Research Article

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An RNA interference biopesticide reduces reproduction of the honey bee parasite *Varroa destructor* by down-regulating embryo development pathways

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Abstract

BACKGROUND: The honey bee parasite, *Varroa destructor*, is a globally devastating pest. Our previous experiments with laboratory mini-hives of bees and Varroa have shown an RNA interference biopesticide consisting of double-stranded RNA specific in sequence to a *V. destructor* calmodulin gene (named Norroa™, with the active ingredient vadescana) can substantially reduce Varroa reproduction. Here, we used a similar experimental design to examine the transcriptomic effects of vadescana on these parasites.

RESULTS: Consistent with previous results, Varroa reproduction was significantly reduced in vadescana treated mini-hives with no effect on foundress survival. Calmodulin expression was only significantly reduced in vadescana-treated mites sampled 5 days after brood cell capping. While RNAseq and qPCR results showed the apparent recovery of gene expression by the foundress mites by the time that bees emerged from the pupal cells, this recovery was insufficient to enable successful Varroa reproduction. Gene ontology results showed that calcium ion binding and cadherin related genes engaged in processes associated with cell adhesion and embryo development were significantly down-regulated in vadescana 2 and 8 g/L treated mites.

CONCLUSION: Our study shows the effects of vadescana on Varroa reproduction occur early in the reproductive cycle, as no offspring were produced by foundress mites sampled 5 days after cell capping and calmodulin expression was significantly reduced in mites sampled from brood cells at this time. These findings inform our understanding of Varroa reproduction by showing that calmodulin-mediated calcium signaling plays a critical role in early embryogenesis and reproduction in these parasites.

Supporting information may be found in the online version of this article.

Keywords: honey bee (*Apis mellifera*); parasite; dsRNA; RNA interference; calmodulin; embryogenesis

1 INTRODUCTION

Varroa destructor (hereafter Varroa) are ectoparasites of the European honey bee (*Apis mellifera*). These mites are consistently reported to be one of the leading causes of colony losses around the world.[1–4] Varroa parasitism has been shown to result in decreased body weight and lipid content of young emerging bees,[5] altered flying, homing, and orientation abilities in foragers, as well as altered neural processes and learning abilities.[6] Varroa are also known to vector or be associated with bee disease-causing viruses such as Deformed wing virus.[7,8] These effects on individual bees can rapidly translate to negative impacts at the colony level.

Varroa feed on the fat body of adult bees while dispersing[9] and on bee hemolymph when reproducing under capped pupal cells.[10] The life and reproductive cycle of Varroa is highly linked with that of their honey bee host. To reproduce, Varroa invade a larval brood cell hours prior to the cell being capped.[11] This invading adult female mite, called the foundress, feeds on the developing pupae and lays her eggs. The first egg is not fertilized and will develop into a male who will mate with his sisters, which develop from subsequent fertilized eggs.[11] By the time the young bee emerges from her cell, up to

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3–4 mature female offspring mites can emerge with her.[12] Synthetic chemical and organic acaricide treatments are currently available for beekeepers to treat Varroa, although existing treatments pose toxicity risks to bees and beekeepers[13,14] and resistance to some of these acaricides is increasing.[15–18] Novel Varroa control methods that are effective, sustainable, and specific are globally needed.

RNA interference-based technologies have been proposed for the control of pathogens and parasites by exploiting the RNAi pathway through the delivery of sequence-specific dsRNA complementary to mRNA transcripts that encode for proteins highly important to a target organism or entity.[19–21] Researchers have explored the use of RNAi-based biotechnology as a biopesticide in apiculture to control several honey bee pests, pathogens, and parasites, including Varroa.[22–32] However, not all organisms are sufficiently susceptible to exogenous dsRNA for RNAi to be a successful pest control strategy, even when gene expression is altered.[33]

Exposure to Varroa-specific dsRNA through injection and immersion routes have been shown to significantly knockdown target-gene expression in mites.[34–36] Garbian *et al*. (2012) tested 14 Varroa-targeting dsRNA sequences on Varroa survival and gene expression by feeding dsRNA to laboratory mini-hives of bees, larvae and Varroa. Bees in mini-hives fed Varroa-specific dsRNA had significantly fewer mites at the end of the experiment, with treated mites showing a significant reduction in target gene expression.[24] These Varroa-specific dsRNA sequences were later shown to significantly reduce Varroa survival when mites parasitized bees inoculated with a gut symbiont genetically engineered to express these dsRNA sequences.[27] Other studies have used RNAi to identify the function of genes in Varroa mites as well as explore potential RNAi targets for Varroa control. For example, RNAi knockdown of a salivary chitinase significantly reduced Varroa survival and feeding on host pupae, showing an important role in maintaining the feeding wound and preventing host infection by opportunistic pathogens.[22] Knockdown of a pheromone receptor transcription factor (PRTF) gene that is highly expressed in Varroa forelegs has been shown to cause a reduced ability to reach a host.[36] RNAi knockdown of two neural peptides identified in the Varroa synganglion, B-type allatostatin and crustacean hyperglycemic hormone, resulted in 85% and 55% reduction in relative gene expression, respectively, after 48 h, which coincided with a significant reduction in mite mortality compared to control treated mites.[35] These studies have supported the promising use of RNAi for Varroa control by demonstrating that Varroa are susceptible to RNAi effects through multiple routes of exposure to dsRNA. Additionally, a recent field trial testing effects of applying a mixture of dsRNA targeting Varroa acetyl-CoA carboxylase, Na +/K+ and endochitinase genes to honey bee hives achieved a 33%–42% reduction in average mite infestation rates relative to sugar and non-targeting dsRNA controls, respectively.[37]

Recently, GreenLight Biosciences, Inc. (Medford, MA, USA) has developed a dsRNA product (brand name: Norroa™) with an active ingredient specific to a 372 bp region of a *Varroa destructor* calmodulin gene (LOC111247793, with the common name vadescana). The dsRNA active ingredient is provided to honey bees in a sucrose solution, which is used by nurse bees to make brood food and deposited into brood cells. Upon invasion of a brood cell just prior to cell capping, mites enter the brood food at the bottom of the cell.[38] Mites infesting worker bee cells can remain in the brood food for up to 6 h, while mites infesting drone cells may be released from the food up to 20 h after cell capping.[38] This immersion in brood food is hypothesized to be the main route of Varroa exposure to vadescana.[39]

Calcium is an essential signaling molecule for inter- and intracellular communication. Calcium levels are tightly regulated by a variety of calcium-binding proteins that buffer intracellular calcium levels as well as translate changes in calcium levels into cellular responses. Calmodulin is a calcium-binding protein known to play critical roles in processes such as muscle contraction, regulation of cytoskeletal elements, neurotransmitter release, egg activation and apoptosis.[40–42] In invertebrates specifically, calmodulin has shown to be transferred to insect oocytes where it is suggested to play a role in triggering pathways critical for vitellogenesis and embryogenesis.[43,44] Calmodulin has been investigated as an RNAi target for other pests including the brown planthopper (*Nilaparvata lugens*).[45] RNAi knockdown of calmodulin in *N. lugens* resulted in an inability to properly molt, increased mortality, impaired ovary development, and female infertility.[45]

We have previously published results showing exposure to two concentrations (2 g/L and 8 g/L) of vadescana nearly completely inhibits reproduction of Varroa sampled from emerging adult bees.[39] However, the molecular processes by which vadescana impacts Varroa are unknown. Here we build upon our previous work using experiments with the mini-hive rearing system described in McGruddy *et al*. (2024) to sample Varroa earlier in their reproductive cycle and closer to initial exposure to vadescana. We provide additional survival and reproduction data of vadescana-treated Varroa sampled 5 days after brood cell capping (referred to as Early uncapping experiments) and include the survival and Varroa reproduction data from our initial study (*i.e*., when mites were sampled from emerging bees after completing their reproduction cycle, reffered to as Late uncapping experiments) for the purpose of comparison. Our primary goal in this study was to determine the transcriptomic effects of exposure to two concentrations of vadescana on Varroa sampled at different times in their reproductive cycle. RNA-sequencing (RNA-seq) was conducted on pools of foundress mites collected from brood cells 5 days after cells were capped or from emerging adult bees. Quantitative PCR (qPCR) was also used to corroborate changes of calmodulin expression from RNA-seq analysis of collected Varroa mites.

2 MATERIALS AND METHODS

2.1 Mini-hives and experimental design

The mini-hives were the same as described in McGruddy *et al*. (2024). Briefly, they were modified full-depth, five-frame, corflute honey bee nucleus boxes (Ecrotek, https://www.ecrotek.co.nz; Figs S1–S3). Wax frames for bees within these hives were 23 cm by 21.5 cm (Fig. S1(A)). A plexiglass divider was taped into place down the middle of the box to separate brood and foraging chambers (Fig. S1(B)). Four mini-hive replicates per treatment were conducted for both late and early uncapping experiments.

