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Apoptosis-mediated *vasa* down-regulation controls developmental transformation in Japanese *Copidosoma floridanum* female soldiers

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ABSTRACT

Copidosoma floridanum is a polyembryonic, caste-forming, wasp species. The ratio of investment in different castes changes with environmental stressors (e.g. multi-parasitism with competitors). The vasa gene was first identified in *Drosophila melanogaster* as a germ-cell-determining factor, and *C. floridanum* vasa (*Cf-vas*) gene positive cells have been known to develop into reproductive larvae. *Cf-vas* seems to control the ratio of investment in *C. floridanum* larval castes. In this study, we identified environmental factors that control *Cf-vas* mRNA expression in Japanese *C. floridanum* by examining *Cf-vas* mRNA expression under competitor (*Meteorus pulchricornis*) venom stress; we treated the male and female morulae with *M. pulchricornis* venom. We also assessed the effects of multi-parasitism of Japanese *C. floridanum* with *M. pulchricornis* and found an increasing number of female soldier larvae. The results showed that several amino acid sequences differ between the Japanese and US *Cf-vas*. Quantitative reverse transcription polymerase chain reaction (qRT-PCR) showed that Japanese *Cf-vas* mRNA is expressed in both male and female larvae and pupae, but mRNA expression decreases in adults. *Cf-vas* mRNA expression significantly decreased, while *C. floridanum* dronc (*Cf-dronc*) mRNA expression increased, in female morulae after *M. pulchricornis* venom treatment at 20 h and 0 h of the culture period, respectively. Females and males showed different *Cf-vas* or *Cf-dronc* mRNA expression after *M. pulchricornis* venom treatment. Therefore, *M. pulchricornis* venom could affect the ratio of investment in different female castes of Japanese *C. floridanum* by decreasing *Cf-vas* mRNA expression via apoptosis.

1. Introduction

Copidosoma floridanum is a polyembryonic egg-larval parasitoid of Lepidoptera (Godfry and Cook, 1997). A single parasitoid egg can divide to produce >2000 separate individuals, which are clones with the same gene (Ode and Strand, 1995). At ~12 h after the female oviposits into the host insect eggs, maternal embryos form at the 2-cell stage in 2 h and the 8-cell stage in 4–5 h. The morula embryos separate from the amniotic membrane, and a polymorula forms. Each embryo constituting this polymorula develops into a larva (Fig. 1; Iwabuchi, 1991; Grbić et al., 1998).

C. floridanum larvae are divided into two castes: reproductive larvae, which are fertile, and soldier larvae, which are sterile larvae (infertile larvae) (Fig. 1). Reproductive larvae enter morphogenesis when the host insect reaches the pre-ultimate larval stage; then they consume the host tissue and finally kill the host insect, form a pupa, and emerge as adults (Strand, 1989a; Baehrecke and Strand, 1990). Soldier larvae have an important role in defence against a competitor (Cruz, 1981). They eliminate allogeneic or other parasitic wasps by killing them with their large mandibles (Godfray, 1994).

In Hymenoptera, only polyembryonic wasps such as *C. floridanum* have both male and female soldier larvae (also known as precocious larvae) (Strand, 1989a). They have different characteristics. Japanese

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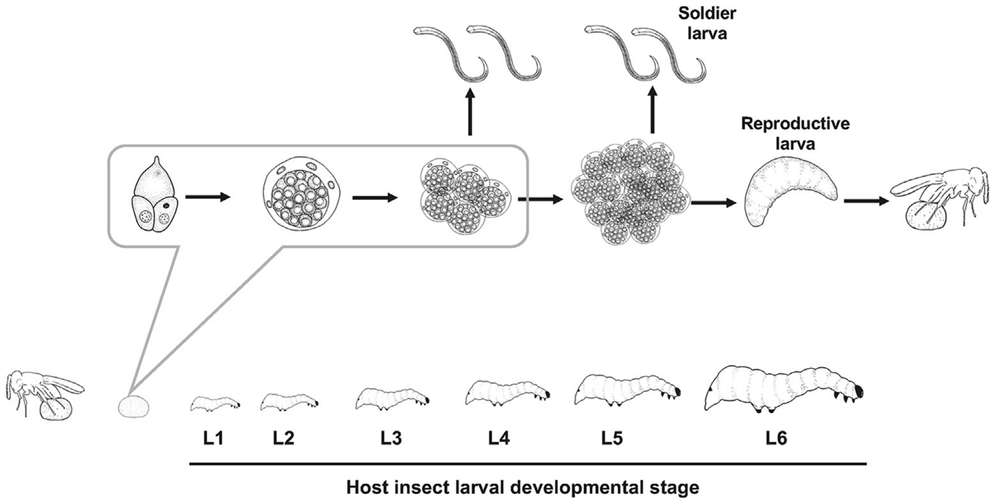

Fig. 1. Life cycle of *C. floridanum*. *C. floridanum* female oviposit into host eggs, and maternal embryos are formed at the 2-cell stage in 2 h, the 8-cell stage in 4–5 h, and, after ~60 h, morula embryos are separated by the amniotic membrane, and a polymorula is formed (until L1 host larvae). Male and female soldier larvae (precocious larvae) appear from the polymorula, and they are dead by the host larval L6 developmental stage. Reproductive larvae enter morphogenesis when the host reaches the L6 developmental stage; then they kill the host, form a pupa, and emerge as adults. Therefore, *C. floridanum* forms castes.

