (ASW; *Listronotus bonariensis* Kuschel) (Coleoptera: Curculionidae), which caused NZ$200M in annual losses before the establishment of an effective control measure (Prestidge et al., 1991). The wasp stings adult weevils and oviposits into the body cavity, with the host surviving until the wasp larva emerges. While initial parasitism rates were as high as 90% three years post-release (Barker & Addison, 2006), continued monitoring has revealed significant declines in parasitism (Goldson & Tomasetto, 2016; Popay et al., 2011; Tomasetto et al., 2017).

This biocontrol decline is hypothesised to be a result of the ASW evolution of resistance to *M. hyperodae*, due to the initial parasitism rates imposing a strong selective pressure. This would be one of the first examples of evolved resistance to previously successful biocontrol, though genomic investigations of ASW populations are yet to reveal a genetic mechanism for this decline (Harrop et al., 2020). Despite its value as a biocontrol agent in NZ, limited genetic analyses have been performed on *M. hyperodae* thus far, and little is known about traits that play key roles in either the initial success or decline of this system. In this study, we aimed to characterise two such traits in *M. hyperodae*, the venom complement and the asexual reproductive mode.

Parasitoid venoms play key roles in ensuring successful parasitism, and the venom complement of *M. hyperodae* likely contributed to the high parasitism rates initially

observed post-release. Parasitoid venom is required to manipulate the host during parasitism, e.g. by preventing immune responses to the parasitoid egg (which is one of the most well-characterised parasitism resistance mechanisms) and providing nutrition for the developing egg (Asgari & Rivers, 2011). Venom is particularly important for koinobiont adult endoparasitoids such as *M. hyperodae*, where the egg is oviposited into the host hemocoel and develops within the surviving adult host, as these manipulations must be long-lasting (Moreau & Asgari, 2015). Investigation of *M. hyperodae* venom has been limited, with eight components identified with dideoxy-sequencing (three of which were subject to mass spectrometry), and their expression patterns across other tissues not characterised (Crawford et al., 2008). An SDS-PAGE gel of *M. hyperodae* venom also contained far more than eight bands (Crawford et al., 2008), with parasitoid wasp venoms often containing 70 or more venom proteins (Danneels et al., 2010; Scieuzo et al., 2021; Sim & Wheeler, 2016; Yan et al., 2016), so this is unlikely to represent the full venom complement.

Unlike venom, the reproduction mechanism of *M. hyperodae* has been detrimental to biocontrol success. While ASW reproduces sexually and has a considerable genetic diversity (Harrop et al., 2020), *M. hyperodae* reproduce asexually via thelytokous parthenogenesis and went through a severe bottleneck upon introduction to NZ (Goldson et al., 1993). Modelling shows this asymmetry in reproduction strategies and genetic diversity contributed to the biocontrol decline and evolution of resistance (Casanovas et al., 2018). However, during the importation and rearing of *M. hyperodae* for release as a biocontrol agent four impotent males were collected (Goldson et al., 1990), indicating *M. hyperodae* has the potential to reproduce sexually in their home range. Sexually reproducing *M. hyperodae* could be used to increase genetic diversity and for selective breeding programs to increase biocontrol efficacy.

To provide a more comprehensive investigation of both *M. hyperodae* venom and parthenogenesis we used RNA-seq to *de novo* assemble a transcriptome and survey gene expression across multiple tissues. A meta-transcriptomic analysis detected viral genes expressed in *M. hyperodae* but did not reveal common parthenogenesis-causing bacteria. Differential gene expression analysis identified both new venom components and improved assembly and annotation of previously characterised components. Further investigation of

the expression pattern of calreticulin also revealed this core parasitoid venom component is not venom-specific in *M. hyperodae*, with expression in the follicle cells of the ovaries. Core meiosis genes were present in the transcriptome, with the highest expression in the ovaries indicating *M. hyperodae* asexual reproduction involves meiosis, retaining the potential for sexual reproduction.

Methods

Library preparation for RNA-seq

M. hyperodae were collected from Lincoln, New Zealand in batches from November to March 2019/2020 and 2020/2021 and were dissected in PBS under a Leica dissection microscope using fine-tipped forceps. Three replicates of twenty pooled individuals were collected for the head, thorax, ovaries, venom gland and reservoir, and remaining abdomen tissue. Three replicates of ten pooled pupae were collected, all three days after cocoon formation. Samples were stored at -80°C, before extraction, and RNA was extracted using a hybrid of Trizol (Ambion) and RNeasy mini kit (Qiagen) methods. RNA purity was assessed using a Nanodrop 2000, and RNA integrity was measured using the Agilent 5200 Fragment Analyzer System, with all samples passing these quality assessments. Samples were then prepared for sequencing using the low-input paired-end (2 x 100bp) Illumina stranded mRNA platform and sequenced on an Illumina HiSeq 2500 by Otago Genomics Facility (https://www.otago.ac.nz/genomics/index.html). An additional head, abdomen and two ovary RNA-seq samples from two previous RNA-seq runs prepared using the same extraction and library preparation protocols were also used in the analyses below.

Pre-processing of RNA-seq samples for transcriptome assembly

BBDuk v38.90 (Bushnell, 2014) was used to quality trim and remove Illumina sequencing adapters, using default settings with trimq set to 15, and ftl=1 to remove the T overhang added by the Illumina stranded mRNA library platform. FastQC v11.9 was then used to ensure quality trimming was successful and no further issues remained with samples. Kraken2 v2.1.2 (Wood et al., 2019) was then used to taxonomically classify reads against the

Kraken standard database (downloaded 17th May 2021), and to identify potentially contaminated samples, given the low input RNA concentrations.

De novo M. hyperodae transcriptome assembly and quality assessment

Before assembly, BBMerge v38.90 (Bushnell et al., 2017) was used to merge overlapping reads using the 'very strict' setting. *De novo* transcriptome assembly was performed with Trinity v2.12.0 (Grabherr et al., 2011) using default settings with output from BBMerge for all samples retained after Kraken2 analysis (excluding one contaminated venom replicate). Transcript redundancy was reduced by retaining the longest transcript assembled for each gene. BUSCO v5.2.1 (Simão et al., 2015) was then used to assess transcriptome completeness using the hymenoptera_odb10 lineage. The scripts used for this pipeline are available in an open-source repository at https://github.com/sarahinwood/mh-transcriptome with a Snakemake-managed workflow (Köster & Rahmann, 2012).