The honey bees and larvae were obtained from donor hives located at Victoria University of Wellington. These hives did not receive a spring treatment for Varroa prior to the experiment, resulting in no exposure to acaricides for >4 months. Varroa for addition to the mini-hives were obtained from additional colonies within the Wellington region. To obtain mini-frames of similarly aged larvae from the donor hives, Ceracell™ Nicot Queen Introduction Cages (Auckland, New Zealand) were used to trap queens and 10–15 attendant workers on one side of a mini-frame for 24 h. For each of the 32 mini-hives ($n = 4$ replicates per treatment for early and late uncapping time), one mini-frame of second instar larvae (5 days after egg laying) was transferred to each mini-hive

with approximately 300 nurse bees from the same donor colony. Immediately prior to the introduction of mini-frames, 40–60 actively moving Varroa were collected by the sugar shake method and transferred to the bottom of the mini-hive brood chamber (Fig. S2). Mini-hives were maintained in a temperature-controlled room at 31 °C and approximately 60–65% relative humidity (Fig. S3). Additional details on maintaining mini-hives in the lab can be found in File S1. Treatments were applied immediately to mini-hives after they were brought into the lab.

Double-stranded RNA treatments were manufactured by GreenLight Biosciences, Inc. (Medford, MA, USA) in a 60% w/w sucrose solution. A previous study included a bioinformatic examination of potential-off target effects on the honey bee genome, with none detected.[46] Treatments were in a single 500 mL pouch with perforated holes on the upward-facing side to allow bee foraging (Fig. S1(D)). Four treatments were used in the experiments: (1) 2 g/L vadescana treatment; (2) 8 g/L vadescana treatment; (3) non-targeting dsRNA at 2 g/L; and (4) sugar control of 60% w/w sucrose solution. The dsRNA concentrations we used are similar to previous RNAi studies observing gene knockdown in Varroa (*e.g.*, the 1 g/L used in Becchimanzi *et al.*[22]) and to those applied in other successful pest management approaches using dsRNA (*e.g.*, those used in Colorado potato beetle control with the biopesticide Calantha[47]). The vadescana dsRNA sequence and non-targeting dsRNA sequence of the MPK4a soybean (*Glycine max*) gene are provided in Table S1.

2.2 Experimental sampling

Mini-frames of brood were monitored daily through plexiglass windows in the mini-hives (Fig. S3). For the early uncapping mini-hives, mini-frames were removed 5 days after capping to sample Varroa early in their reproductive cycle and close to treatment exposure. For the late uncapping mini-hives, mini-frames were removed either 12 days after most brood cells were capped or when young bees began emerging from cells.[39]

Mini-hives of bees were anesthetized with CO_2 to remove the frames. All capped cells in the brood frame were individually uncapped under a stereo microscope. Pupae were removed, and their age and survival were recorded. Bees were aged based on morphological characteristics.[48] Foundress mite survival was recorded as counts of alive and dead mites in each cell. Counts of infested and non-infested cells in each mini-hive replicate are provided in Table S2. Foundress reproduction was recorded for each infested cell as counts of female and male offspring. Counts of foundress mite eggs were also recorded. The average number of brood cells examined per replicate mini-hive in the trial was 202 (range 31–485) (Table S2).

All foundress mites were collected from experimental mini-hives, distinguished from female offspring by their darker exoskeleton, and stored at −80°C until molecular analysis. For late uncapping mini-hives, foundress mites were not separated based on host age, and therefore collected mites were from hosts of different ages and different stages within the mite reproductive cycle. Foundresses from early uncapping mini-hives were pooled based on pupae host age, with only mites from brood cells of 5 days post capping pupae used for molecular analyses.

To determine if Varroa survival differed significantly between treatments, the proportion of living foundress mites collected from brood cells for each mini-hive was analyzed using a generalized linear model fitted with a quasibinomial distribution (to account for overdispersion). To assess Varroa reproduction only infested brood cells with a single foundress mite and from brood cells of 5 days post capping age pupae were included in analyses, as fecundity of individuals can decline when multiple foundresses are present[49] and only Varroa mites from 5 days post capping were used in molecular analyses. The impact of vadescana treatment on Varroa reproduction, measured as the total number of offspring produced per infested cell, was analyzed using a permutational analysis of variance conducted using R statistical software 4.3.1[50] with the lmPerm[51] package, given the non-parametric nature of the data. Dunn *post hoc* tests for pairwise comparisons were used to identify treatments that differed significantly from each other. The survival and varroa reproduction data from the late uncapping experiments is taken from McGruddy *et al.* (2024) and is included here for comparison with the early uncapping analyses shown here, which have not been reported previously.

2.3 RNA extractions of pooled foundress Varroa mites

For mites collected from late uncapping mini-hives, RNA was extracted from 37 samples, each consisting of seven foundress mites from the same mini-hive (n = 8–11 samples per treatment). Due to low RNA concentration, all RNA from 24 of these samples (n = 6 per treatment) was sent for RNA-seq and 13 samples were reserved for confirming *V. destructor* calmodulin expression by qPCR.

For the early uncapping mini-hives, RNA was extracted from 30 samples, each consisting of seven foundress mites collected from brood cells 5 days after cell capping (n = 7–8 samples of pooled mites per treatment). RNA from 24 samples was sent for RNA-seq (n = 5–7 per treatment) and RNA from 20 samples was used for qPCR to confirm calmodulin expression levels (n = 4–6 per treatment), with some samples used for both RNA-seq and qPCR. Table S3 lists the samples used for RNA-seq and qPCR from mini-hives in early and late uncapping experiments. Due to the early and late uncapping experiments occurring in different years, RNA samples were sent for separate RNA-seq runs and therefore analyzed as separate datasets, although both datasets were processed using the same pipeline described below.

RNA was extracted from Varroa mites by pooling seven randomly selected foundress mites in 1.5 mL reinforced tubes (Sarstedt, Germany) containing approximately 10 0.5 mm stainless steel beads, two 2.3 mm stainless steel beads and 600 µL of TRIzol reagent (Life Technologies, Carlsbad, CA, USA). Samples were homogenized in a Precellys® Evolution homogenizer (Bertin Instruments, Mon-tigny-le-Bretonneux, France) at 6800 rpm for two cycles of 30 s with a 15 s pause followed by incubation at room temperature for 10 min. Direct-zol™ MicroPrep (Zymo Research, Irvine, CA, USA) kit was then used to extract RNA from Varroa homogenate using manufacturer's instructions with DNase treatment and an additional column wash step with 350 µL of RNA Wash buffer prior to the final RNA Wash before elution in water. Samples were eluted in 15 µL of DNase/RNase-free water and RNA concentration was measured on NanoPhotometer NP80 (Implen, München, Germany) and RNA was stored at −80 °C until further analysis.

Samples sent for RNA-seq were dried using GentegraRNA™ tubes (Gentegra, Pleasanton, CA, USA) and sent *via* Custom Science (Auckland, New Zealand). Sequencing was conducted using 150 base pair paired-end sequencing run on a NovaSeq 6000 platform (Illumina, San Diego, CA, USA). Varroa RNA used for qPCR was reverse transcribed into cDNA using qScript® SuperMix (Quanta Biosciences, Beverly, MA, USA) and prepared to a final concentration of 50 ng/µL.

2.4 Quantitative PCR of calmodulin expression

Quantitative PCR was performed to confirm the results of RNA-seq, which showed expression of two calmodulin genes in Varroa were influenced by the vadescana treatment. One set of qPCR primers (VdCaM618_F and VdCaM714_R) were designed to amplify a 97 nucleotide (nt) long product of the *V. destructor* calmodulin-like (LOC111247793) mRNA based on the NCBI sequence XM_022799184. The second set of qPCR primers (VdCaM_F2 and R2) were designed to amplify 103 nt product of a *V. destructor* calmodulin (LOC111247608) mRNA based on the NCBI sequence XM_022798728. Two validated *V. destructor* internal reference genes, NADHase and SDHA, were chosen for normalization of calmodulin expression.[52] Primer sequences and efficiencies are provided in Table S4.