C. floridanum soldier larvae appear in the host insect from the first larval developmental stage (Uka et al., 2013), while US *C. floridanum* male soldier larvae appear in the host insect from the last larval developmental stage (Grbic et al., 1997). Japanese *C. floridanum* female soldier larvae are more active than their male counterparts (Uka et al., 2016). If both sexes parasitise (mixed brood) in the same host insect, US *C. floridanum* will produce more females than males (Grbic et al., 1992). These differences might lead to environmental competition between *C. floridanum* male and female soldier larvae.

The ratio of investment in different castes changes with environmental stressors (e.g. multi-parasitism with competitors). These environmental stressors cause an increase in the number of soldier larvae (Harvey et al., 2000; Watanabe et al., 2012). For example, when a host insect parasitised by *C. floridanum* females is exposed to high temperature, the number of soldier larvae significantly increases at the last larval developmental stage (L6) of the host insect (Takano et al., 2019). However, the number of male soldier larvae does not increase (Takano et al., 2019). In addition, the number of soldier larvae gradually increases till the L6 developmental stage of the host insect under multi-parasitism with *Microplitis demolitor* (Harvey et al., 2000). Furthermore, the number of female soldier larvae increases under multi-parasitism with *Glyptapanteles pallipes* (Yamamoto et al., 2007). Therefore, *C. floridanum* appears to increase the number of soldier larvae to avoid environmental stressors (e.g. multi-parasitism and high temperature) and decreases the number of reproductive larvae with a ‘trade-off’ for leaving their offspring. However, the molecular mechanism of such a trade-off is unclear, as is the reason why the number of *C. floridanum* female soldier larvae increases when the host insect is exposed to environmental stressors.

The *vasa* gene was first identified as a germ cell–determining factor in *Drosophila melanogaster* (Eddy, 1975; Kotaja and Sassone-Corsi, 2007), and studies have reported that *vasa* messenger RNA (mRNA) is expressed in various organisms' primordial germ cells (PGCs) and reproductive tissues (Styhler et al., 1998). *C. floridanum* vasa (*Cf-vas*) was first identified in US *C. floridanum* (Donnell et al., 2004; Zhurov et al., 2004) and was localised from one blastomere at the 2-cell stage by immuno-histostaining using anti *C. floridanum* vasa antiserum (Donnell et al., 2004; Gordon and Strand, 2009). Interestingly, reproductive larvae did not develop when *Cf-vas*-expressing blastomeres were disrupted at the 4-cell stage (Zhurov et al., 2004). *Cf-vas* may only be expressed if germ cells will develop into reproductive larvae, not if embryos (not germ cells) will develop into soldier larvae. Therefore, *Cf-vas* can control the ratio of investment in *C. floridanum* larval castes. In addition, *C. floridanum* is an interesting model insect for demonstrating that in social insects, the ratio of investment in larval castes is regulated by environmental factors; however, how these environmental factors control *Cf-vas* mRNA expression is unclear.

We identified and characterized *Cf-vas* in Japanese *C. floridanum*, whose characteristics differ from those of US *C. floridanum* (Grbic et al., 1997; Uka et al., 2013). To identify environmental factors regulating *Cf-vas* mRNA expression in this study, we focused on *Meteorus pulchricornis*, a competitor of *C. floridanum*, during natural parasitism of their hosts, Lepidoptera. The *M. pulchricornis* female injects her venom through the ovipositor into the host insect to protect her eggs from encapsulation by the host's haemocytes after she oviposits (Vinson and Iwansch, 1980). *M. pulchricornis* (competitor) venom includes VLPs, which reduce the host's encapsulation ability by inducing apoptosis of its haemocytes (Suzuki and Tanaka, 2006). Suzuki et al., showed that hemocyte causes apoptosis when purified VLPs are added. Conversely, apoptosis does not occur when venom fluid without VLPs is added with haemocytes (Suzuki and Tanaka, 2006). Thus, we examined *Cf-vas* mRNA expression under *M. pulchricornis* venom stress, and we examined the expression of *Cf-dronc* mRNA (*C. floridanum* Death regulator Nedd2-like caspase) under *M. pulchricornis* venom stress. *Cf-Dronc* is *Dronc* homolog which is essential for caspase-dependent cell death in *D. melanogaster* (FlyBase, FBgn0026404).

2. Materials and methods

2.1. Rearing of parasitoids and their hosts

The encyrtid parasitoid *C. floridanum* (synonym: *Litomastix maculata*) and its natural host, *Chrysodeixis eriosoma* (Lepidoptera, Noctuidae), were maintained at 25 °C in a 16 h light/8 h dark photoperiodic cycle, as previously described (Iwabuchi, 1991, 1995). Parasitism by *C. floridanum* was accomplished by exposing the host's eggs (aged 8–14 h) to non-mated or mated parasitoid females.

2.2. Morula culture

C. floridanum non-mated females oviposited only male eggs into host insect eggs adhered to 5 × 5 mm^2 paraffin paper in a 9 cm glass Petri dish (Becton Dickinson, Oxnard, CA, USA). *C. floridanum* mated females oviposited both male and female eggs into host insect eggs. To sex discrimination, we observed their abdomen movements during oviposition under a stereoscopic microscope to select only female eggs (Strand, 1989b).

The embryo culture of parasitised eggs were surface-sterilised by submersion in 70% ethanol for 10 min and then dried under a stream of clean air in a laminar flow cabinet. *C. floridanum* embryos at the 2-cell stage, which were enveloped by the egg chorion, were removed at 3 h post-parasitism by dissecting their host eggs in a serum-free, MGM-450 culture medium (modified as previously described; Iwabuchi, 1991). We used morula-stage embryos developed from the 2-cell stage in vitro. Studies have shown that morula formation is complete by 24 h post-parasitism (Grbić et al., 1998). In this study, embryonic cells were completely enveloped by the extraembryonic syncytium when the egg chorion was shedded, so these embryos were considered morula-stage embryos within 10 h (Nakaguchi et al., 2006). The embryos were transferred individually to the culture medium in a 35 mm Petri dish (Becton Dickinson). A host embryo (aged 30–35, 45–55 or 60–65 h) and a parasitoid embryo (morula-stage) were cultured in a hanging droplet of 20 μl culture medium at 25 °C. The morulae were observed under a phase-contrast microscope.