M. hyperodae transcriptome annotation

Functional annotation of the *M. hyperodae* transcriptome was performed using the Trinotate pipeline v3.2.0 (Bryant et al., 2017). BlastX v2.9.0 (Altschul et al., 1990) was used against the UniProtKB/SwissProt database (Boutet et al., 2007) downloaded on February 3rd 2020. This was used to generate KEGG (Kanehisa et al., 2012), GO term (Ashburner et al., 2000) and eggNOG (Powell et al., 2012) annotations. Transdecoder v4.0.0 was then used to predict protein-coding regions within transcripts. Transdecoder output was then used for a BlastP v2.9.0 (Altschul et al., 1990) search against the same UniProt database to retrieve the same information as BlastX, by Hmmer v3.1b2 (Finn et al., 2011) to identify protein domains using the Pfam database (Finn et al., 2014) downloaded 3rd February 2020, and by SignalP v4.1 (Petersen et al., 2011) to predict signal peptides. Annotations were loaded into an SQLite database. A Python 3 wrapper for this annotation is available in an open-source repository at https://github.com/sarahinwood/trinotate_pipeline.

Reciprocal BlastX for viral transcripts

As many insect viruses are not present in the UniProtKB/Swiss-prot database used for Trinotate annotation, an alternate method was used to investigate the virome of *M. hyperodae*. This involved performing a BlastX v2.12.0 search (with an E value of 1E-05 to minimise false-positive results) of all transcripts against the non-redundant (nr) database (Pruitt et al., 2007) downloaded on May 16th, 2021. This search was restricted to viral entries in the database by using TaxonKit 0.8.0 (Shen & Ren, 2021) to produce a list of all viral taxonomy identifiers at a species level or below, and restricting the BlastX search to this list using the -taxidlist option. Any gene with a significant BlastX result to a virus was then used in a subsequent BlastX search against the whole nr database to remove genes with better non-viral hits. All transcripts with significant hits that had sequence identity over 90% were then subject to a BlastN v2.12.0 search against the nucleotide database (downloaded March 16th, 2022), with an E-value threshold of 1E-05.

RNA-seq analysis pipeline

All samples were *quasi*-mapped against the length-filtered *M. hyperodae* transcriptome using Salmon v1.5.1 (Patro et al., 2017) with default settings. DESeq2 v1.30.1 (Love et al., 2014) was used to create the DESeqDataSet (DDS) object, by importing Salmon output files using tximport v1.18.0 (Soneson et al., 2016) in R v4.0.4 (R Development Core Team, 2020). A blind variance stabilising transformation (VST) was performed on the DDS object, which was used for principal component analysis (PCA). Differentially expressed genes (DEGs) were identified by filtering DESeq2 results with the arbitrary alpha threshold value of 0.05 for all analyses, and a log fold change magnitude of greater than 1 for Wald tests.

DESeq2 v1.30.1 (Love et al., 2014) was used to investigate differential expression between adult tissue samples. Iterative pairwise Wald tests were carried out comparing the tissue of interest to each other adult tissue separately, using the design ~Flowcell+Batch+Tissue. This design controlled for the effect of different sequencing runs, and variance between sample batches for each sequencing run, as batches were collected consecutively across two summers and this was shown to cause tighter clustering of some head, thorax, and

abdomen samples in the PCA than tissue did (particularly for batch two where the head and thorax are directly on top of one another, supplementary figure 1). For each tissue, results from each comparison were overlapped and genes detected as significantly differentially expressed in all comparisons were retained as a list of tissue-specific genes. Result tables (including $\log_2$ fold-changes and adjusted P-values) were retained from the venom vs ovary comparisons, after being subset to only contain genes differentially expressed in all pairwise comparisons.

Heatmaps of expression were generated using VST normalised data with pheatmap v1.0.12 (Kolde, 2019) for comparisons of genes of interest, with unsupervised clustering of genes and samples based on expression patterns. Enrichment of gene ontology terms in tissue-specific gene lists was investigated using ClusterProfiler v3.18.1 (Yu et al., 2012).

Venom-specific analyses

A BlastN v2.9.0 (Altschul et al., 1990) search was carried out for the sequences from Crawford et al. (2008) against the *M. hyperodae* transcriptome database (with an E value of 1E-5), retaining the Trinity 'genes' with the best hit to each previously characterised venom component.

BlastX v2.12.0 (Altschul et al., 1990) homology searches of any DEGs without Trinotate annotation, as well as Crawford venom gene transcripts, and all venom DEGs with signal peptides, were performed (with an E value of 1E-5 to minimise false-positive results) against the non-redundant database (Pruitt et al., 2007), downloaded May 16th 2021. Results were first filtered to remove uncharacterised or hypothetical annotations that provided no information on gene function before the result with the lowest E-value (and highest bit score in the case of a tie) was selected for any genes with results.

Ovary-specific analyses

To identify core meiosis genes, Trinotate BlastX, BlastP and Pfam annotations were searched for the following genes: CORT (cell cycle regulation), REC8 (sister chromatid cohesion), SPO11, MND1, HOP2 and DMC1 (meiotic interhomolog recombination), and MSH4 and

MSH5 (crossover resolution) (Schurko et al., 2010; Schurko & Logsdon, 2008; Tvedte et al., 2017). The resulting hits were then filtered based on whether Blast hits or Pfam domains indicated a hit to a meiosis-specific gene or a homolog. Any genes with these meiosis-specific annotations were subject to a BlastX v2.12.0 search against the nr database (with an E value of 1E-5), with expression patterns also investigated. Given a lack of hits to REC8 and DMC1, the *M. hyperodae* transcriptome was the subject to a BlastX v2.12.0 search (with an E value of 1E-5) against sequences for these genes used in a previous investigation of Hymenopteran meiosis genes (Tvedte et al., 2017) to further investigate their presence. Any transcripts with a significant hit were then searched against the non-redundant database with BlastX v2.12.0 (with an E value of 1E-5), and results were filtered to retain the best hits after the removal of hypothetical or uncharacterised hits.

After the presence of these genes in the ovary-specific DEG list was investigated, a likelihood ratio test (LRT) was performed, with the design ~Flowcell+Batch+Tissue and reduced model of ~Flowcell+Batch, to determine whether expression patterns of meiosis-specific genes were significantly influenced by tissue. The scripts used for this differential expression analysis pipeline are available in an open-source repository at https://github.com/sarahinwood/mh-rnaseq with a Snakemake-managed workflow.

Hybridisation chain reaction for Calreticulin expression

Hybridisation chain reaction (HCR) (Choi et al., 2016, 2018) was used to investigate the expression of calreticulin in the ovaries of ten *M. hyperodae*. Dissected ovaries were fixed for five minutes by rocking at room temperature in a 1:1 mix of heptane and 4% formaldehyde in PBS (phosphate buffered saline). The lower heptane layer was replaced with 100% ice-cold methanol and incubated for five minutes. The upper formaldehyde layer was then removed, and tissue was washed in ice-cold methanol three times for five minutes, before being stored at -20 °C.