Samples were analyzed in duplicate and no template controls were included for each target. Each reaction was made to a final volume of 20 μL and contained 10 μL of PowerUp™ SYBR™ Green Master Mix (Applied Biosystems/Thermo Fisher Scientific, Waltham, MA, USA), forward and reverse primers each to a final concentration of 0.03 μM, and cDNA to a final concentration of 1 ng/mL. Reactions were run in 96-well plates on a QuantStudio 7 Flex Real-Time PCR platform (Applied Biosystems/ThermoFisher Scientific, Massachusetts, USA) using the following standard cycling conditions: 2 min at 50 °C; 2 min at 95 °C followed by 40 cycles of 15 s at 95 °C; 15 s at 58.5 °C; and 30 s at 72 °C. A melt curve was included to check for specificity. Relative expression of calmodulin genes was calculated from cycle threshold (Ct) values using the $2^{-\Delta\Delta Ct}$ method.[53] Differences in calmodulin expression between mites from different treatments was analyzed using ANOVA of relative expression values. Tukey *post hoc* tests were used to assess multiple comparisons between treatment groups. Statistical analysis was conducted in R 4.3.1.[50]

2.5 Differential gene expression analysis

Early and late RNA-seq datasets were processed separately using the same analysis pipeline. Quality of raw RNA-seq reads was assessed using FASTQC 0.11.7.[54] Reads were then cleaned using Trimmomatic 0.39,[55] performed under the following conditions: ILLUMINACLIP 2:30:10 SLIDINGWINDOW: 4:20 MINLEN: 25 and read quality was reassessed with FASTQC 0.11.7. Reads were further processed to determine and remove any vadescana-derived sequences present in RNA-seq sample libraries. Four single nucleotide deletion sites are present in the vadescana dsRNA sequence compared to the calmodulin-like mRNA sequence. Reads were mapped to the vadescana sequence using BBmap[56] and sam output files were double-checked manually to ensure files only contained reads absent of insertions at these four single nucleotide sites (*i.e.* mapping perfectly to the vadescana dsRNA sequence), as these were determined to be vadescana-derived. In the rare case that reads with an insertion were found in the output these were determined to be mRNA derived and were not included in the list of vadescana-derived reads. Using the 'filterbyname' function in BBmap (File S2) we removed these vadescana-derived reads from their respective sample libraries. The *V. destructor* reference transcriptome was downloaded from NCBI database (GCF_002443255.1_Vdes_3.0) (https://www.ncbi.nlm.nih.gov/genome/annotation_euk/Varroa_destructor/100/). HISAT2 2.1.0[57] was used to index the *V. destructor* reference transcriptome and align processed reads to it. Salmon 1.1.0[58] was then used for transcript quantification in alignment-based mode using the bam files as input.

Differential gene expression analysis was conducted using DESeq2 1.40.2[59] in the statistical software R 4.3.1.[50] Differentially expressed genes were identified as genes with a log fold change significantly greater than log2 (1) ($P < 0.05$) between the treatment pairwise comparisons: vadescana 2 g/L *vs*. non-targeting dsRNA, vadescana 8 g/L *vs*. non-targeting, vadescana 2 g/L *vs*. sugar; and vadescana 8 g/L *vs*. sugar.

To specifically investigate differences in calmodulin expression between treatments, genes with a calmodulin annotated product were selected from the RNA-seq datasets. Three genes in the *V. destructor* genome have annotated calmodulin products: 'calmodulin'; 'calmodulin-like'; and 'calmodulin-A-like' corresponding to NCBI genes LOC111247608, LOC111247793, and LOC111244832, respectively. The calmodulin-like (LOC111247793) gene was used to design the vadescana sequence used in these experiments. Kruskal–Wallis tests were performed for each calmodulin related gene on normalized gene counts obtained from DESeq2 using the counts function after running the 'estimateSizeFactors' function on the DESeq object to determine if expression of the individual genes was significantly different ($P < 0.05$) between treatments. Dunn's *post hoc* test with Benjamini & Hochberg correction for multiple comparisons[60] was used to test which treatments had significantly different calmodulin gene expression.

2.6 Gene ontology analysis

Gene Ontology (GO) annotations for *V. destructor* genes were retrieved from swissprot (https://www.uniprot.org/). DIAMOND 2.0.7[61] was used to blast the *V. destructor* genome to the swissprot database and GO annotations were retrieved from genes based on homology to *Drosophila melanogaster*. Gene Ontology analyses were performed in topGO Bioconductor package version 2.58.0.[62] Gene to GO annotations were formatted to fit the topGO custom annotation format. Significantly up-regulated and down-regulated genes between the treatment pairwise comparisons (vadescana 2 g/L *vs*. non-targeting dsRNA, vadescana 8 g/L *vs*. non-targeting, vadescana 2 g/L *vs*. sugar; and vadescana 8 g/L *vs*. sugar) were used as the testing gene lists. The gene universe was defined as all the genes used to perform differential expression analysis in DESeq2 for which there were associated GO terms. Enrichment analysis was performed using a Fisher's exact test. The weight algorithm was implemented in topGO to reduce node redundancy and give preference toward more specific GO terms for all comparisons, except for the 2 g/L vadescana *vs* sugar control comparison where the classic algorithm was used due to few significant GO terms.

3 RESULTS AND DISCUSSION

3.1 Vadescana affects early stages of mite reproduction

Previous experiments have shown vadescana to substantially reduce the number of offspring produced during a reproductive cycle, while foundress survival was not affected by vadescana.[39] Consistent with these results, the proportion of living Varroa recovered from each mini-hive was not significantly affected by 8 g/L (*t*-value = 1.030, $P = 0.323$) or 2 g/L vadescana treatments (*t*-value = 1.214, $P = 0.518$) compared to sugar control treated mites sampled in our early uncapping experiments (Fig. 1). However, vadescana treatment significantly decreased the number of mite offspring produced 5 days after cell capping ($F_{3,275} = 68.25$, $P < 0.001$), confirming the substantial effects of vadescana on foundress reproduction occurs early in their reproductive cycle (Fig. 2(C)). The permutational analysis of variance

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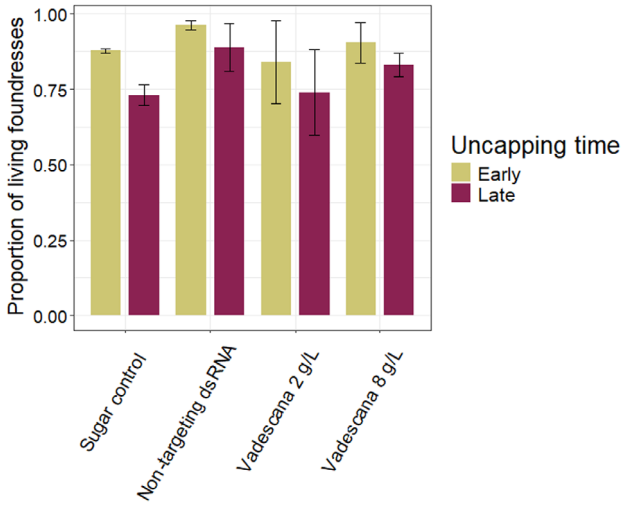

Figure 1. The proportion of living foundress mites recovered from each mini-hive was similar between treatments. Yellow bars show data from early uncapping experiments (5 days after pupal bee cell capping) and maroon bars show foundress survival taken from McGruddy *et al.* 2024 (~12 days after cell capping when adult bees began to emerge). Error bars show standard error from the mean.

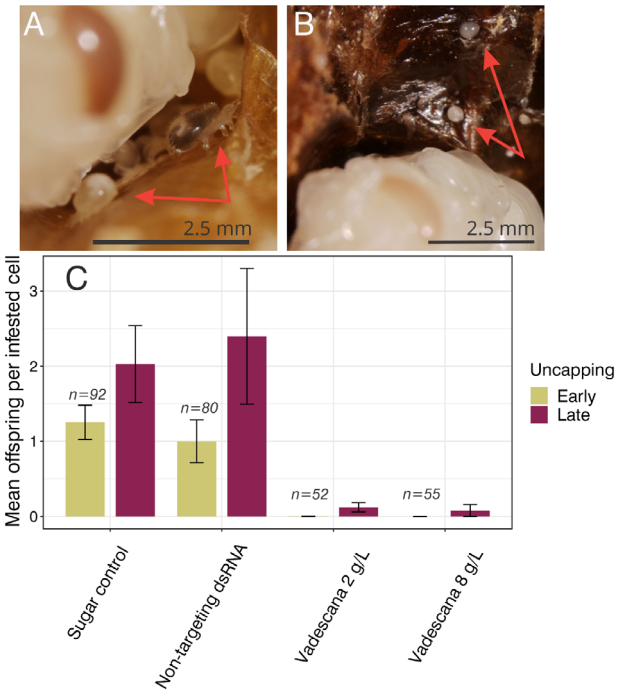

Figure 2. The influence of the vadescana treatments on mite reproduction. Photos show infested brood cells with pupae of similar age from (A) offspring mites (red arrows) in an uncapped brood cell from a sugar control treated mini-hive with; and (B) two misshapen eggs (red arrows) produced in an uncapped brood cell from vadescana 8 g/L treated mini-hives. Graph (C) shows varroa reproduction as the mean number of offspring per infested cell (where n = number of infested brood cells with a single foundress mite for early uncapping mini-hives). 'Offspring' includes mature and/or immature mites, consisting of females and males. Reproduction of mites sampled from brood cells 5 days after cell capping (Early uncapping) is shown in yellow. Data in the maroon bars is from McGruddy *et al.* (2024) and presented here for comparison. Error bars show standard error from the mean.