2.3. *Purification of total RNA and cDNA synthesis from morulae, and whole-body samples*

After 20 h of parasitism of *C. floridanum*, male and female morulae were used as morula samples. Larval, pupal and adult whole-body samples were dissected from L6 host larvae. These samples were stored at −80 °C in 750 μL of TRIzol LS reagent (Thermo Fisher Scientific, MA, USA) until total RNA purification. The male or female morulae (n = 3), the male or female larval whole body (n = 5), the male or female pupal whole body (n = 5), and the male or female adult whole bodies (n = 5) were homogenised using 750 μL of TRIzol LS reagent, then 200 μL of chloroform was added, and the mixture was vortexed for 1 min. The supernatant was collected after centrifugation at 12,000×*g* for 10 min and used for total RNA purification using NucleoSpin® RNA XS (Macherey-Nagel, GmbH & Co, Düren, Germany) according to the manufacturer's instructions. The purified total RNA was processed for complementary DNA (cDNA) synthesis using a PrimeScript™ 1st strand cDNA Synthesis Kit (Takara Co. Ltd., Tokyo, Japan).

2.4. *cDNA cloning of C. floridaum vasa from Japanese C. floridanum male morulae*

In this study, we used cDNA from male morulae. *C. floridanum vasa* cDNA sequences were amplified by polymerase chain reaction (PCR) using KOD-plus-Neo polymerase (Toyobo Co. Ltd., Tokyo, Japan) with specific primers (5′-aacaaccgacacgtgcgga-3′, 5′-tcattcatcagtcaaggaactt-3′). Next, the amplified products were cloned into the cloning vector Topo-p2T (Invitrogen, Carlsbad, CA, USA) and then transformed into *Escherichia coli* XL-1 Blue (Toyobo Co. Ltd.). The purified vectors were then processed for sequencing by dideoxynucleotide chain termination using the ABI PRIZM 3100 Genetic Analyser (Applied Biosystems, Tokyo, Japan). We confirmed *Cf-vas* cDNA sequence using two cDNA library sequences from different male morula.

2.5. *A phylogenetic analysis*

Bark homologs of the following species were obtained from NCBI databases (https://www.ncbi.nlm.nih.gov/gene/): *C. floridanum* (NP_001302515.1, XP_014219851.1), *Nasonia vitripennis* (XP_001603956.3), *Apis mellifera* (NP_001035345.1), *Bombyx mori* (NP_001037347.1), *Tribolium castaneum* (EFA07550.1), *D. melanogaster* (NP_723899.1), *Homo sapiens* (NP_077726.1). The phylogenetic analysis was conducted using MEGA ver. 7 with neighbor-joining method.

2.6. *RNA-seq analysis*

At 20 h after parasitism of *Thysanoplusia intermixta* eggs, we collected morulae and isolated the total RNA from male morulae using TRIzol® LS Reagent (Thermo Fisher Scientific) and a PureLink® RNA Extraction Kit (Thermo Fisher Scientific) according to the manufacturer's instructions. Next, we used the Agilent TapeStation 2200 (Agilent Technologies, Santa Clara, CA, USA) to assess RNA quality. We constructed paired-end sequencing cDNA libraries with total RNA from Japanese *C. floridanum* male morulae using a TruSeq Stranded mRNA Sample Preparation Kit (Illumina Inc., San Diego, CA, USA) according to the manufacturer's instructions. The libraries were sequenced (75 bp, paired-end) on the Illumina NextSeq500 platform, and FASTQ files were assessed using Trim Galore! v0.4.5 (https://www.bioinformatics.babraham.ac.uk/projects/trim_galore/). The *C. floridanum* genome (GCF_000648655.2) sequence is available in the National Center for Biotechnology Information (NCBI) genome database (https://www.ncbi.nlm.nih.gov/genome/annotation_euk/Copidosoma_floridanum/101/). We aligned the obtained FASTQ files to the genomic reference sequence using HISAT2 v2.1.0 with default parameters (Kim et al., 2015). We also sorted and indexed the binary alignment map (BAM)-formatted files, generated by HISAT2 using SAMtools ver.1.9 (Li et al., 2009), and then converted them to the Wiggle track format (WIG) using bam2wig software (https://github.com/MikeAxtell/bam2wig). This allowed us to visualise the density of reads mapped to the specific region of interest (Liao et al., 2013, 2014). Finally, we generated a histogram using TIBCO Spotfire Desktop v7.6.0 under the 'Better World' programme licence (TIBCO Spotre, Inc., Palo Alto, CA, USA; http://spotfire.tibco.com/better-world-donation-program/). In addition, we deposited the sequence data (FASTQ files) to the DNA Data Bank of Japan (DDBJ) Sequence Read Archive (accession no. DRA008013).