Hybridisation was performed using HCR version 3 (Choi et al., 2016, 2018). Ovaries were rehydrated in successive five-minute 75%, 50% and 25% methanol washes. Ovaries were then washed 3x for five minutes in PTw (PBS + 0.1% Tween 20). Ovaries were pre-hybridised

in 500 µL of 30% probe hybridisation buffer (30% formamide, 5x sodium chloride sodium citrate (SSC)) for 30 minutes at 37 °C. The probe solution, containing 1 µL of calreticulin probe in 500 µL probe hybridisation buffer, was then added to tissues and incubated overnight at 37 °C. Excess probes were removed by washing with probe wash buffer (30% formamide, 5X SSC, 9 mM citric acid (pH 6.0), 0.1% Tween, 50 µg/mL heparin) four times for 15 minutes each at 37 °C. Samples were then washed with 5X SSCT (5X SSC, 0.1% Tween 20) three times for 5 minutes each at room temperature.

In this study, Calreticulin was used with fluorophore 488. The hairpin mixture was prepared by incubating 2 µL of each 3M hairpin stock at 95 °C for 30 seconds in separate tubes, then left to cool at room temperature for 30 minutes. To prevent bleaching hairpins, all subsequent steps were carried out in the dark. 100 µL of amplification buffer (5X SSC, 0.1% Tween 20, 10% dextran sulphate) was added to hairpins, and the amplification buffer with tissue was replaced with the hairpin mixture and incubated overnight at room temperature. The hairpin solution was then removed, and tissues were washed with 5x SSCT as follows; 2x 5 minutes, 2x 30 minutes, 1x 5 minutes, and then stored in 70% glycerol. Tissues were mounted on slides and imaged using an Olympus BX61 Fluoview FV100 confocal microscope with FV10-ASW 3.0 imaging software.

Results

M. hyperodae transcriptome assembly.

Before assembly, taxonomic classification of reads was performed with Kraken 2 to ensure samples were not contaminated during preparation. We expected samples would contain predominantly unclassified reads as the Kraken2 database does not contain insects. Kraken 2 revealed most samples had between 90.0% to 96.8% of reads unclassified, though detected significant contamination in one venom replicate with 29.8% reads unclassified, 52.1% reads classified as bacterial, and 15.0% reads classified as human. This venom replicate (Mh_venom_3) was removed from all subsequent analyses.

Sequencing from the remaining samples generated 36.8-68.7 million reads per sample, with trimming retaining over 99.7% of reads (Supplementary table 1). *De novo* assembly with Trinity resulted in 202,655 transcripts sorted into 120,072 'genes' with a GC content of 32.9%. BUSCO analysis indicates that our transcriptome has a high level of completeness though many BUSCO genes were duplicated (C:94.0% [S:5.5%, D 88.5%] F: 2.9%, M:3.1%). This was reduced when the assembly was filtered to retain only the longest isoform per gene without a large decrease in complete BUSCO genes (C:92.2% [S:81.6%, D:10.6%), F:3.6%, M:4.2%) indicating BUSCO redundancies were due to the assembly of multiple transcript isoforms per gene. Therefore, further analyses used the longest isoform per gene only.

A BlastX search against the UniProtKB/Swiss-prot database as part of the Trinotate pipeline found significant hits for 21.5% of Trinity genes. Transdecoder predicted complete coding sequence in 18.7% of genes, and SignalP predicted signal peptides in 5,430 of these genes. Significant hits to Pfam protein domains were found for 11.6% of genes, with associated GO terms for 7.4%.

Preliminary investigation of *M. hyperodae* microbiome and virome.

Parthenogenesis in insects can be induced by intracellular bacteria, such as those in the *Wolbachia*, *Cardinium* and *Rickettsia* genera (Ma & Schwander, 2017), with the potential for sexual reproduction retained (e.g. Arakaki et al., 2000; Stouthamer et al., 1990). As well as the impact of the microbiome on sexual reproduction, the virome of parasitoids can play a critical role during parasitism, where endogenous viral genes or proteins from polydnaviruses (PDVs) or virus-like particles (VLPs), and/or exogenous viral infections which can assist in host manipulation (Drezen et al., 2017; Ye et al., 2018). We investigated the bacterial and viral content of reads and the assembled transcriptome. While our libraries were prepared with poly(A) enrichment, such enrichment during RNA library preparation did not prevent the detection of *Wolbachia* infection in *Nasonia vitripennis* ovaries in previous studies (Sim & Wheeler, 2016). Kraken2 classified 4.9% of reads from the remaining samples, with 2.0% bacterial and 0.1% viral. Bacterial reads were predominantly classified as the following phyla: *Proteobacteria* (0.72%), *Firmicutes* (0.48%), *Actinobacteria*

(0.25%), *Bacteroidetes* (0.14%), *Cyanobacteria* (0.04%), *Fusobacteria* (0.01%), *Spirochaetes* (0.01%), and *Tenericutes* (0.01%). The *Wolbachia*, *Rickettsia* and *Cardinium* genera, all known to have caused parthenogenesis in other insects (Ma & Schwander, 2017), have 0.0% reads classified to them (15528, 2445 and 117 reads respectively), and there were no Trinotate annotations from these genera despite their inclusion in the database used for annotation. This is consistent with previous failed attempts to revert asexuality in *M. hyperodae* with antibiotic and heat treatments (Phillips, 1995).

Investigating viral gene content in the *M. hyperodae* transcriptome using a reciprocal BlastX search found 132 genes with significant hits. Many hits were to dsDNA viruses and most had protein sequence identity below 80%, unlikely to represent detection of characterised viruses (Supplementary figure 1, Supplementary table 2). This viral gene search did not reveal any strong PDV gene candidates or other viral genes with expression patterns specific to the venom and/or ovaries that could have indicated a similar role in parasitism. A genome assembly would be required to more extensively survey for endogenous viral elements.

Of the 16 BlastX hits with protein sequence identity above 90%, representing detection of previously identified viruses, nine were from *Iflaviridae,* a viral family known to infect insects. A BlastN search of these transcripts revealed they represent fragmented genomes for Deformed wing virus (DWV) and Moku virus (Supplementary table 3), which have not previously been described as infecting parasitoid wasps. Mean transcripts per million (TPM) were highest for the longest contig of the viral genome, at 2.73 for DWV and 5.53 for Moku virus. Both DWV and Moku virus are known to infect Hymenoptera (Dalmon et al., 2019; Highfield et al., 2020; Mordecai et al., 2016; Wilfert et al., 2016). DWV is well characterised in its negative impacts on honeybees (Koziy et al., 2019), though the impacts of DWV infection in other host species, particularly when it is not vectored by *Varroa* leading to high viral titres, are not known. The impact of Moku virus infection, in general, is also unknown.

Differential gene expression analysis.

Salmon mapping rates against the length-filtered transcriptome ranged between 79.9% and 87.2%. Principal component analysis (PCA) revealed the combination of PC1 and PC2 (which together accounted for 77% of the variance between samples) grouped samples based on tissue identity (Supplementary figure 1). The iterative Wald test results resulted in 782 DEGs in venom (604 with BlastX annotation), and 585 DEGs in the ovaries (412 with BlastX annotation) (Supplementary tables XX). GO enrichment analysis with ClusterProfiler detected significant enrichment of two gene ontology (GO) terms and six Pfam protein domains in the venom list and two GO terms and three Pfam domains in the ovary list (Supplementary figures 3-4).