showed that vadescana treatment significantly decreased the number of mite offspring produced per cell for the early uncapping experiments ($F_{3,275} = 68.25$, $P < 0.001$; Fig. 2(C)). No significant difference between offspring produced from 2 g/L and 8 g/L vadescana treated mini-hives was observed (Table S5). Notably, no female mite offspring were found in infested cells across all early uncapping mini-hives treated with 2 g/L or 8 g/L vadescana (Fig. S4), suggesting that the very few immature female offspring observed in brood cells of emerging bees from late uncapping vadescana-treated mini-hives[39] may be due to foundress mites recovering from vadescana treatment later in their reproductive cycle. This recovery is unlikely to be functionally relevant as immature female offspring are not believed to survive outside the brood cell and live to contribute to a reproductively viable Varroa population.[63] While a statistically significant difference in offspring produced per infested cell was observed between the sugar control and non-targeting dsRNA treatment groups (Table S5), this result appears to be driven by results from one outlier mini-hive in the non-targeting dsRNA treatment group where mean offspring per infested brood cells was 0.27 compared to between 1.92 and 1.36 mite offspring for the other non-targeting dsRNA mini-hives (Table S2), which is within the average reported reproductive rates for Varroa mites.[63]

The few eggs that were observed in vadescana-treated mini-hives typically had an irregular shape and discoloration (Figs 2(B) and 3(B)). These eggs were more fragile compared to those laid by mites from sugar control or non-targeting dsRNA treated mini-hives and would disintegrate when gently moved with a paintbrush, while eggs from mites in control treatments could easily be transferred intact. Consistent with previous observations (Fig. 3(F)), foundress mites in the early uncapping experiment that were collected from brood cells in vadescana-treated mini-hives typically had a secreting white mass from the genital orifice (Fig. 3(D)). We have hypothesized this secreted mass to be degraded or malformed eggs.[39] The effects of vadescana on mite gene expression, which are discussed in the following sections, further support this hypothesis. Characterization of the peptide content of the white secretion would help further confirm the identity of this secretion.

3.2 Calmodulin expression in Varroa mites

Three genes with annotated calmodulin products were investigated to determine if vadescana treatments affected gene expression in mites from early and late uncapping mini-hives. These genes were calmodulin-like protein (LOC111247793), calmodulin (LOC111247608), and calmodulin-A like (LOC111244832). The peptide sequence of the annotated 'calmodulin' is identical to other calmodulins in other arthropods and has been shown to have the highest expression out of the three calmodulin-annotated products.[64] The 'calmodulin-like' sequence has only one amino acid difference compared to the 'calmodulin' sequence despite a significant nucleotide difference (83% identical). The expression of this paralog was previously shown to be ~10× less than the expression of 'calmodulin' and peaks during the pre-lay reproductive stage,[64] suggesting a specialized role in varroa reproduction. The final paralog, 'calmodulin-A-like', is only 71% identical at the amino acid level to 'calmodulin', was difficult to align at the nucleotide level for a percent identity calculation and seems to be a more ancient paralog and has the lowest expression of the three.[64]

Vadescana applied at both 2 or 8 g/L reduced calmodulin-like and calmodulin expression in mites 5 days after cell capping. Calmodulin-like (LOC111247793) gene expression was

significantly affected by vadescana treatment in the early uncapping RNA-seq data (Kruskal–Wallis $df = 3$, $\chi^2 = 18.58$, $P < 0.001$); comparing 2 g/L vadescana-treated and non-targeting dsRNA treated mites showed a significant reduction in calmodulin-like expression ($Z = -3.593$, $p_{adj} = 0.001$) confirming differences were due to vadescana treatment and were not a non-specific effect of dsRNA exposure (Table S6). Mites from 8 g/L treated mini-hives also reflected this result showing significantly reduced calmodulin-like expression when compared to non-targeting dsRNA ($Z = -3.667$, $p_{adj} = 0.001$) treated mites. While calmodulin-like qPCR expression levels among early uncapping samples appeared lower in vadescana treated mites compared to sugar or non-targeting dsRNA controls, this difference was not statistically significant ($F_{3, 16} = 2.516$, $P = 0.095$; Fig. 4). We can only speculate as to why this inconsistency occurred between qPCR and RNA-seq results, which perhaps could be due to the overall low expression of this gene that may influence the efficiency of qPCR analysis.[65,66]

Calmodulin (LOC111247608) expression in the RNA-seq data was significantly affected by vadescana treatment (Kruskal–Wallis $df = 3$, $\chi^2 = 17.579$, $P < 0.001$). *Post hoc* tests showed that for the early uncapping experiments, significantly lower calmodulin expression was observed in the vadescana 8 g/L ($Z = -3.361$, $p_{adj} = 0.002$) and 2 g/L ($Z = -2.978$, $p_{adj} = 0.004$) treated mites compared to sugar treatment. Similarly, significantly lower calmodulin expression was observed in the vadescana 8 g/L ($Z = -2.888$, $p_{adj} = 0.004$) and 2 g/L treatments ($Z = -2.490$, $p_{adj} = 0.010$) compared to non-targeting dsRNA treated mites (Fig. 4; Table S6). Calmodulin qPCR expression results were consistent with RNA-seq results and showed a significant effect of vadescana treatment for the early uncapping samples ($F_{3, 16} = 4.447$, $P = 0.020$; Fig. 4). Post-hoc tests did not reveal any significant differences between specific treatment groups after adjusting for multiple comparisons, however relative expression of calmodulin in vadescana 2 and 8 g/L samples was reduced compared to sugar (−0.5 fold-change) and non-targeting (−0.4 fold-change) samples (Table S7).

Mites sampled from emerging bees showed no effect of vadescana on calmodulin ($F_{3, 9} = 2.205$, $P = 0.157$) or calmodulin-like ($F_{3, 9} = 2.388$, $P = 0.136$) expression in qPCR results (Fig. 4). Consistent with qPCR results, calmodulin-like expression in late uncapping RNA-seq data was also not significantly affected by vadescana treatment ($df = 3$, $\chi^2 = 2.147$, $P = 0.543$). This result may indicate that the influence of vadescana treatment on calmodulin-like expression wanes in mites emerging from brood cells on adult bees. However, calmodulin expression was significantly lower in RNA-seq data of 2 g/L ($Z = 2.939$, $p_{adj} = 0.010$) and 8 g/L ($Z = 2.122$, $p_{adj} = 0.050$) treated mites sampled from emerging bees when compared to non-targeting dsRNA treated mites.

These findings show an effect of vadescana on expression of two calmodulin genes in Varroa, a result possibly due to there being sufficient sequence similarity between the two *V. destructor* calmodulin genes to result in suppression of both mRNA transcripts.[46] These genes share 83% pairwise identity at the dsRNA target site, with one 23 nucleotide stretch showing a perfect match between the mRNA transcripts at the dsRNA target region (Fig. S6). While it is possible that this small region of identity could be responsible for direct knockdown of both copies of calmodulin, especially if Varroa possesses a mechanism of siRNA-amplification as has been suggested,[67] it is also possible that knockdown of 'calmodulin' (LOC111247608) is an indirect effect (*e.g.*, secondary change in regulation of other genes due to decreased levels of calmodulin-like).

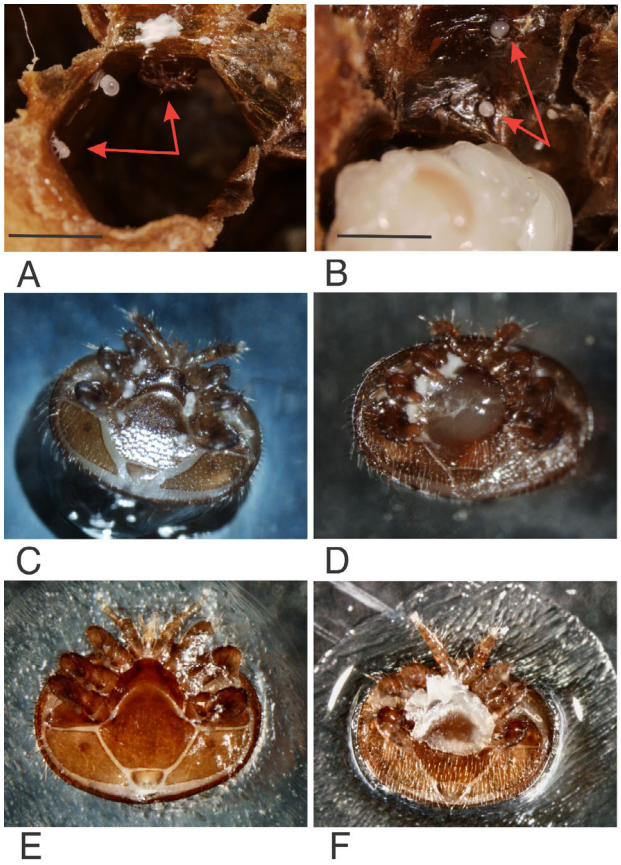

Figure 3. Differences in mite reproductive activity and foundress phenotypes between mites in the sugar control (Left panel: A, C, E) and vadescana 8 g/L treatment (Right panel: B, D, F). (A) Uncapped brood cell 5 days post capping showing foundress mite, male mite and egg. (B) Uncapped brood cell of similar age pupae showing two eggs, with the top egg disintegrated. (C, D) Foundress mites from brood cells 5 days post capping. (E, F) Foundress mites from brood cells of emerging bees. (D, F) A white secretion or encrusted mass observed above the genital plate of foundress mites from vadescana treated mini-hives, these were hypothesized to be parts of disintegrated eggs. Scale bars in photos A – B show 2.5 mm and Varroa in photos C – D are approximately 1.5 mm wide.