2.7. *qRT-PCR*

To quantify RNA expression levels, we used 0.5 μg of total RNA purified from male and female larvae, pupae and adults and total RNA from male and female morulae for cDNA synthesis. Quantitative reverse transcription polymerase chain reaction (qRT-PCR) was performed on a Step One plus Real-Time PCR System (Applied Biosystems, Foster City, CA, USA) using the delta-delta Ct method in 20 μL reaction volumes with 0.5 μL of the cDNA template and primers (Table 1) using a KAPA SYBR Fast qRT-PCR Kit (Nippon Genetics Co., Ltd., Tokyo, Japan) according to the manufacturer's instructions. *C. floridanum* glyceraldehyde-3-phosphate dehydrogenase (Cf-gapdh; DDBJ accession no. M0090) was used as an endogenous reference to standardise RNA expression levels. All data were calibrated against universal reference data, and relative quantification (RQ) values represented the relative expression level against a reference sample. All samples were assayed in triplicate as biological replications.

2.8. *Preparation of M. pulchricornis venom*

We used *M. pulchricornis* as a competitor (Supplementary Fig. 1A). Seven days after eclosion, adult individuals were surface-sterilised using 70% ethanol for 10 min. The ovipositor was pulled with forceps in KPBS (56.1 mM K_2HPO_4, 54.7 mM KH_2PO_4, 24.7 mM glucose) to expose the venom reservoir (Supplementary Fig. 1B), which was picked up with forceps. Three venom reservoirs were collected in Protein LoBind tubes (Eppendorf Inc., Hamburg, Germany) containing 120 μL of modified MGM-450 medium and ground with forceps. To remove the tissue, homogenised venom reservoirs were centrifuged at 0 °C and centrifuged at 600×*g* for 10 min. The supernatant was used as venom for the experiment.

2.9. *Venom treatment of morulae and detection of Cf-vas*

Each male and female morula was treated with a 20 μL drop of extracted venom for 30 min and then transferred to a 20 μL drop of new modified MGM-450 medium. Male and female morulae, treated with a 20 μL drop of modified MGM-450 for 30 min, were used as controls. The morulae were cultured at 25 °C for 0–20 h till total RNA purification. Total RNA was subjected to qRT-PCR to determine *Cf-vas* expression.

Table 1
qRT-PCR primers used in this study.

Primer	Forward	Reverse
Cf-gapdh	5′-GCGAGCGGTTCTATCATTCC-3′	5′-CTCAGCACCAGACGACCAATAC-3′
Cf-vas	5′- AATACGCCACGAAACTATGACTCCC-3′	5′- CTACCAACAATTCCCACAGCTACGA-3′
Cf-dronc	5′- CGGAAATTCAAGGCACTGAT-3′	5′- GGTTGTTCGGAATCAGGATG-3′

qRT-PCR, quantitative reverse transcription polymerase chain reaction; *Cf-gapdh*, *Copidosoma floridanum* glyceraldehyde-3-phosphate dehydrogenase; *Cf-vas*, *Copidosoma floridanum* vasa; *Cf-dronc*, *Copidosoma floridanum* dronc.

2.10. Effect of injecting *M. pulchricornis* venom into *C. floridanum* larvae

One host egg was used for parasitizing by *C. floridanum* and then maintained until day 1 of the L6 developmental stage. We injected 1 μL of extracted *M. pulchricornis* venom intra-haemocoelically into host larvae on day 1 of the L4 developmental stage using a 10 μL glass capillary (Drummond Scientific Company, PA, USA). As a control, we injected 1 μL of KPBS intra-haemocoelically into host larvae on day 1 of the L4 developmental stage using a glass capillary. The host larvae were maintained individually in a plastic cup (90 BL RISUPACK, Gifu, Japan) at 25 °C until day 1 of the L6 developmental stage. The host was anesthetised using 35% ethanol for 10 min and then dissected in KPBS, and the number of soldier larvae was counted.

2.11. Effects of *M. pulchricornis* and *C. floridanum* multi-parasitism in the host egg

One host egg was used for parasitizing by *C. floridanum* and *M. pulchricornis* and then maintained until day 1 of the L6 developmental stage. The host larvae were maintained individually, in a 90 BL RISUPACK plastic cup at 25 °C, until day 1 of the L6 developmental stage. The host was anesthetised using 35% ethanol for 10 min and then dissected in KPBS, and counted the number of soldier larvae in the host body where experimental parasitism was successful. The number of individuals used in this experiment was as follows; Only male-brood of *C. floridanum* is parasitoid to the host (Success of parasitism; n = 7), male-brood of *C. floridanum* and *M. pulchricornis* are parasitoid to the host (Success of