Improved characterisation of *M. hyperodae* venom reveals further venom components & expression of calreticulin in the ovaries.

As venom components are secreted, they are expected to contain signal peptides in their amino acid sequence. Of the 741 venom DEGs upregulated in venom samples, 83 were predicted to encode a signal peptide by SignalP, 64 of which had BlastX annotations (Supplementary table 4). The absence of a signal peptide on a putative venom gene could be a result of incomplete transcript assembly, therefore the number of venom genes with signal peptides may be higher than this.

A BlastN search was used to identify the best transcriptome hits for the previously sequenced *M. hyperodae* venom components, given their expression patterns had not been investigated. The best transcript hit to venom protein 5 (TRINITY_DN1215_c1_g1_i10) was removed at this stage as Blast results showed it was a chimeric transcript, and the second-best hit instead retained. The transcript hits for venom proteins 3, 4, 5 and 10 were all in the venom-specific protein list, giving more certainty to their function in *M. hyperodae*. Venom protein 1 is still considered a venom-specific candidate as it was only excluded from this list due to an adjusted P-value of 0.08 between venom and ovary samples (Table 1**Error! Reference source not found.**) which may have been lower with an additional venom replicate, and was one of the most abundant proteins in *M. hyperodae* venom (Crawford et

al., 2008). Investigation of both these previously identified as well as newly characterised *M. hyperodae* venom-specific gene annotations revealed candidates involved in processes commonly manipulated during parasitism.

Parasitoid egg nutrition. After parasitism of a host by a koinobiont endoparasitoid wasp, it is critical for the survival of the egg to source nutrients required for development from the host's tissue, while avoiding killing the host (Moreau & Asgari, 2015). *M. hyperodae* venom candidates for this nutritional sourcing include a venom acid phosphatase, found in many other parasitoid venoms, such as *N. vitripennis* (Danneels et al., 2010), *Leptopilina boulardi* (Colinet et al., 2013) and *Pachycrepoideus vindemmiae* where it was hypothesised to play a role in sourcing nutrients from host hemolymph (Yang et al., 2020). *M. hyperodae* venom also contains several lipases, one of which has a signal peptide and has significant enrichment of the lipase domain (Supplementary figure 3). Lipases are generally important in digestion, processing of dietary lipids and lipid transport (Sahu & Birner-Gruenberger, 2013) and are a common parasitoid venom component. Changes in host lipid metabolism have been observed following parasitism by both *N. vitripennis* (Rivers & Denlinger, 1995) and *Euplectrus* parasitoids (Nakamatsu & Tanaka, 2004), with this manipulation likely to provide a nutritional source for the developing egg.

There are 15 proteases with significantly higher expression in venom, including a serine protease (discussed below), two furin-like proteases, aminopeptidase-N, and angiotensin-converting enzyme-like. Both aminopeptidases and angiotensin-converting enzymes have also been found in the venom of the parasitoid *Torymus sinensis* (Scieuzo et al., 2021) and may assist in manipulating host tissue to provide nutrition for the parasitoid egg and/or degradation of host extracellular matrix to increase venom permeabilization (Vaiyapuri et al., 2010).

Table 1 Trinotate Pfam, BlastX and SignalP results for Crawford M. hyperodae venom Trinity genes. Venom protein numbers refer to those from Crawford et al. (2008), Trinity IDs are those identified as best hits for Crawford genes using BlastN, Ovary WT Padj the adjusted P value for a differential expression Wald test between venom and ovary samples, mean TPM the mean of TPM calculated by Salmon for the two venom samples and venom TPM rank the placement of each gene in a list ordered from highest to lowest TPM for DEGs upregulated in venom.

Venom protein #	**Crawford analysis**	**Trinity ID**	**Trinotate Pfam domains**	**Nr BlastX**	**Sequence ID (%)**	**Signal peptide**	**Ovary WT Padj**	**Venom mean TPM**	**Venom TPM rank**
1	Major reservoir protein	TRINITY_DN223_c0_g1	Peptidase family M13 (PF05649.13) Peptidase family M13 (PF01431.21)	Membrane metallo-endopeptidase-like 1 isoform X2 (*Fopius arisanus*)	36.7	No	0.08	3109.8	42
2	Major reservoir protein	TRINITY_DN20_c0_g1	Domain of unknown function 4803 (PF16061.5)	80 kDa venom protein (*Habrobracon hebetor*)	34.4	Yes	<0.05	7353.8	24
3	Major reservoir protein	TRINITY_DN4551_c0_g1	Cysteine-rich secretory protein family (PF00188.26)	Venom allergen-like 5 (*Chelonus insularis*)	50.8	Yes	<0.05	17038.5	12
4	High cDNA	TRINITY_DN43				Yes	<0.05	4076.2	31

	abundance	0_c1_g1							
5	High cDNA abundance	TRINITY_DN21775_c0_g1	Gamma interferon-inducible lysosomal thiol (GILT) reductase PF03227.16)	GILT-like protein 1 (*Chelonus insularis*)	52.7	Yes	<0.05	605.9	169
6	High cDNA abundance	TRINITY_DN301_c0_g1	Calreticulin family (PF00262.18)	Calreticulin (*Cephus cinctus*)	81.1	Yes	0.96	275.7	361
8	High cDNA abundance	TRINITY_DN4668_c0_g1	Hsp20/alpha-crystallin family (PF00011.21)	Protein lethal(2)essential for life-like (*Chelonus insularis*)	79.4	No	1	1356.0	83
10	High cDNA abundance	TRINITY_DN17346_c0_g1		PREDICTED: ion transport peptide-like isoform X2 (*Microplitis demolitor*)	43.9	No	<0.05	4514.2	30

Venom toxins. *M. hyperodae* venom protein 10 is a likely venom toxin, with the best transcript hit annotated as ion transport peptide-like (ITPL) isoform X2, belonging to the Crustacean CHH/MIH/GIH neurohormone family. Ion-transport peptide (ITP) family peptides are neurohormones, well characterized in crustaceans where they play many roles (Webster et al., 2012). ITP peptides have been found in venoms of some spiders, ticks, wasps, and centipedes where they act as toxins called helical arthropod-neuropeptide-derived (HAND) toxins (Undheim et al. 2015; McCowan and Garb 2014). ITPLs have been found in the venom of parasitoid wasp *Tetrastichus brontispae* where they are also hypothesised to function as toxins (Liu, Xu, et al., 2018), though there is no more specific indication of function beyond this. Alongside this toxin is a peroxiredoxin, which have been found in two parasitoid venoms previously with unclear function (Perkin et al., 2015), but are thought to be involved in structural or functional diversification of toxins in snake venom (Calvete et al., 2009).