Vadescana treatment did not affect gene expression of the third calmodulin gene, calmodulin A like, in varroa mite samples from early uncapping. Calmodulin-A like (LOC111244832) expression in the early uncapping RNA-seq data showed no significant differences between vadescana 8 g/L or 2 g/L treated mites compared to the sugar control (8 g/L: $Z = -1.415$, $p_{adj} = 0.157$; 2 g/L: $Z = -1.362$, $p_{adj} = 0.130$) and non-targeting dsRNA treated mites (8 g/L: $Z = 1.246$, $p_{adj} = 0.128$; 2 g/L: $Z = 1.429$, $p_{adj} = 0.229$) (Fig. S5). Similarly, vadescana treatment had no significant effects on Calmodulin-A like expression in RNA-seq data from the late uncapping experiment (Kruskal–Wallis $df = 3$, $\chi^2 = 2.587$, $P = 0.460$).

3.3 Differential gene expression of foundress Varroa mites

In addition to analysis of calmodulin expression, we examined the effects of vadescana on overall gene expression patterns in Varroa using RNA-seq. A multidimensional scaling analysis of DESeq2 normalized gene counts of early-uncapping experiment samples showed gene expression of vadescana 2 g/L and 8 g/L treated mites was distinct from each other and mites from control

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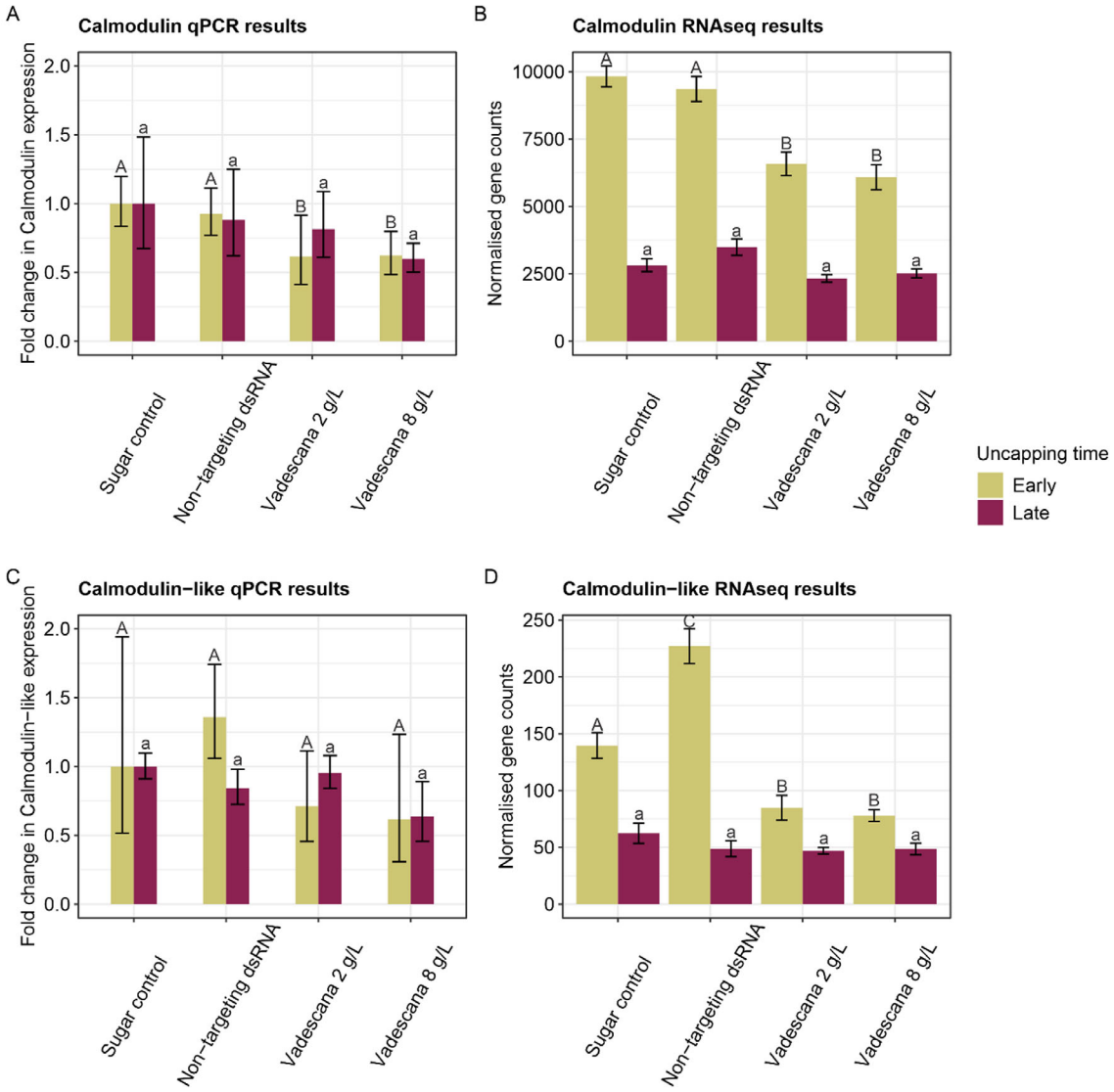

Figure 4. Relative expression of calmodulin LOC111247608 (A, B) and calmodulin-like LOC111247793 (C, D) genes based on qPCR (A, C) and RNA-seq (B, D) data from early (yellow) and late (maroon) uncapping experiments. Significant differences between treatments are indicated by letters above each bar for the early (upper case) and late (lower case) uncapping experiments.

treated mini-hives, due to clear clustering and separation of samples (Fig. 5(A)), an effect that was most pronounced for 8 g/L treatment. This result was expected given the importance of calmodulin-mediated calcium signaling in regulating a variety of processes.[68] However, gene counts of samples from the two control treatments (sugar and non-targeting dsRNA) overlapped in this analysis indicating similar gene expression patterns. For the late uncapping analysis, transcriptional profiles of mite samples from 2 g/L and 8 g/L vadescana treatments clustered closely together with some separation from control treated mite samples (Fig. 5(B)), indicating similar transcriptional effects of vadescana between the two concentrations when mites were sampled from emerging bees.

Vadescana 2 g/L treated mites sampled in the early uncapping trials had 256 (43 up- and 213 down-regulated) differentially expressed genes (DEGs) compared to non-targeting dsRNA treated mites (Fig. 6(A)), with the vadescana-targeted calmodulin-like protein (1.4-$\log_2$ fold decrease) being the second most significantly differentially expressed gene (File S3). The top 5 DEGs in vadescana 2 g/L samples compared to non-targeting dsRNA samples were: fat-like cadherin-related tumor suppressor homolog (0.6-$\log_2$ fold decrease), calmodulin-like protein, uncharacterized LOC111246802 (1.6-$\log_2$ fold decrease), atrophin-1-like protein (1.1-$\log_2$ fold decrease), LOC111251776 (4.6-$\log_2$ fold decrease). Between vadescana 8 g/L and non-targeting dsRNA treated mite samples, 582 genes were significantly differentially expressed (158 up and 424 down-regulated) with calmodulin-like protein (1.5-$\log_2$ fold decrease) appearing in the top 20 DEGs (Fig. 6(B)).