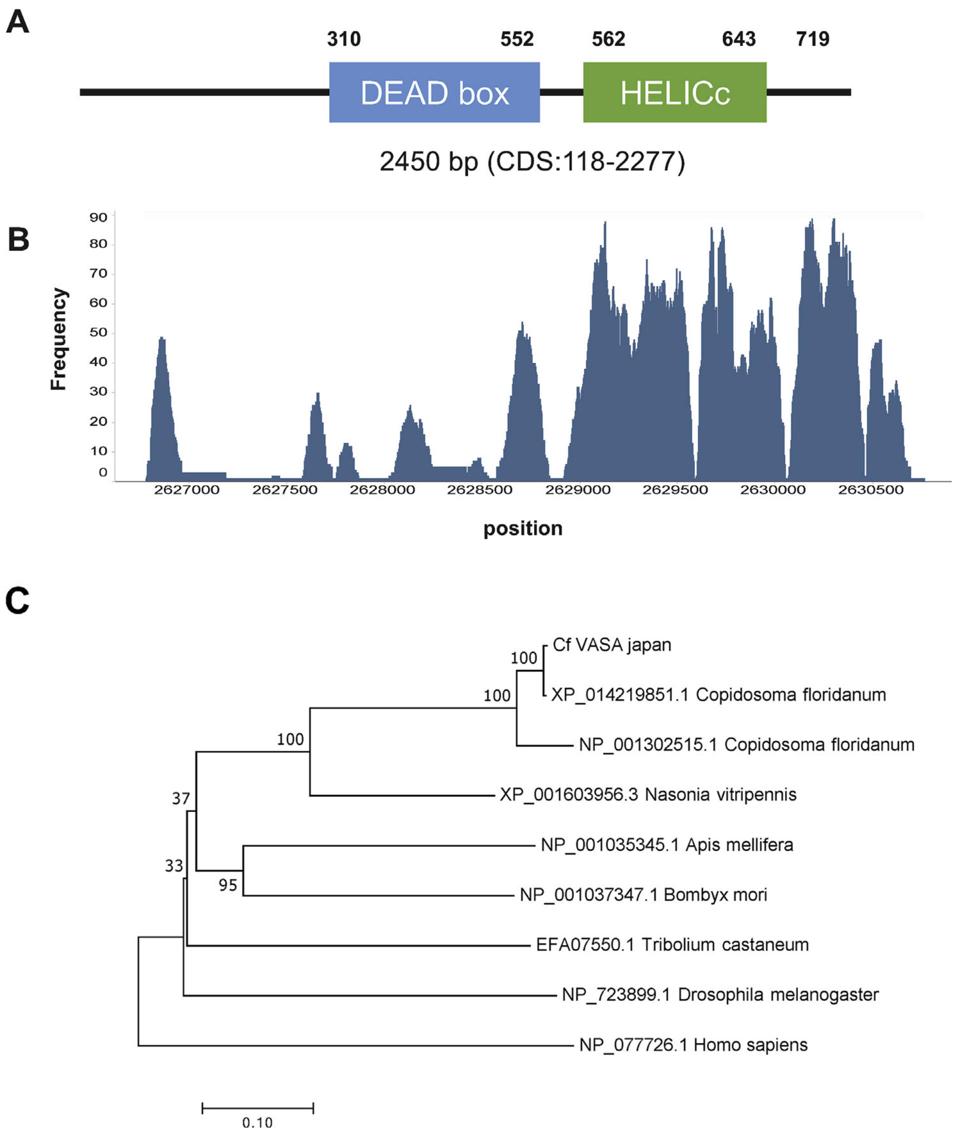

Fig. 2. (A) The putative domain structure of Japanese *Cf-vas*. The upper digits show the amino acid position. Light-blue box, DEAD box; green box, HELICc domain. (B) Verification of *Cf-vas* mRNA expression by RNA-seq analysis. Histograms show the frequency of Japanese *Cf-vas*. *Cf-vas*, *Copidosoma floridanum* vasa; RNA-seq, RNA sequencing; mRNA, messenger RNA. (C)
The phylogenetic tree of Japanese *Cf-vas* and Vasa proteins of other species. Amino acid sequences with NCBI IDs of the Vasa proteins used in this study are shown in this phylogenic tree. *Cf-vas*, *Copidosoma floridanum* vasa; NCBI, National Center for Biotechnology Information.

parasitism; n = 10), Only female-brood of *C. floridanum* is parasitoid to the host (Success of parasitism; n = 16), and female-brood of *C. floridanum* and *M. pulchricornis* are parasitoid to the host (Success of parasitism; n = 9).

2.12. *Statistical analysis*

Statistical analysis was performed using Microsoft Excel and Student's *t*-test.

3. Results

3.1. *cDNA cloning of Cf-vas from Japanese C. floridanum morulae*

We obtained the *Cf-vas* sequence from the NCBI genome database (gene ID 106647823), which yielded two *Cf-vas* sequences among *C. floridanum* genes. The *Cf-vas* sequence from Japanese *C. floridanum* was confirmed through cDNA cloning, which showed that the open reading frame was 2,450 nucleotides long, encoding a protein comprising 719 amino acids, with a molecular weight of 76,576 Da and a putative isoelectric point of 5.01. *Cf-vas* contained a DEAD-like helicase super-family (DEXDc) domain (SMART; SM000487) at position 310G-522S and a helicase super-family c-terminal (HELICc) domain (SMART; SM000490) at position 562D-643G (Fig. 2A). We verified *Cf-vas* mRNA expression using RNA sequencing (RNA-seq) at single-nucleotide resolution (Fig. 2B) and found that *Cf-vas* expression of the C-terminus was significantly higher than the N-terminus (Fig. 2B). However, we could not find other *Cf-vas* isoform sequences.

Japanese *C. floridanum Cf-vas* showed 87% similarity to US *C. floridanum Cf-vas* (gene ID 106647823), 62% similarity to *Nasonia vitripennis vasa* (gene ID 100120300), 51% similarity to *Bombyx mori vasa* (gene ID 692765), 47% similarity to *D. melanogaster vasa* (gene ID 26067080), 47% similarity to *Apis mellifera vasa* (gene ID 410692) and 52% similarity to *Tribolium castaneum vasa* (gene ID 641597).

We found that position 685 in US *C. floridanum* was a tyrosine residue but changed to a serine residue in Japanese *C. floridanum* (Supplementary Fig. S2, green arrow). The N-terminal of several amino acid residues was different between US and Japanese *C. floridanum* (Supplementary Fig. S2, red arrows). We verified the Japanese *Cf-vas* cDNA sequences using our RNA-seq data (Supplementary Figs. 3–A). However, the transcript data did not match the US *Cf-vas* sequence (Supplementary Figs. 3–B). Therefore, we decided the Japanese *Cf-vas* cDNA sequence.