Parasitism of an adult host requiring alternate venom function. As most parasitoids attack the egg or larval stages of their hosts, parasitoid venom components have not been investigated as having roles in affecting an adult host. This may indicate that known venom components include only some of the functions that venoms play during parasitism, or that the functions of venom components have diversified between parasitoids that attack egg/larval stages and those which attack adults. *M. hyperodae* venom contains two chitinases, enzymes that are capable of disrupting and digesting chitin, the major constituent of insect exoskeletons (Kramer & Koga, 1986). Hypotheses of venom function for chitinases to date are specific to parasitoids of egg/larval host stages, where they play a role in disrupting development (Cônsoli et al., 2005). It may instead play a role in breaking physical barriers to better source nutrition for the developing egg or to facilitate the spread of venom and other injected factors.

M. hyperodae venom is here also shown to contain metalloproteases (e.g., venom protein 1), a common parasitoid and snake venom component, with significant enrichment of the metalloendopeptidase activity GO term and two metalloprotease domains in the venom-specific gene list (Supplementary figure 3). In parasitoids, metalloproteases have been shown to alter host development (Z. Lin et al., 2019; Price et al., 2009), though with *M. hyperodae* attacking adult ASW such alteration does not occur. Parasitoid metalloproteases

may also inhibit haemocyte accumulation, thereby preventing encapsulation of the parasitoid egg (Danneels et al., 2010; Parkinson et al., 2002) providing an alternative function for this venom component.

Manipulation of the innate immune system. With improved assembly and annotation of the best hit for *M. hyperodae* venom protein 5, the function of this venom component in venom is clearer. The transcript is predicted to contain a Gamma interferon-inducible lysosomal thiol (GILT) reductase domain and had a significant Blast hit to GILT-like protein 1. GILT-like proteins have been found in the venom of the parasitoid *Pteromalus puparum* (Yan et al., 2016). GILT-like 1 in *Drosophila melanogaster* is involved in immune responses to bacterial infection, with RNAi knockdown resulting in increased bacterial loads 24 hours after infection (Kongton et al., 2014).

Alongside this, another venom-specific gene has a hit to waprin-Thr1-like proteins from several parasitoids and other hymenopteran species, with the best hit from *Diachasma alloeum* with 52.6% amino acid sequence identity, though has not been documented in any parasitoid venoms thus far. Snake venoms contain waprin toxins which have been demonstrated to have roles in protease inhibition, the innate immune system and antimicrobial activity (Hagiwara et al., 2003; Nair et al., 2007). A waprin-Thr1-like protein was found in a proteo-transcriptomic analysis of the wood wasp *Sirex nitobei*, where it was hypothesised to be involved in preventing bacterial growth during colonization of a symbiotic fungus. Therefore *M. hyperodae* venom contains two different components that act to stimulate the innate immune system, to potentially prevent bacterial infection of the host through the wound created during oviposition.

Prevention of cellular immune response to parasitoid egg. One of the critical roles of venom components, as well as PDVs in some parasitoids, is to prevent the host immune system from recognising and mounting a cellular immune response against the parasitoid egg. Many *M. hyperodae* venom-specific genes have functions in other parasitoid venoms involved in the manipulation of this immune response. This includes cathepsin, which is a protease involved in the regulation of autophagy in lepidopteran insects (Saikhedkar et al., 2015). Cathepsin has hypothesised roles in parasitoid venoms of blocking host immunity,

production of venom components and fat body degradation for nutrient mobilisation (Becchimanzi et al., 2017; Heavner et al., 2013).

There are multiple venom components with roles in disrupting the prophenoloxidase (PPO) cascade, which plays a critical role in the melanisation of parasitoid eggs by the host. *M.* hyperodae venom contains a superoxide dismutase (SOD), which has been demonstrated in the venoms of parasitoids *L. boulardi* and *Scleroderma guani* to prevent melanization of the parasitoid egg by disrupting the PPO cascade (Colinet et al., 2011; Liu, Huang, et al., 2018). There is also a venom serine protease, and two serine protease inhibitors (serpins) annotated as antichymotrypsin and chymotrypsin inhibitor. Serine proteases have been found in a wide variety of parasitoid venoms and can play a critical role in preventing melanization of the parasitoid egg by blocking prophenoloxidase (PPO) cascade activation (Thomas & Asgari, 2011; Zhang et al., 2004). Serpins again have been found in many venoms, where they form complexes with serine proteases and assist in the regulation of the PPO cascade to prevent melanisation of the parasitoid egg (Gettins, 2002; Kanost & Gorman, 2008; Yan et al., 2017).

Calreticulin is not venom-specific in *M. hyperodae*. Notably missing from the venom-specific gene list given its presence in many parasitoid venoms, is calreticulin. *M. hyperodae* venom protein 6 was annotated as calreticulin but it was excluded from the venom-specific gene list due to having no significant difference in gene expression between the venom and ovaries (Table 1). Calreticulin is one of the best-studied parasitoid venom components, with RNAi knockdown in *N. vitripennis* revealing it prevents the host immune system from responding to the parasitoid egg (Siebert et al., 2015; Wang et al., 2013; Zhang et al., 2006).

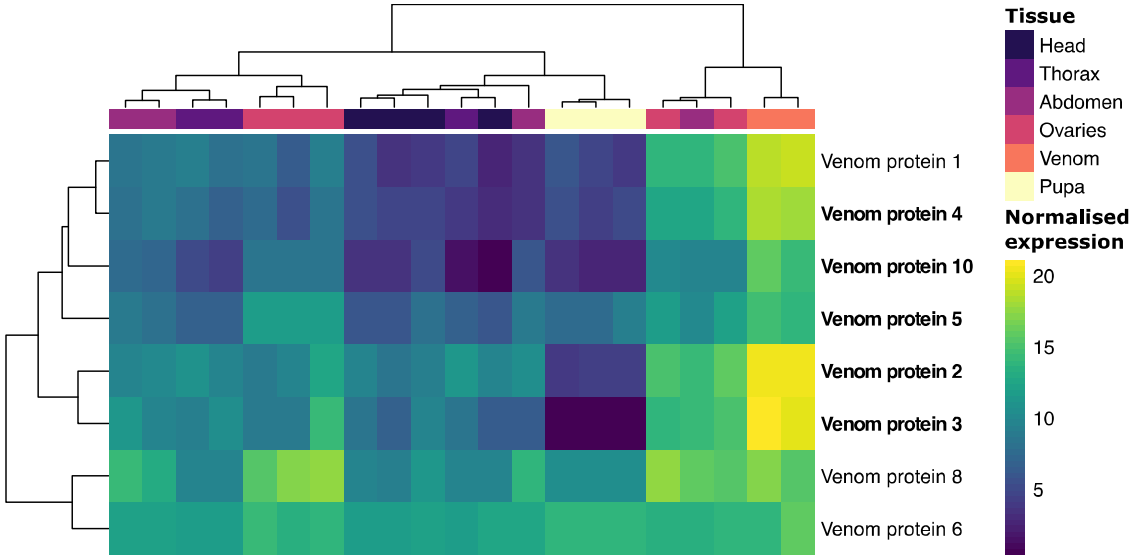

Figure 1 A clustered heatmap showing VST normalized expression for the best hits to previous M. hyperodae venom genes from Crawford et al. (2008). Genes significantly differentially expressed in all pairwise Wald tests and the LRT test are bolded. Pupa samples are included in the heatmap, to indicate continued expression of venom proteins 6 and 8 in pupa, though were not included in the differential expression tests.