Identifying DEGs shared between the treatment comparisons found 188 shared DEGs between 2 g/L vadescana *vs* non-targeting dsRNA and 8 g/L vadescana *vs* non-targeting dsRNA treatment comparisons (File S3), suggesting these differences are due to vadescana treatment and not due to dsRNA concentration. Among this list of shared DEGs were delta B-protein (2 g/L: 0.70-$\log_2$ fold decrease; 8 g/L: 0.97-$\log_2$ fold decrease), neurogenic locus Notch protein-like (2 g/L: 0.88-$\log_2$ fold decrease; 8 g/L: 0.99-$\log_2$ fold decrease), and segmentation polarity homeobox protein engrailed-like (2 g/L: 1.07-$\log_2$ fold decrease, 8 g/L:

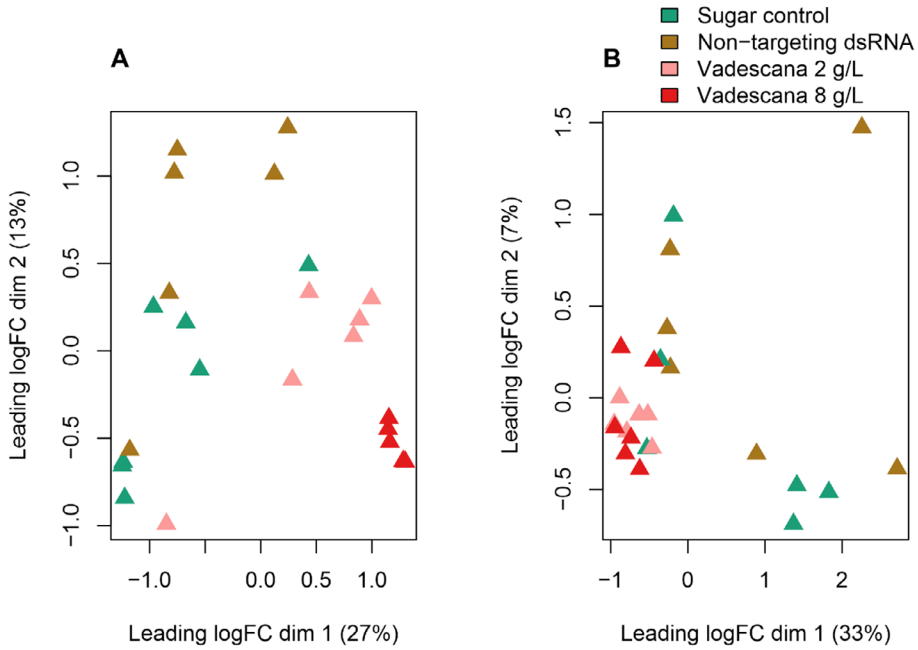

Figure 5. Multidimensional scaling plots of DESeq2 normalized gene counts of RNA-seq samples from the (A) early and (B) late uncapping datasets. Triangles represent different pooled foundress mite samples colored by treatment group; sugar control (early $n = 7$, late = 6), non-targeting dsRNA (early $n = 6$, late = 6), 2 g/L vadescana (early $n = 6$, late = 6), 8 g/L vadescana (early $n = 5$, late = 6). The first two dimensions of both MDS plots account for 40% of the variation in the data for the early and late uncapping experiments.

1.22-$\log_2$ fold decrease), which were significantly down-regulated in vadescana treated mites. The importance of these genes in mite oogenesis has also been previously suggested in a transcriptomic and proteomic study of reproductive diapause in the two-spotted spider mite (*Tetranychus urticae*).[69] Knockdown of *Notch* and *Delta* genes by RNAi in spider (*Cupiennius salei*) embryos showed defects in embryos through impaired expression of the segmental marker *engrailed*.[70]

Comparing vadescana treated mite samples to sugar control treated mites collected 5 days after cell capping showed 42 (12 up- and 32 down-regulated) DEGs in 2 g/L treated mites (Fig. 6(C); File S3) and 961 (534 up- and 427 down-regulated) DEGs in vadescana 8 g/L mites (Fig. 6(D); File S3). Ten DEGs were shared between vadescana 8 g/L and 2 g/L treated mites compared to controls (sugar and non-targeting dsRNA), with all 10 DEGs significantly down-regulated. These 10 DEGs included calmodulin-like protein and calmodulin, as well as protocadherin-like wing polarity protein stan, cadherin 23-like protein, probable endochitinase protein, protein atonal-like, ras-related protein Rab-23-like, histone H3, and two uncharacterized proteins LOC111254255 and LOC111244581. The conserved knockdown in cadherin-related proteins as well as Rab-23-like protein in vadescana-treated samples compared to both control treatments is consistent with the suppression of calmodulin, as calmodulin is known to regulate cadherins and Rab-like GTPases.[71–73] Cadherin family calcium-dependent adhesion proteins are known to play important roles in early embryogenesis processes.[74,75] Additionally, knockdown of cadherins has also been shown to disrupt convergent extension during embryo development.[76] Rab-like small GTPases can act as regulators of hedgehog signaling, an essential process of embryonic development.[77]

The RNAseq analysis of vadescana-treated mites in our study is consistent with results of other transcriptomic studies investigating changes in gene expression that occur during the varroa life cycle. Muntaabski *et al.* (2023) analyzed the transcriptomes of mites sampled from brood cells 4 days post capping and compared mites that had successfully reproduced to those that had not reproduced (non-reproductive). Functions associated with embryonic segmentation were over expressed in reproductive mites compared to non-reproductive mites and the authors proposed that the protein patch homolog 1-like gene, a membrane receptor involved in the hedgehog signaling pathway, and transcription factor AP-1 associated with Wnt signaling pathways are highly important for successful reproduction in mites.[64] More recently, RNAi induced silencing of patch homolog 1-like and AP-1 transcription factor genes have been shown to both result in mite infertility.[78] Results from our study are consistent with these findings and further suggest that expression of these genes are influenced by calmodulin-mediated calcium signaling, as expression of transcription factor AP-1 was significantly reduced in 2 and 8 g/L vadescana-treated mites, and protein patch homolog 1-like gene also significantly down-regulated in 8 g/L treated mites.

Differential gene expression analysis of mites collected from emerging bees (late uncapping experiment) showed different effects of vadescana on gene expression than those observed in the early uncapping analysis (Fig. 7; File S3). Most notable, calmodulin-like protein was not significantly differentially expressed in vadescana treated mites. We acknowledge that it's impossible to distinguish between this difference in vadescana effects between early and late uncapping datasets being due to the difference in sampling time or due to lane and sequencing run effects of conducting the RNA-sequencing separately and conducting the experiments in different years and using different hives. We had not expected to need both late and early uncapping experiments, with trials performed in different runs and years. But after the late-uncapping results suggested a possible recovery of calmodulin-like expression we repeated the experiments with an earlier sampling period.

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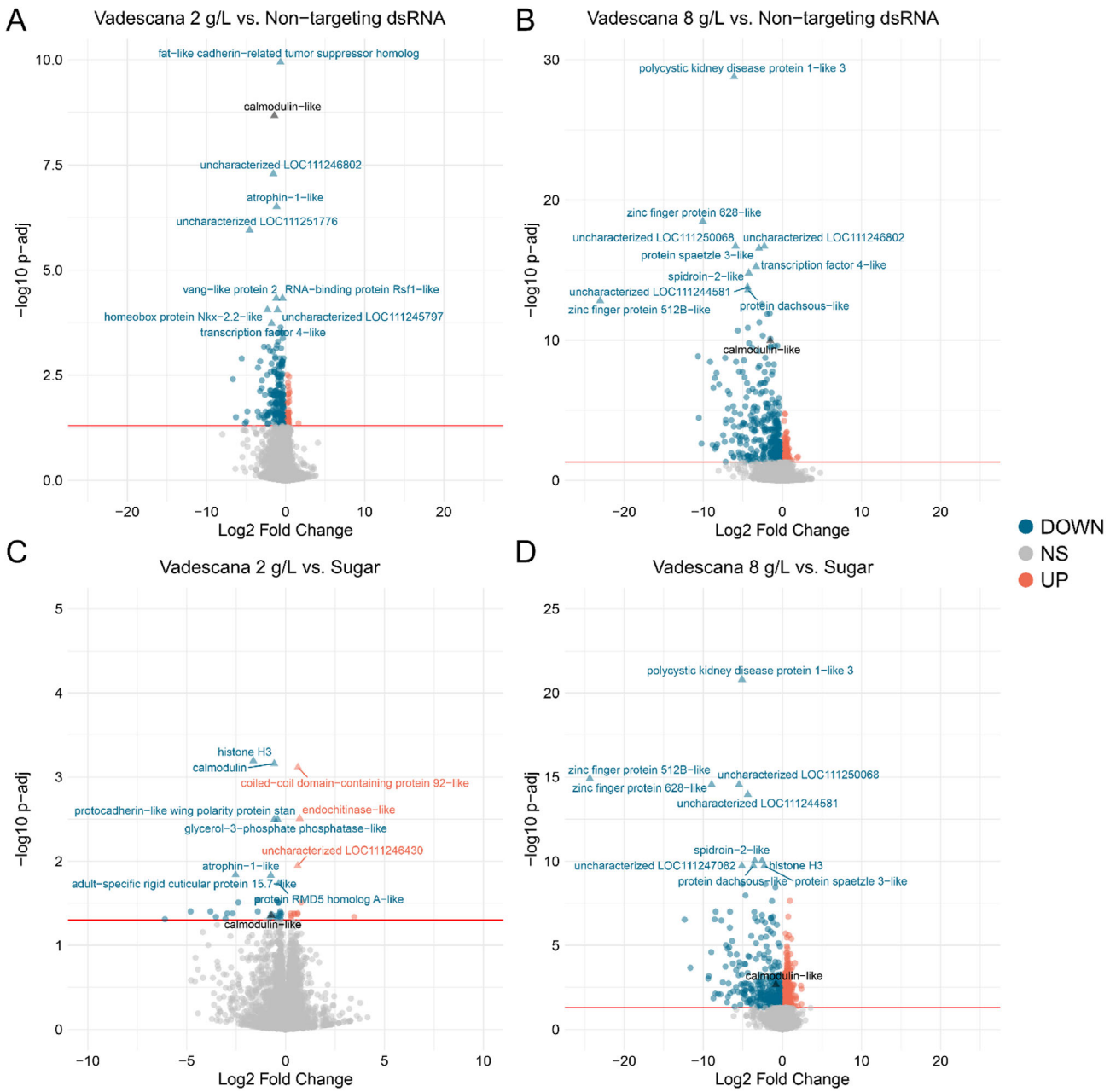