Cf-vas was close to *N. vitripennis vasa* on the phylogenetic tree (Fig. 2C). (The nucleotide sequence reported in this study has been submitted to the GeneBank/DDBJ SAKURA Data Bank [accession no. LC460265]).

3.2. *Cf-vas mRNA expression in all developmental stages*

qRT-PCR showed that *Cf-vas* mRNA is expressed in all developmental stages. *Cf-vas* mRNA expression increased in female pupae compared to female larvae, while *Cf-vas* expression decreased in male and female adults compared to larvae (Fig. 3).

3.3. *Increase of female soldier larvae in C. floridanum with M. pulchricornis multi-parasitism*

M. pulchricornis is a competitor parasite of *Chrysodeixis eriosoma*. Multi-parasitism of *M. pulchricornis* and *C. floridanum* into the host eggs had a different effect on the number of *C. floridanum* male and female soldier larvae. There was no significant difference in the increase in the number of male soldiers in multi-parasitism and *C. floridanum* male parasitism ($p = 0.56$); however, there was a significant difference in the increase in the number of female soldiers in multi-parasitism and *C. floridanum* female parasitism ($p = 5.88\,E{-}11$) (Fig. 4).

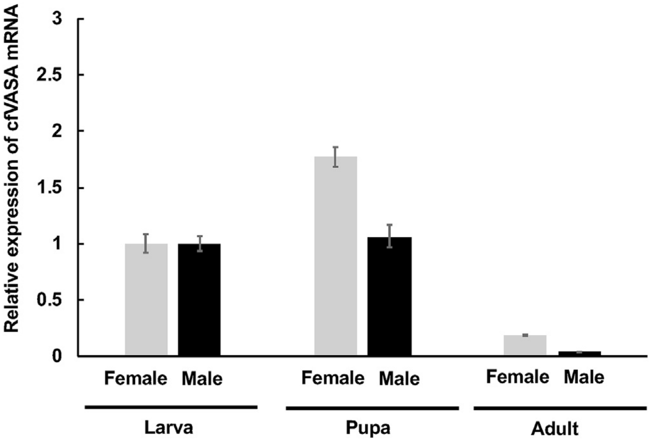

Fig. 3. Expression of Japanese *Cf-vas* in different developmental stages of *C. floridanum*. Whole bodies were used for qRT-PCR for each developmental stage. RQ values of male and female larvae, pupae, and adults represent the relative expression level compared to the reference sample. Error bars indicate relative minimum/maximum expression levels against mean RQ values. *Cf-gapdh* was used as an endogenous control. *Cf-vas*, *Copidosoma floridanum* vasa; qRT-PCR, quantitative reverse transcription polymerase chain reaction; RQ, relative quantification; *Cf-gapdh*, *Copidosoma floridanum* glyceraldehyde-3-phosphate dehydrogenase.

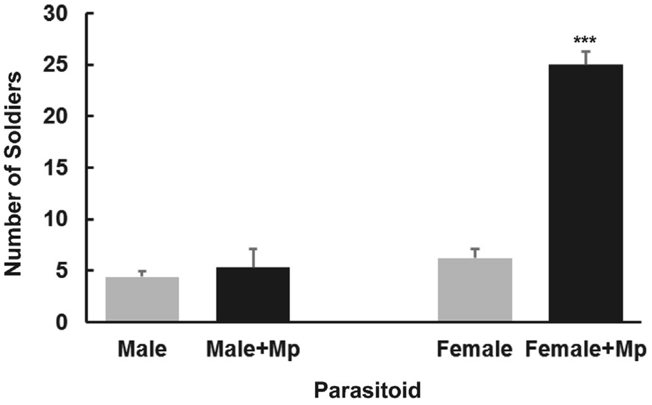

Fig. 4. Number of *C. floridanum* soldier larvae with *M. pulchricornis* multi-parastitoid. One host egg was parasitised by *C. floridanum* and *M. pulchricornis* and then maintained until day 1 of the L6 developmental stage. The number of soldier larvae was counted and plotted on a graph. Male indicates only male-blood of *C. floridanum* is parasitoid to the host (Success of parasitism; n = 7). Male + Mp indicate male-blood of *C. floridanum* and *M. pulchricornis* are parasitoid to the host (Success of parasitism; n = 10). Female indicates only female-blood of *C. floridanum* is parasitoid to the host (Success of parasitism; n = 16). Female + Mp indicate female-blood of *C. floridanum* and *M. pulchricornis* are parasitoid to the host (Success of parasitism; n = 9). Statistically significant differences determined by Student's *t*-test are indicated as $^{***}P < 0.001$.

3.4. *Effect of M. pulchricornis venom treatment on Cf-vas and Cf-dronc mRNA expression*

After *M. pulchricornis* venom treatment, compared to controls, the average number of female soldier larvae appears to be slightly higher than the value in the control groups in the. However, there was no significant difference in the increase in the number of male soldiers in the venom treatment, and there was a no significant difference in the increase in the number of female soldiers in the venom treatment (Table 2).

C. floridanum males and females had different responses to *M. pulchricornis* venom in relation to *Cf-vas* and *Cf-dronc* mRNA expression. *M. pulchricornis* venom treatment increased *Cf-vas* mRNA expression in male morulae (Fig. 5A) but decreased *Cf-vas* mRNA expression in

Table 2
Number of soldier larvae on day 1 of the L6 developmental stage *M. pulchricornis* venom injection.