Adjusted P-values for both venom proteins 6 and 8 are above 0.9, with expression of these genes comparable to or higher in ovary samples (Figure 1**Error! Reference source not found.**). This highlights the benefit of performing a comparison to expression levels in other tissues. The expression patterns of venom proteins 6 and 8 indicate they may either have multiple functions in different tissues or be involved in the same function in venom glands, the venom reservoir, and the ovaries. Both genes also appear to have moderate expression in pupae, while most other venom-specific genes have little to none.

Given the critical role that calreticulin plays in preventing the host immune response in other parasitoid species, we investigated its RNA expression pattern in the venom gland and ovaries. HCR revealed that calreticulin RNA in the venom gland is in cells on the outside layer arranged around the central lumen (Figure 2), while no expression was detected in the venom reservoir (not shown). This is as expected given a previous histological examination of *M. hyperodae* venom apparatus (Crawford et al., 2006).

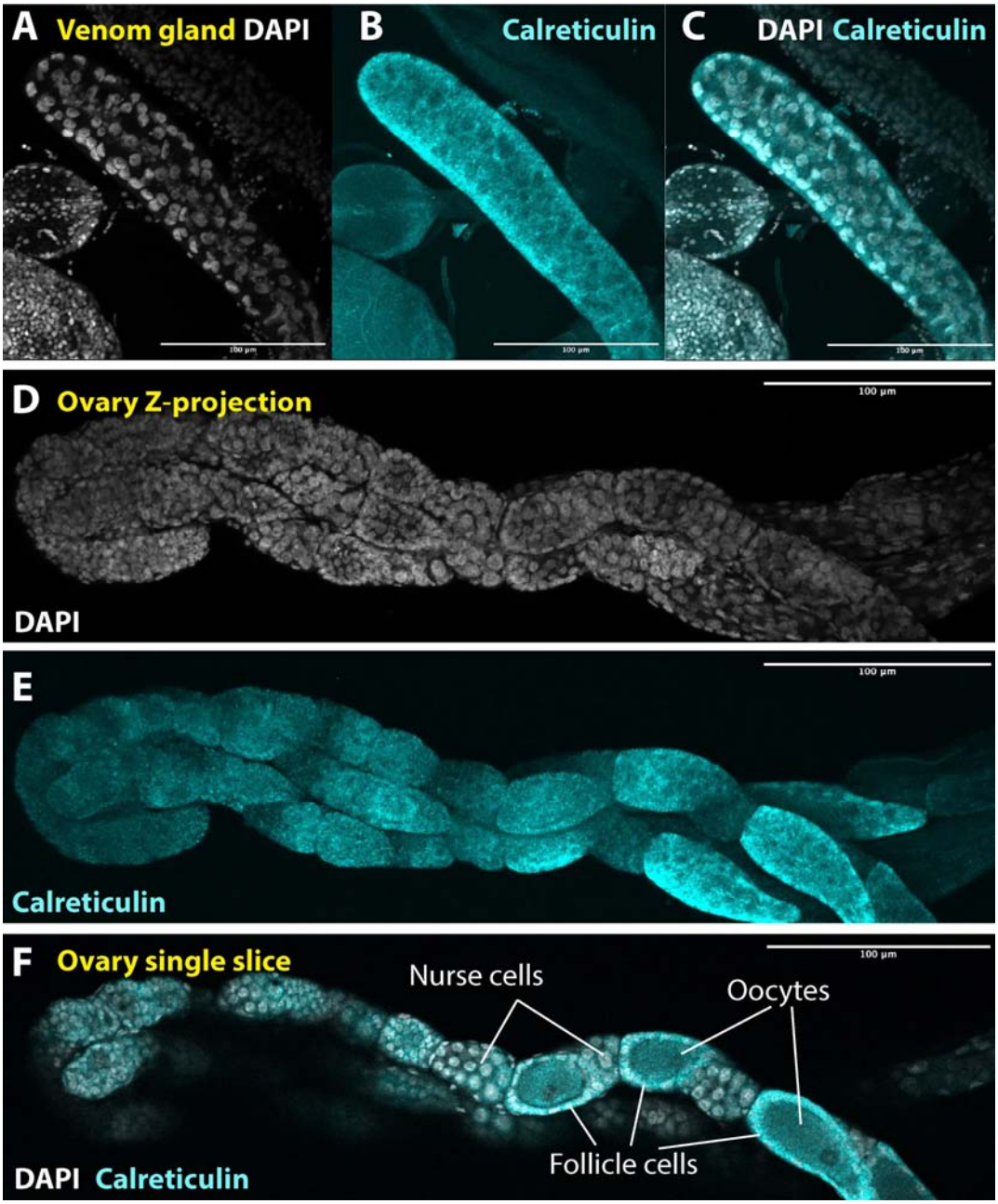

Figure 2 Calreticulin expression in the venom glands and ovaries of M. hyperodae, as determined using hybridisation chain reaction.

In the ovaries, calreticulin expression is largely restricted to follicle cells surrounding eggs and was not detected in the oocyte or nurse cells. Calreticulin expression has been detected in the ovaries of *N. vitripennis* (Sim & Wheeler, 2016), *P. puparum* (Wang et al., 2013), *Cotesia rubecula* (Zhang et al., 2006) and *Toxoneuron nigriceps* (Laurino et al., 2016), though

expression patterns across the different cell types in the ovaries have not been investigated before. In these examples it was concluded that such expression may be due to calreticulin being involved in basic cellular processes, for example as a molecular chaperone, or due to its role as a core immune response gene.

Eggs from the parasitoid *Hyposoter didymator* can evade encapsulation without their associated PDV due to proteins present on the egg exochorion, which are acquired from follicle cells (Dorémus et al., 2013). Proteins with similarity to a venom serpin that prevented melanization of the parasitoid egg have also been detected on the egg surface in *Cotesia chilonis*, with transcription of this egg serpin mainly in follicle cells (Teng et al., 2021). While calreticulin was not one of the proteins detected in these analyses, this suggests an alternate role for calreticulin expression in the follicle cells of *M. hyperodae* ovaries, where calreticulin protein may be deposited onto the *M. hyperodae* egg exochorion to assist in evading the host immune system.

Ovary gene expression provides insight into the mechanism of parthenogenesis.