Figure 6. Volcano plots of significantly up (red) and down (blue) regulated genes from the early uncapping experiment between (A) vadescana 2 g/L and (B) 8 g/L compared to non-targeting dsRNA treated mites and (C) vadescana 2 g/L and (D) 8 g/L compared to sugar water treated mites. Labelled genes show the top 10 differentially expressed genes for each treatment comparison, with the down-regulated calmodulin-like gene target shown in black.

Of the 10 228 analyzed genes, mites from vadescana 2 g/L treated mini-hives showed 64 (7 up- and 57 down-regulated; Fig. 7(A)) and 280 (97 up and 183 down-regulated; Fig. 7(C)) DEGs compared to sugar control and non-targeting dsRNA treated mites, respectively. Mites collected from brood cells of emerging bees in vadescana 8 g/L treated mini-hives showed 220 (38 up- and 185 down-regulated; Fig. 7(B)) and 311 (85 up- and 226 down-regulated; Fig. 7(D)) DEGs compared to sugar control and non-targeting dsRNA treated mites, respectively. Forty DEGs were shared across the four treatment comparisons with all 40 genes significantly down-regulated in vadescana treated mites. Sixteen of these 40 shared DEGs were cuticle proteins, with cuticle protein 21-like (LOC) conserved among the top 10 DEGs across all comparisons. Other cuticle associated proteins were also among the 40 shared DEGs, including two genes for glycine-rich proteins and two obstructor E-like proteins.

Although direct RNAi effects on calmodulin-like expression were not detected in late uncapping mites, differential gene expression results suggest a significant and lasting effect of vadescana treatment on Varroa mites. Knockdown of calmodulin in the brown planthopper, *Nilaparvata lugens*, has led to an inability for insects to molt, suggesting that calmodulin may be an important regulator of cuticle formation.[45] It is possible that dysregulation of calmodulin expression interferes with cuticle biosynthesis in mites. Conducting further bioassays on the effects of vadescana on expression of cuticle proteins in Varroa mites is

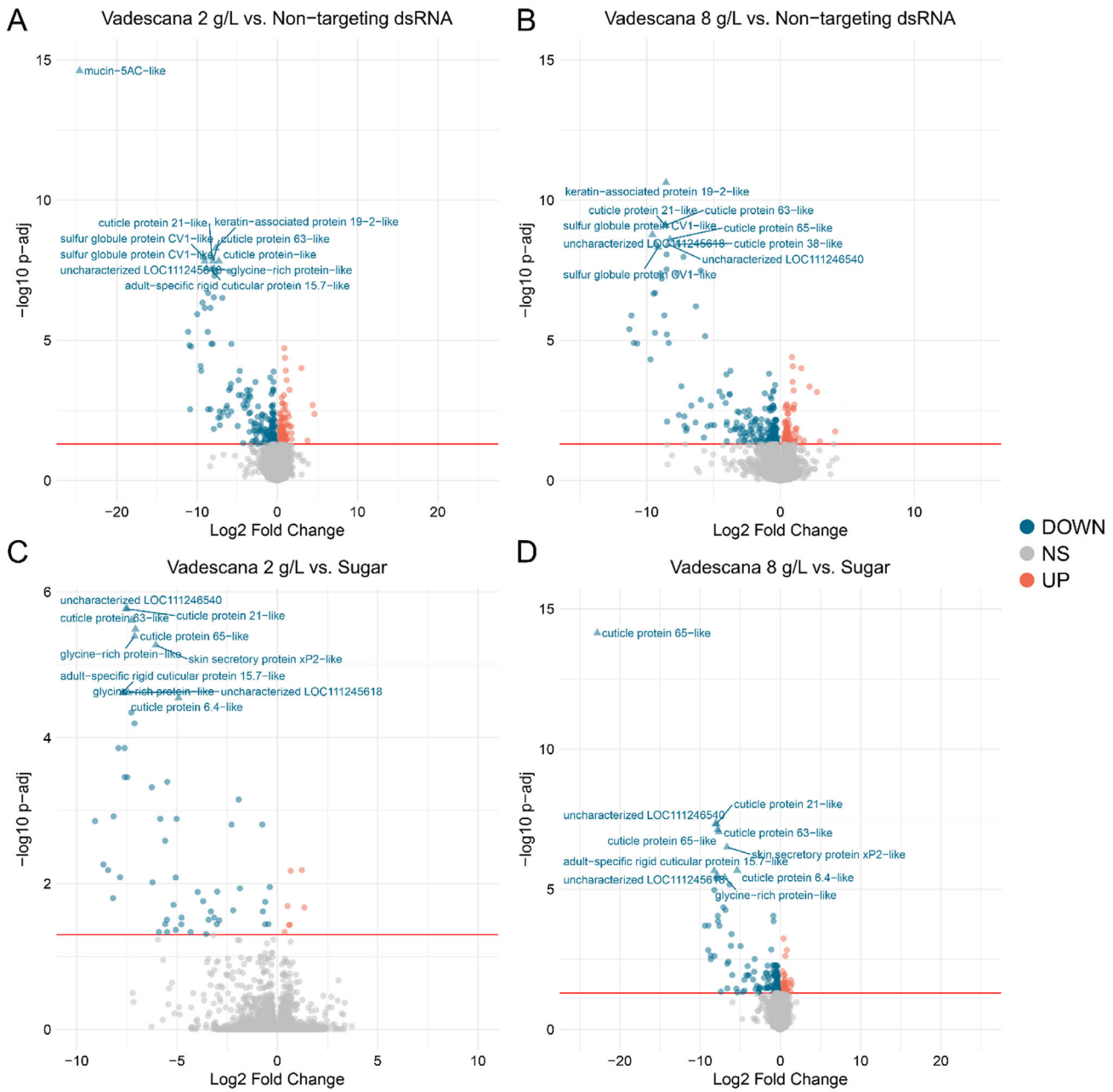

Figure 7. Volcano plots of significantly over (red) and under (blue) expressed genes from the late uncapping experiment between (A) vadescana 2 g/L and (B) 8 g/L *vs.* non-targeting dsRNA treated mites and (C) vadescana 2 g/L and (D) 8 g/L *vs.* sugar water treated mites. Labelled genes show the top 10 differentially expressed genes for each treatment comparison.

necessary to confirm these results. The cuticle is highly important for protecting arthropods against pathogen infection[79] and environmental chemicals. For example, knockdown in acari cuticle proteins has been shown to increase cuticle permeability and mite sensitivity to acaricides.[80] Understanding whether vadescana-treated mites emerging from brood cells are more susceptible to pathogens such as entomopathogenic fungi[81] or other miticides could be a productive avenue for future research.

3.4 Gene ontology

A Gene Ontology (GO) enrichment analysis was conducted to understand what functional components were affected by vadescana treatment compared to control treatments (sugar and non-targeting dsRNA). Our focus in this analysis was the results from the early uncapping trials. GO terms were obtained for a total of 7163 *V. destructor* genes, with 7153 identified in our dataset as significantly differentially expressed and used for the analysis. A complete list of significantly enriched biological process and molecular function GO terms and the differentially expressed genes engaged in these GO terms can be found in File S4.