Experiment	Control (female)	Venom (female)	Control (male)	Venom (male)
1	52	108	22	22
2	54	60	61	12
3	39	53	36	43
4	96	35	39	62
Mean ± SD	60.3 ± 24.7	64.0 ± 31.2	39.5 ± 16.1	34.8 ± 22.3

Data indicate the number of soldier larvae after each treatment.

female morulae (Fig. 5B) after 20 h culture period. In contrast, *M. pulchricornis* venom treatment increased *Cf-dronc* mRNA expression in male morulae after 20 h culture period (Fig. 6A) but increased *Cf-dronc* mRNA expression in female morulae immediately (Fig. 6B).

In summary, *Cf-vas* mRNA expression decreased in Japanese *C. floridanum* male and female adults. However, *Cf-vas* mRNA expression was maintained in both male and female larvae and pupae. Male morulae developed more rapidly in a culture medium compared to female morulae (data not shown). *Cf-vas* mRNA expression differed between males and females in all developmental stages—it was maintained in male larvae and pupae, while it increased in female pupae.

4. Discussion

In this study, cDNA cloning, phylogenic tree analysis, and protein motif analysis showed that Japanese *Cf-vas* is different from US *Cf-vas*. The N-terminal of 10 amino acid sequences is different between the Japanese and US *C. floridanum*. In addition, the tyrosine residue at position 685 in US *C. floridanum* is a serine residue in Japanese *C. floridanum*, which leads to different characteristics and is related to amino acid phosphorylation. This substitution might contribute to different functions of Japanese *Cf-vas*.

Japanese *C. floridanum* male and female *Cf-vas* might play different roles during development. The *vasa* gene is an RNA helicase, which controls translational regulation of genes involved in germ cell development (Dehghani and Lasko, 2015; Hay et al., 1988). US *Cf-vas* is related to germ cell development in reproductive larvae (Donnell et al., 2004). Previous studies have reported that *vasa* plays a role in wound-healing in sea urchin embryos (Yajima and Wessel, 2015) and in meiosis promotion (Yajima and Wessel, 2011), the piwi-interacting RNA (piRNA) pathway (Dehghani and Lasko, 2016), pattern formation of the posterior region, and PGC formation in *D. melanogaster* embryos (Hashimoto et al., 2008; Poon et al., 2016). In addition, *VASA* (also known as *DDX4*) is involved in tumour formation in humans (Pek and Kai, 2011; Xiol et al., 2014). Thus, *Cf-vas* could also relate to reproductive larvae development, and Japanese *Cf-vas* also shares similar functions with US *Cf-vas*.

The caste ratio shifts adaptively because of multi-parasitism of *C. floridanum* with competitors (Harvey et al., 2000). Survival depends on the number of soldier larvae. Soldier larvae can attack multi-parasitoids to protect reproductive larvae. US *C. floridanum* male and female soldier larvae have different characteristics. In US *C. floridanum*, male soldier larvae appear in the L5 developmental stage, and they are non-aggressive compared to female soldier larvae (Giron et al., 2007).

Caste structure is different between *C. floridanum* males and females. The number of female soldier larvae, but not male soldier larvae, increased in the *M. pulchricornis* multi-parasitoid, a phenomenon also observed in US *C. floridanum* (Harvey et al., 2000).

In the Braconidae, venom suppresses host immunity for parasitism success. The laid eggs need to escape from encapsulation by the host's haemocytes (Vinson and Iwansch, 1980). The VLPs including *M. pulchricornis* venom induce apoptosis of the host's haemocytes to prevent encapsulation (Suzuki and Tanaka, 2006). However, *M. pulchricornis* venom fluid without VLPs does not cause the apoptosis of the host haemocytes (Suzuki and Tanaka, 2006).

In the study, multi-parasitism of *C. floridanum* and *M. pulchricornis* could induce the down-regulation of *Cf-vas* mRNA, and the up-regulation of *Cf-dronc* mRNA. These genes could be associated with the increasing soldier larvae in the *C. floridanum* larvae. *D. melanogaster Dronc* plays a key role in stress-induced apoptosis and contains a caspase recruitment domain (CARD), which is similar to caspase-9 (Daish et al., 2004; Dorstyn et al., 1999, 2002).

Soldier larvae originate from morulae without germplasm (Zhurov et al., 2004). In addition, experimental elimination of *Cf-vas*-positive cells at the 4-cell stage increases soldier larvae (Zhurov et al., 2004). Xenopus vasa-like gene 1 (XVLG1), a vertebrate vasa homolog, is also required for the formation of PGCs (Shimaoka et al., 2017), and *XVLG1* mRNA down-regulation induces PGC apoptosis (Shimaoka et al., 2017). In addition, it has been demonstrated that the loss of vasa is associated with germ-cell apoptosis in mice, and *D. melanogaster* (Styhler et al., 1998; Tanaka et al., 2000). We speculate that *Cf-vas* down-regulation induced PGC apoptosis by the up-regulation of *Cf-dronc* in the female morula. Therefore, *M. pulchricornis* venom would induce apoptosis in *C. floridanum* morula.