The ovary DEG list contained 198 genes with significantly higher expression in the ovaries (Supplementary table 5), many of which were involved with various processes during cell cycle turnover as would be expected during gametogenesis. This included ten genes involved in the Piwi-interacting RNA (piRNA) silencing pathway, including two ovary-specific genes annotated as piwi-like protein SIWI, one as Argonaute-3 (AGO3), three RNA helicases, and one as protein vreteno. piRNA silencing is critical for transposable element silencing in the germline of the sexually reproducing *D. melanogaster*, with loss of this piRNA silencing causing severe gametogenesis defects (H. Lin & Spradling, 1997).

Ten ovary-specific genes in our dataset are involved directly in cell cycle progression. These include genes involved in the regulation of the G1 to S transition during the cell cycle (CCNE, a cyclin), chromosome condensation (CND2, RCC1, HMGI-C), sister chromatid cohesion (ESCO2, STAG1), chromosome segregation (ESPL1), DNA replication and regulation (DPOA, DPOD), and post-replicative DNA break and mismatch repair (MCM9). This is indicative of gametogenesis occurring in the ovaries, though none of these genes are specific to mitosis

or meiosis. The list also included a gene annotated as MARF1, a meiosis regulator and mRNA stability factor, which may indicate meiosis is occurring during *M. hyperodae* gametogenesis.

There are also three DEGs (MCM5, AGO and ESG) involved in endoreduplication, where cells repeat the S phase of mitosis so that DNA is replicated without cell division occurring, increasing cell ploidy. Endoreduplication in *Drosophila* follicle and nurse cells in the ovary is critical for egg production, with disruption leading to sterility (Lilly & Sptadling, 1996; Maines et al., 2004).

Meiosis-specific genes. To determine whether *M. hyperodae* has retained the potential for sexual reproduction, it is key to determine the mode and cause of parthenogenesis. Parthenogenesis can be broken down into two main mechanisms, automixis and apomixis. Automixis relies on meiosis to produce eggs, using one of several strategies to restore eggs to diploidy, while apomixis involves the production of eggs without meiosis occurring (Tvedte et al., 2019). These two mechanisms can be distinguished by the expression of meiosis-specific genes, which are required for and only expressed during meiosis (Ramesh et al., 2005). In an organism using apomixis, there is no evolutionary constraint acting on genes with meiosis-specific functions, as demonstrated in model organisms, and it is expected that the sequence and function of these genes would not be conserved (Schurko et al., 2010; Schurko & Logsdon, 2008). Loss of these meiosis-specific genes leads to obligative parthenogenesis, where an organism can no longer reproduce sexually, in contrast to facultative parthenogenesis where an organism retains the potential for sexual reproduction.

To identify the mode of parthenogenesis in *M. hyperodae* we identified these meiosis-specific genes in the *M. hyperodae* transcriptome and investigated their expression patterns. Investigation of Trinotate annotations and a subsequent BlastX search for DMC1 and REC8 revealed hits for all genes present in the *M. hyperodae* transcriptome (Table 2). Multiple transcripts were found with Trinotate annotations for MND1 and REC8. Both had one transcript over 2000 bp which was retained for analysis, with other transcript hits below 550 bp and incomplete.

All but DMC1 had a transcript with a Transdecoder protein prediction and were predicted to contain Pfam domains consistent with the gene, providing further confidence in the identification of these genes. The DMC1 transcript is short and likely incomplete, and an investigation of its expression pattern reveals much lower expression compared to the other meiosis-specific genes (Figure 3). DMC1 has previously been demonstrated to be absent in *D. melanogaster* and most Hymenoptera investigated, indicating it is not required for meiosis in these species (Tvedte et al., 2017).

None of the meiosis-specific genes had significant results in all iterative pairwise Wald tests against other tissues, with some having no significant results against any tissue, and others not against venom and/or abdomen samples. However, six of the eight genes were significant in the tissue LRT test (with 28,406 total DEGs from this test) indicating that their expression pattern was significantly influenced by tissue type (Supplementary table 6).

Table 2 BlastX annotation, Transdecoder protein prediction status, and Pfam results for genes annotated as core meiosis genes.

Meiosis gene ID	Length (bp)	BlastX annotation	Sequence identity (%)	BlastX E-value	BlastX hit accession	Protein prediction	Trinotate Pfam domains
TRINITY_DN1194_c0_g1_i3	4322	Protein cortex (*Diachasma alloeum*)	42.30	4.47E-125	XP_015109840.1	Complete	Anaphase-promoting complex subunit 4 WD40 domain (PF12894.7) WD domain, G-beta repeat (PF00400.32)
TRINITY_DN22299_c0_g1_i1	555	DMC1-like isoform X2 (*Chelonus insularis*)	48.31	1.25E-20	XP_034948447.1	No prediction	
TRINITY_DN19819_c0_g1_i18	1657	HOP2 homolog (*Chelonus insularis*)	57.14	1.83E-67	XP_034943941.1	Complete	TBPIP/Hop2 winged helix domain (PF07106.13)
TRINITY_DN7176_c0_g2_i3	2069	MND1 homolog (*Diachasma alloeum*)	67.98	1.15E-90	XP_015126850.1	Complete	Mnd1 HTH domain (PF03962.15) Leucine zipper with capping helix domain

							(PF18517.1)
TRINITY_DN11335_c0_g1_i21	5853	PREDICTED: MSH4-like (*Microplitis demolitor*)	53.38	0	XP_008554338.1	Complete	MutS domain II (PF05188.17) MutS domain III (PF05192.18) MutS family domain IV (PF05190.18) MutS domain V (PF00488.21)
TRINITY_DN56398_c0_g1_i1	2971	MSH5-like (*Diachasma alloeum*)	54.95	0	THK33018.1	Complete	MutS domain III (PF05192.18) MutS domain V (PF00488.21)
TRINITY_DN86016_c0_g1_i1	2081	REC8-like protein (*Ooceraea biroi*)	27.90	8.82E-17	EZA54708.1	Complete	N terminus of Rad21 / Rec8 like protein (PF04825.13)
TRINITY_DN57836_c	1267	PREDICTED: SPO11	47.40	1.49E-113	XP_01429	3' partial	Type IIB DNA topoisomerase

0_g1_i1		(*Microplitis demolitor*)			6694.1		(PF04406.14) PAS domain (PF13426.7)