Gene ontology results showed that vadescana 2 and 8 g/L treated mites were significantly down-regulated in the calcium ion binding (GO:0005509) molecular function. As well as calmodulin and calmodulin-like proteins, other DEGs associated with this term included several cadherin related genes such as protocadherin-like wing polarity protein stan, fat-like cadherin-related tumor suppressor homolog, dachsous-like protein and cadherin-23-like protein (File S4). These cadherins were also

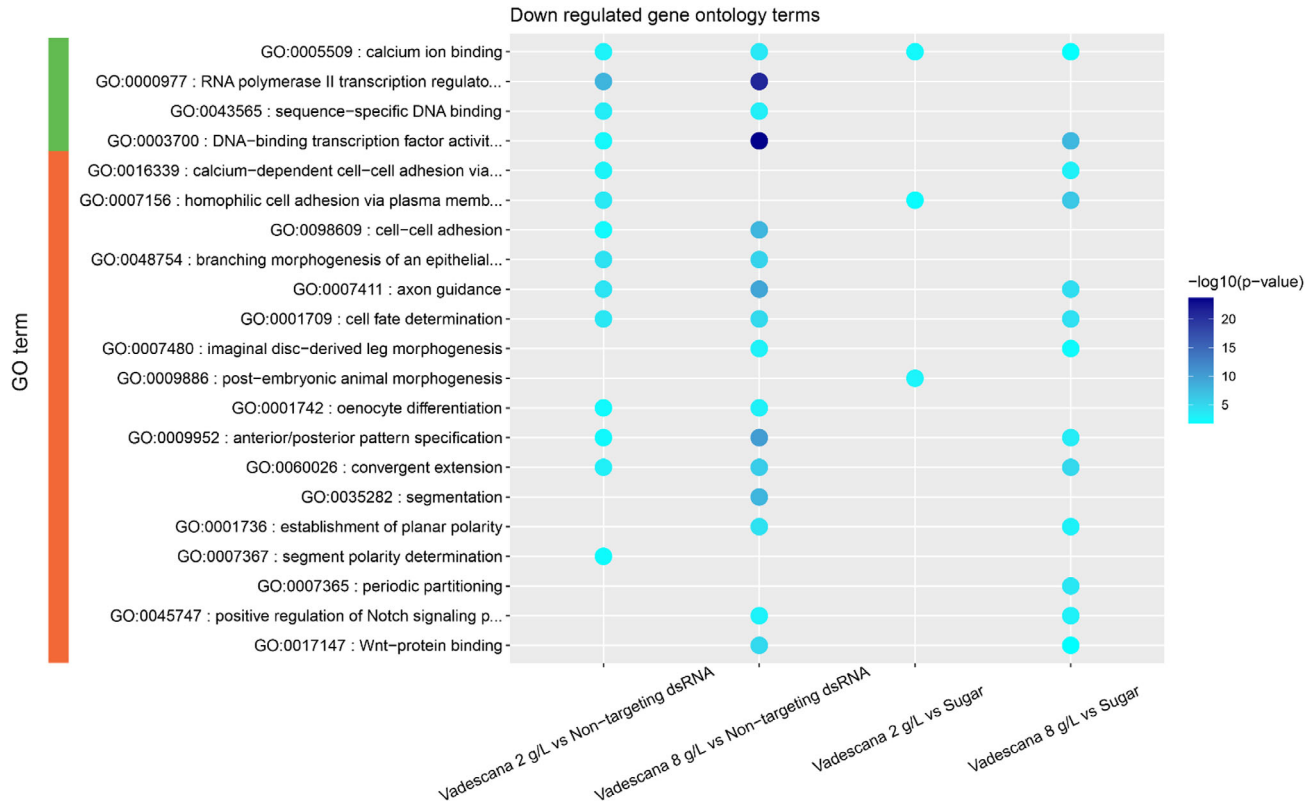

Figure 8. Significant Gene Ontology (GO) terms of down-regulated differentially expressed genes from vadescana 2 g/L *vs.* non-targeting dsRNA, vadescana, 8 g/L *vs.* non-targeting dsRNA, vadescana 2 g/L *vs.* sugar, and vadescana 8 g/L *vs.* sugar treatment comparisons from early uncapping trials. Presence of a circle in the column of each comparison indicates the GO term is significant for that comparison. Molecular function ontology terms are indicated by the green bar and biological process terms by the orange bar. The color gradient of the circle shows −log10 p-value from the weight algorithm in topGO with darker colors being more significant.

engaged in down-regulated biological processes associated with cell adhesion and early embryo development processes of pattern specification, polarity, segmentation, and embryo morphogenesis in vadescana-treated mites (Fig. 8). The specific biological processes associated with embryogenesis, which were significantly down-regulated in vadescana treated mites, included segment polarity determination (GO:0007367), convergent extension (GO:0060026), anterior/posterior pattern specification (GO:0009952), post-embryonic animal morphogenesis (GO:0009886), and oenocyte differentiation (GO:0001742).

Up-regulation of functions associated with DNA replication, transcription and translation have been identified as key features of egg laying foundresses likely due to oogenesis and cell division.[82] Interestingly, these molecular processes such as RNA polymerase II activity and DNA-binding were significantly down-regulated in 2 g/L and 8 g/L vadescana-treated mites (Fig. 8), reflecting a dysregulation in embryo development. On the other hand, up-regulation of metabolic processes has also been identified as an important feature in transcriptomic analysis of reproductive *vs.* non-reproductive foundresses.[82] Yet, genes associated with these processes were not significantly differentially expressed in vadescana-treated mites compared to mites from control treated mini-hives, indicating the differences in gene expression presented here are not consistent with the phenomenon of foundresses naturally failing to reproduce.

Gene ontology analysis found little agreement in up-regulated biological processes and molecular functions identified in vadescana 2 g/L treated mites compared to non-targeting dsRNA treated mites and those identified in 8 g/L treated mites compared to non-targeting dsRNA treated mites (File S4). Therefore, it was difficult to confidently associate specific up-regulated processes and functions with vadescana treatment. However, up-regulated DEGs in mites from vadescana 2 and 8 g/L treated mini-hives compared to mites from sugar treated mini-hives were engaged in ion transport related biological processes such as sodium ion transport (GO:0006814). Consistent with this result symporter activity (GO:0015293) was also commonly up-regulated in vadescana-treated mites compared to mites from sugar control treated mini-hives. It is possible this result reflects an investment in maintaining a stable ion charge across the cell membrane in vadescana treated mites due to accumulation of calcium ions in the cell consistent with knockdown in calcium binding.

4 CONCLUSION

Our findings demonstrate that effects of vadescana on reproduction occur early in the Varroa reproductive cycle and disrupt genes associated with embryo development through knockdown in calmodulin-mediated calcium signaling, highlighting the importance of this process for embryogenesis in Varroa. Calmodulin is a critical calcium signaling molecule with roles in regulating a variety of processes among eukaryotes[68] and suggested to play a role in triggering invertebrate embryogenesis.[43-45,83] Calcium fluxes have been detected throughout embryo development for several species, as well as in the cytoplasm of

tick oocytes,[84,85] and is thought to be important in triggering processes such as yolk mobilization in insects, convergent extension, pattern formation and neural development.[76,86–89] Calmodulin-like proteins may be important in regulating calcium-signaling during these processes[71] and has shown to be transferred to insect oocytes from surrounding epithelial cells *via* gap junctions, where it is implicated in triggering pathways essential for vitellogenesis.[43,44] In Varroa, most stages of embryogenesis including segmentation and limb differentiation occur while embryos are inside the foundress mite prior to oviposition.[90] Given that Varroa show a highly accelerated oogenesis and embryogenesis that enables rapid reproduction under the capped brood cell,[90] tight control over the timing of embryo developmental stages may be crucial and sensitive.

Although effects of vadescana on calmodulin expression appear to wane as they remain in the brood cell, Varroa reproductive activity does not sufficiently recover during this specific reproduction cycle to enable successful reproduction.[39] These findings show the transient effects of RNAi on gene expression and would suggest vadescana-treated Varroa emerging from brood cells with adult bees are likely to be capable of reproducing in the future, as varroa can undergo multiple reproductive cycles.[63] Results from the experiments presented here characterized the transcriptional effects of an RNAi-based technology developed for Varroa control to link the phenotypic effects of vadescana with differences in gene expression and provides valuable insight to genes associated with Varroa reproduction, which could have a broader benefit for our understanding of acari reproduction and RNAi-based control methods.

FUNDING INFORMATION

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DATA AVAILABILITY STATEMENT

Genetic data: Raw RNA-sequencing data generated for this study have been deposited NCBI with the BioSample accessions SAMN44301127 – SAMN44201174.

CONFLICT OF INTEREST

Brian Manley, Kenneth Narva, Emma De Neef, Eric R. L. Gordon, Upendra K. Devisetty and Katie Youngs, are employed by GreenLight Biosciences Inc., the developer of vadescana. The other authors declare no conflict of interest.

SUPPORTING INFORMATION

Supporting information may be found in the online version of this article.

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