Therefore, we believe *M. pulchricornis* venom causes a change in female morulae morphology via apoptosis. *M. pulchricornis* venom leads to apoptosis of *Cf-vas*-positive cells, and *Cf-vas* mRNA down-regulation is

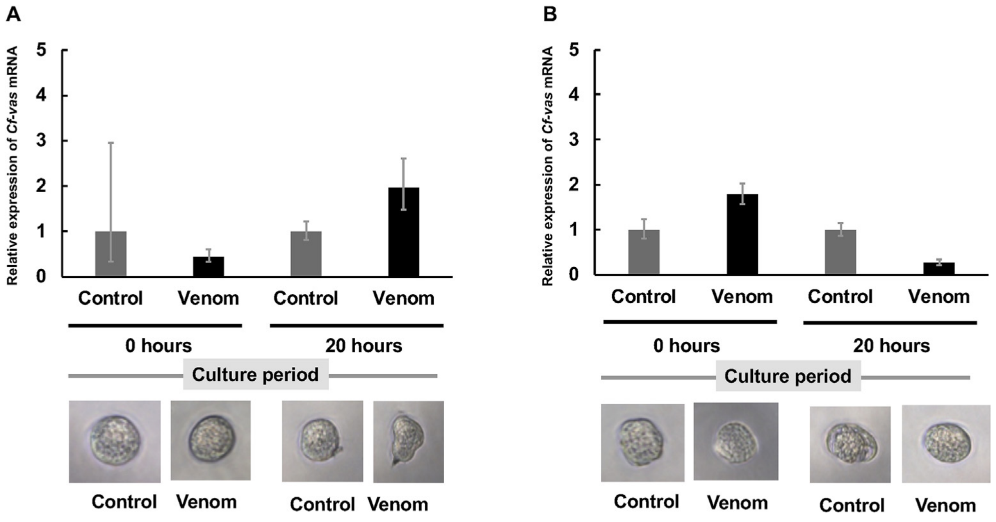

Fig. 5. Japanese *Cf-vas* mRNA expression in *M. pulchricornis* venom–treated *C. floridanum* male and female morulae. mRNA expression in the (A) male and (B) female morulae treated with *M. pulchricornis* venom for 30 min and then cultured in modified MGM-450 medium for 0 and 20 h, respectively ($n = 3$ each). Control and *M. pulchricornis* venom–treated groups were plotted as RQ values. Error bars indicate relative minimum/maximum expression levels against the mean RQ values. *Cf-gapdh* was used as an endogenous control. Technical replication was performed in triplicate. Lower panel: cultured morulae in each treatment group. *Cf-vas*, *Copidosoma floridanum* vasa; mRNA, messenger RNA; RQ relative quantification; *Cf-gapdh*, *Copidosoma floridanum* glyceraldehyde-3-phosphate dehydrogenase.

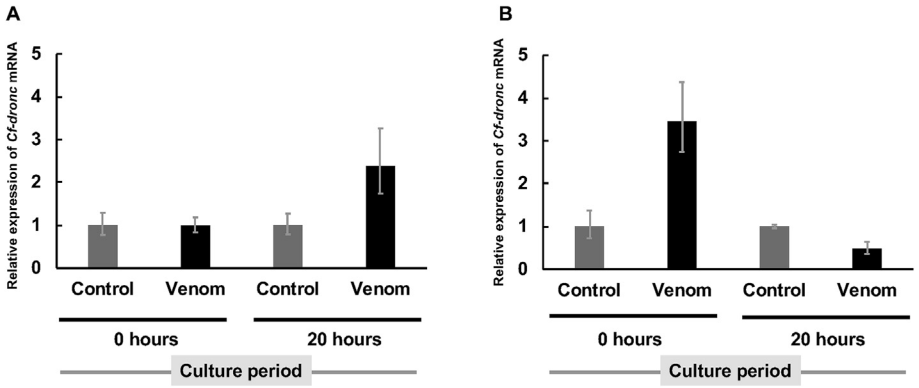

Fig. 6. Japanese *Cf-dronc* mRNA expression in *M. pulchricornis* venom–treated female morulae. The mRNA expression in female morulae was treated with *M. pulchricornis* venom for 30 min and then cultured in modified MGM-450 medium for 0 and 20 h ($n = 3$ each). Control and *M. pulchricornis* venom–treated groups were plotted as RQ values. Error bars indicate relative minimum/maximum expression levels against mean RQ values. *Cf-gapdh* was used as an endogenous control. Technical replication was performed in triplicate. *Cf-dronc*, *Copidosoma floridanum* dronc; mRNA, messenger RNA; RQ relative quantification; *Cf-gapdh*, *Copidosoma floridanum* glyceraldehyde-3-phosphate dehydrogenase.

seen in female morulae. Embryo cells with high and low resistance to *M. pulchricornis* venom coexist in the morula, and low resistance embryo cells which express *Cf-vas* might be excluded from the morula embryo through apoptosis, increasing the number of soldiers. However, this does not clarify why male morulae responded differently to *M. pulchricornis* venom in this study.

5. Conclusions

M. pulchricornis venom would induce apoptosis in *C. floridanum* female morulae by increasing *Cf-dronc* mRNA expression and decrease *Cf-vas*-positive cells. As a result, *M. pulchricornis* venom could induce to change the caste structure in *C. floridanum* morulae by decreasing *Cf-vas* mRNA expression.

The molecular mechanism of the caste structure changes in *C. floridanum* males is different than the females because their responses to *M. pulchricornis* venom are different. Therefore, we need to find other factors which cause the caste structure changes in males.

In future, we will investigate how *Cf-Vas* and *Cf-dronc* are related to caste transdetermination using a knockdown strategy in *C. floridanum*. Injecting dsRNA into *C. floridanum* eggs using current injection systems is challenging due to the tiny sizes. Therefore, it is necessary to develop a novel injection system for injecting into tiny eggs such as *C. floridanum* eggs. *C. floridanum* is one of the few examples which demonstrate that, in social insects, investment in soldier castes is regulated by environmental factors, and this study's findings will contribute to our understanding of this phenomenon.

Availability of data and materials

The nucleotide sequences for cfVASA was submitted to DDBJ/ENA/GenBank (Accession No. LC460265). The RNA-seq reads supporting the conclusions of this article are available in the Sequence Read Archive (SRA) with accession ID DRA008013, https://www.ncbi.nlm.nih.gov/sra/.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ydbio.2019.09.005.

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