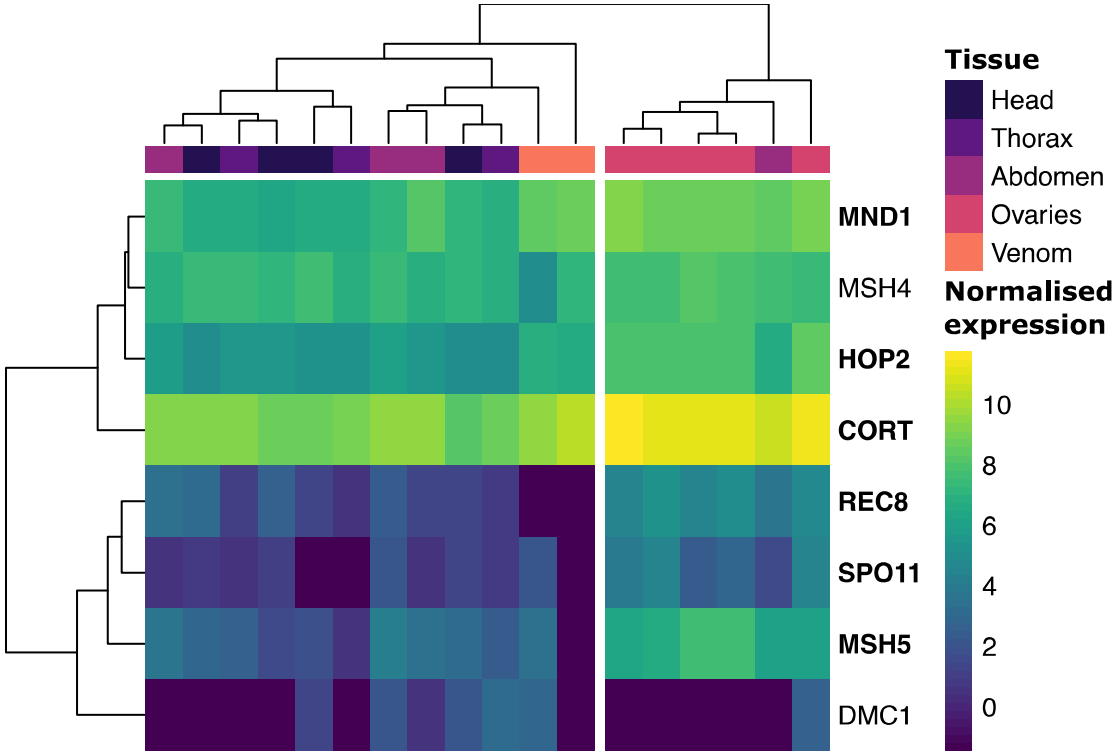

Figure 3. A clustered heatmap showing VST normalized expression for all meiosis-specific genes identified in the M. hyperodae transcriptome. Genes that were significantly differentially expressed in the tissue LRT analysis are indicated in bold.

When samples were clustered in an unsupervised manner based on meiotic gene expression, all ovary samples clustered together (with an abdomen sample), against all remaining tissues (Figure 3). The heatmap also shows that the meiosis-specific genes which were significant in the LRT test have the highest expression in the ovaries. The inclusion of an abdomen sample within this clade might be explained by incomplete dissection of ovarian tissue from this sample.

Discussion

Given the significant economic value of *M. hyperodae* as a biocontrol agent for the ASW in New Zealand, we need a better understanding of key traits that influence the success and later failure of this biocontrol system. Here we have characterised the full venom complement of *M. hyperodae*, and investigated the expression pattern of calreticulin,

demonstrating expression in the follicle cells which may serve to protect the egg from the host immune system during parasitism and development. Both this characterisation and the wider understanding of the full *M. hyperodae* venom complement are beneficial in better understanding this biocontrol system. Parasitism dissections on ASW have not detected an increase in encapsulation of *M. hyperodae* eggs (Tomasetto et al., 2017) despite this being one of the most common parasitoid resistance mechanisms documented (Colinet et al., 2013). This may be explained by the multiple *M. hyperodae* venom components working in tandem to prevent such a response, such as in the regulation of autophagy and the PPO cascade, as well as the potential role of calreticulin in the follicle cells of the ovaries.

Parasitised ASW also have their internal organs consumed by the developing egg, though the posterior of the digestive system is left intact (Loan & Lloyd, 1974), and have significantly reduced flight capacity (Goldson et al., 1999). Consumption of host tissues likely explains reduced flight capability, with enzymes such as lipases and proteases allowing for the mobilisation of host nutrients. After parasitism ASW have regressed ovaries and a reduction in testes size (Loan & Lloyd, 1974), leading to reduced fecundity (Barratt et al., 1996). The cause of this reproductive sterilisation is not known, with no currently identified venom components demonstrating a clear link to reproductive manipulation.

The parthenogenetic reproduction of *M. hyperodae* is critical to better understand if the efficacy of biocontrol is to be maintained or improved. There is no indication so far of what is causing *M. hyperodae* to reproduce asexually, though preliminary investigation of the microbiome has not yet revealed parthenogenesis causing endosymbionts, such as *Wolbachia*. The meiosis-specific genes and their expected expression pattern imply that parthenogenetic reproduction in *M. hyperodae* is either automictic, involving meiosis, or evolved from a mechanism involving meiosis without sufficient time for pseudogenisation or loss of meiosis-specific genes. *M. hyperodae* asexual reproduction may therefore be facultative, which fits with previous reports of impotent male *M. hyperodae* (Goldson et al., 1990). Given previous detection of heterozygous loci in the *M. hyperodae* genome (Iline & Phillips, 2004) it should be assumed that if parthenogenesis is automictic as this data suggests, *M. hyperodae* must rely on a strategy to restore diploidy that maintains at least some heterozygosity.

Such strategies involve either fusion of two haploid meiosis II products (which retains some heterozygosity), the fusion of two meiosis I products (which retains all heterozygosity) or duplication of the entire genome before meiosis (premeiotic duplication, which retains all heterozygosity). The upregulation of genes involved in endoreduplication in the ovaries, which increases cell ploidy, provides a putative mechanism for this premeiotic duplication, though endoreduplication plays an important role in nurse and follicle cells in *Drosophila*, so expression patterns would need to be investigated to confirm a role in *M. hyperodae* automixis.

Conclusions

With the first genomic analysis of *M. hyperodae* since the onset of biocontrol decline, we have characterised two traits with significant roles in the initial success (venom) or eventual failure (parthenogenesis) of this biocontrol system. Investigating gene expression across multiple tissues expanded the *M. hyperodae* venom complement to 83 genes while also revealing that calreticulin is not venom specific. Expression localised in the follicle cells of the ovaries may result in the deposition of calreticulin on the egg exochorion to protect the egg in the host. We also show that core meiosis genes are present in the *M. hyperodae* transcriptome and have expression patterns consistent with conserved function. This implies that *M. hyperodae* parthenogenesis is automictic and that they have likely retained the potential for sexual reproduction.

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Author Contributions

SNI; Sample collection, molecular biology, bioinformatics, *in-situ* hybridisation, imaging, manuscript drafting. TWRH; Bioinformatics support and analysis. PKD; Funding, project conception, supervision, manuscript drafting.

Data availability

All samples used in the analysis (excluding the contaminated Mh_venom_3) are hosted at the National Center for Biotechnology Information Sequence Read Archive (NCBI SRA) database with the accession PRJNA841753. The length-filtered transcriptome assembly is hosted at the NCBI Transcriptome Shotgun Assembly (TSA) database under the same accession. This assembly has a reduced number of transcripts, after those with UniVec sequencing primer hit or those labelled as contaminated during the submission process were removed. Scripts used for analyses are hosted in GitHub repositories as specified in the methods.

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